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Research Article

ASSESSMENT OF THE GENOTOXIC AND MUTAGENIC EFFECT OF AL-TAIF POMEGRANATE (*PUNICA GRANATUM L*) PEEL EXTRACT ALONE AND COMBINED WITH MALATHION AND ATRAZINE PESTICIDES IN LIVER OF MALE ALBINO MICE

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ABSTRACT

In our previous studies, we report the antioxidant, hepatoprotective and nephroprotective potential of of Al-Taif Pomegranate (*Punica granatum L*) extracts against toxicity induced by Malathion (Mal) and Atrazine (Atra) pesticides in male albino mice. Hereby, we assess the genotoxic and mutagenic potential of Al-Taif Pomegranate (*P. granatum L*) peel extract (PPE) alone and combined with Atra and Mal pesticides in the liver of male albino mice. Our results report PPE genotoxicity and its failure to significantly decrease the genotoxic effect of the pesticides Mal and Atra. Genotoxic potential was reported by using Comet assay, in which fifty isolated comets were randomly selected and used to measure tail length, % DNA of tail and tail moment for each group in comparison with the negative control group. Moreover, PPE combined (Mal and Atra) groups show DNA point of mutation in P53 exon 5, that was detected by the highly sensitive and accurate assay single-strand conformation polymorphism (SSCP), represented by an extra third band in comparison with the negative control group. This mutation was not detected by direct sequencing, means that it is a low-frequency mutation. In conclusion, our results report Al-Taif PPE as a genotoxic extract and mutagenic in combination with Mal and Atra pesticides. Moreover, the present results also confirm the sensitivity of SSCP technique in detection of point of mutation in comparison to direct sequencing.

Keywords: Malathion, Atrazine, Pomegranate peel extract, Mutagenic, Genotoxic.

INTRODUCTION

Malathion (Mal) and atrazine (Atra) are widely used pesticides, they are considered among the largest groundwater pollutants of the world [1,2]. Mal is a potential hazard to the environment. Adverse effects induced by Mal in mice, reptiles, birds, earthworms, and insects have been reported [3]. It has previously been reported that Mal has the potential to induce lipid peroxidation of cellular membranes through various biochemical processes [4]. During the metabolic process, Mal has shown the potential to cause DNA damage in exposed individuals. Both *in vivo* and *in vitro* studies have reported the genotoxic effects of Mal [5]. It has been reported that the metabolism of mal produces reactive oxygen species that lead to the onset of oxidative stress [6,7].

On the other hand, in laboratory studies, excessive exposure to Atra is detrimental to several organ systems, including the immune [8], reproductive [9], and nervous systems. It has been classified as a restricted use pesticide due to its potential for contamination. It is slightly to moderate toxic to human and other animals. Symptoms of Atra has been reported to cause cancer, reproductive abnormalities, poisoning include abdominal pain, diarrhea and vomiting, eye irritation, and skin reactions [10-12]. In addition, Meisner *et al.* [13] generated data from mammalian *in vivo* and *in vitro* test models show that higher doses (20 ppm) of Atra beyond the environmental concentrations can induce genotoxicity.

Nowadays, there is an increased demand for using plants or their extracts in therapy instead of using synthetic drugs, which might have adverse effects and consequently might be more dangerous than the disease itself [14]. *Punica granatum* L. (Punicaceae), commonly known as pomegranate, is a shrub or a small tree native to the Mediterranean region [15]. A number of biological activities such as antitumor, antibacteria and antidiarrhea have been reported for extracts from different parts of *P. granatum* [16-18]. Furthermore, antioxidant activity accompanied with radio protective and anti-fibrotic properties of *P. granatum* peel extract have been demonstrated recently [19,20].

Therefore, this study was undertaken to explore the genotoxic and mutagenic effect of Al-Taif Pomegranate (*P. granatum L*) extracts alone and combined with pesticides, Mal and Atra, on male albino mice.

METHODS

Animals

This study was performed on 70 mature male mice, weighing about 35-45 g bw. Animals were obtained from the animal house of the King Fahad Center for Medical Research, King Abdul-Aziz University in Jeddah. They were breeding in a well-ventilated room with the temperature ranging between 22°C and 25°C and maintained under standardized conditions away from any stressful conditions with 12/12 light and dark cycle with free access to humidity and were fed dry balanced meal for experimental animals provided by the General Organization for Grain Silos and Flour Mills in Jeddah, with a constant source of water. All experimental procedures and animal maintenance were conducted in accordance with the accepted standards of animal care per cage (Council of Europe, European Convention for the Protection of Vertebrate Animals 2006). We have followed the European Community Directive (86/609/EEC) and National Rules on Animal Care.

Chemicals

Mal and Atra were produced by Misr for Agricultural Development Company, Cairo, Egypt. Atra in the commercial product Cotrazine 80 WP (an 80% wettable powder) was obtained from Alderelm limited UK. Pomegranate was supplied by local market in Al-Taif city, Saudi Arabia, the peel of pomegranate was washed and then dried and then was grinded by using electrical mixer and then was prepared for intra-peritoneal injection for male mice, also we used the juice of the pomegranate fruit.

Experimental protocols

Mice were divided into ten groups, 7 mice/group. Group 1: Negative control group treated with 1 mg/kg bw corn oil/day; Group 2: Mal group (27 mg/kg bw [1/50] of the LD50 for an oral dose) per day in corn oil

via gavage [21]; Group 3: Atra group treated daily with 0.24 ml vehicle suspension of 80% (w/w) Atra equivalent to 120 mg/kg body weight mg/kg bw per day in corn oil gavage [22]; Group 4: Pomegranate peel extract (PPE) group treated with PPE (0.4 g/kg bw/day in corn oil); Group 5: Mal + PPE co-administration group (27 mg/kg bw + 0.4 g/kg bw/day, respectively); Group 6: Mal + pomegranate juice (PJ) co-administration group (27 mg/kg bw/day + 0.4 g/kg bw/day, respectively); Group7: Atra + PPE co-administration group (120 mg/kg bw + 0.4 g/kg bw/day, respectively); Group8: Atra + PJ co-administration group (120 mg/kg bw/day + 0.4 g/kg bw/day, respectively); Group 9: Mal + PPE + PJ and group 10: Atra + PPE + PJ combined co-administration groups treated in the same doses as previously discussed. We did not use a PJ alone for injection because it was known as a safe part of pomegranate fruit.

The doses of either PPE and/or PJ were chosen on the basis of previous studies [23]. The substances were administered in the morning (between 07.00 and 8.30 am) to non-fasted mice. Mice were injected orally with Atra and/or Mal, followed by PPE and/or PJ after 30 minutes daily for successive 30 days as the treatment schedule that previously mentioned. All animals were sacrificed and dissected. The liver tissues were excised and frozen for later molecular evaluation represented by Single-strand conformation polymorphism (SSCP) and Comet assays.

Molecular evaluation

DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from the liver with the Genomic DNA Purification kit (Fermentas) according to the manufacturer's instructions. The quantity of DNA was estimated by absorbance reading at 260 nm and DNA shows more purity was estimated by the ratio of absorbance reading between 260 nm and 280 nm. The resultant extracted DNA was run in a 1.5% agarose gel containing ethidium bromide. The gel was examined and photographed under ultraviolet (UV) light.

For polymerase chain reaction (PCR), P1 forward 5'-tctcttccagtactctcctc-3' and reverse 5'-aggcggtgttgagggcttac-3' were used to amplify P53 exon5 fragment (214 bp) of mouse [24]. The PCR reaction mixture (20 µl) contained: 7 µl sterile water, 1 µl (100 ng/1 µl) extracted DNA, 1 µl forward primer (20 pmol), 1 µl reverse primer (20 pmol) and then 10 µl ×2 master mix (Fermentas, USA), added in a 0.2 ml PCR Eppendorf tube. Cycling was started in the Thermal Cycler (Programmable Thermal Cycler, PTC-100TM thermal cycler, Model 96; MJ Research, Inc., Watertown, MA, USA), with initial denaturation at 94°C for 5 minutes, DNA double-strand denaturation at 94°C for 30 seconds, primer annealing at 58°C for 1 minutes, and primer extension at 72°C for 1 minute, for 30 cycles. Final extension at 72°C for 10 minutes was necessary for complete amplification. PCR products were separated and visualized by electrophoresis on a 1.5% ethidium bromide-treated agarose gel (Sigma, UK) according to the standard protocol described by Sambrook *et al*. [25].

SSCP analysis and sequencing

PCR products were denatured with TE buffer ([26]; diluted 1:10, pH 8.0). 5 µl of each diluted solution was mixed with 5 µl of denaturing-loading dye (95% formamide, 4 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol FF and 0.5 µl 15% ficoll) and the mixture was heated to 94°C for 5 minutes, then chilled on ice for 10 minutes [27]. The denaturated PCR samples were subjected to 9% polyacrylamide gel electrophoresis (acrylamide/bisacrylamide = 49:1, v/v). The gel was stained by shaking for 10 minutes in 100 ml of ×1 TBE with 10 µl ethidium bromide (10 mg/ml) to visualize the DNA bands. The gel was placed on a UV trans-illuminator (Stratagene, USA) and pictures were taken with a Polaroid camera (Polaroid MP4 Land Camera).

Bands that abnormally shifted in the SSCP gel compared with their corresponding normal control were considered to harbor somatic mutations. The PCR products that showed mutation using SSCP were sequenced for detection of point mutation.

Amplification products were purified using the QIA quick PCR purification kit (Qiagen, GmbH, Germany). Cycle sequencing of both strands was performed using the BigDye Terminator Kit version 3.1 (Applied Biosystems, Foster City, CA) on an ABI Prism 3730 Genetic Analyzer automated sequencer. Primers for sequencing are described by Morimura *et al*. [24]. Sequence data was analyzed using the Sequencher 4.1 software package (Gene Codes, MI). If the DNA sequence at a particular location in the DNA differed from the corresponding normal DNA, then it was defined as a somatic mutation.

Comet assay

The alkaline comet assay was performed as described by Singh *et al.* [28]. Conventional frosted microscopic slides were dipped into hot 1.0% normal melting point agarose to one-half of the frosted area and the underside of the slide wiped to remove agarose. A 65 µl of 0.5% low melting point agarose at 37°C was mixed with 10 µl of homogenized liver tissue in cold Hank`s Balanced Salt Solutions, and a coverslip was applied to spread the samples. The slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10mMTris, NaOH to pH 10.0, 1% Triton-100 and 10% dimethyl sulfoxide) at 4°C for 2 hrs to remove cell protein. They were then soaked in a coupling jar containing electrophoresis solution to unwind for 20 minutes and electrophoresed at a constant current of 300 mA, for 35 minutes. After electrophoresis, the slides were neutralized with Tris-HCl buffer at pH 7.5 by three washes for 5 minutes, followed with cold ethanol for 5-10 minutes and left to dry overnight. The slides were stained by placing 80 µl ethidium bromide (20 µg/mL) on each slide and covered with a coverslip for 20 minutes. Then slides were viewed under an epifluorescence microscope (Zeiss epifluoresent) with an attached CCD camera and computer. Images were saved as electronic files and for each sample, 50 isolated comets were randomly selected and measured for comet tail length, %DNA in tail and tail moment using COMETSCORE software based on the definition by Olive and Banáth [29].

Statistical data analysis

Data were expressed as the mean ± standard error (SE). Statistical significances of differences between two groups were determined using Student's t-test. The difference between means at the level of $p<0.05$ was considered as significance. Statistics were carried out using statistical analysis systems program.

RESULTS

Extracted DNA was sufficiently qualified for further PCR amplification (data not included). Fig. 1 represents successful PCR product for negative control and different treated groups at expected molecular weight 214 bp. Primers were specified for P53 exon 5 of the mouse.

Fig. 2 represents PCR-SSCP for negative control and different treated groups. In which, PPE combined groups of Mal + PPE and Atr + PPE + PJ show somatic mutation represented by an extra band in comparison

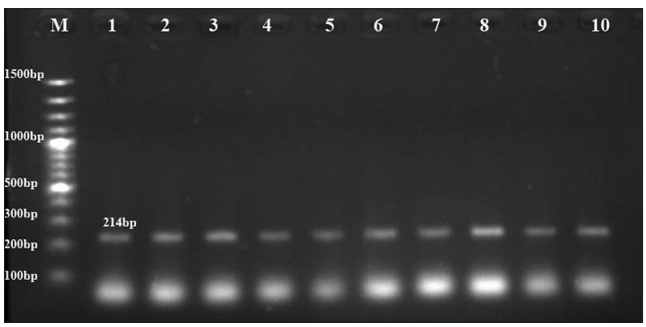

Fig. 1: A 1.5% agarose gel separating polymerase chain reaction (PCR) product for P53 exon 5 (214bp). In which, Lane M, low molecular weight DNA marker (100-1500bp); lane 1, PCR product for negative control group; lanes 2-10, PCR products for different treated groups

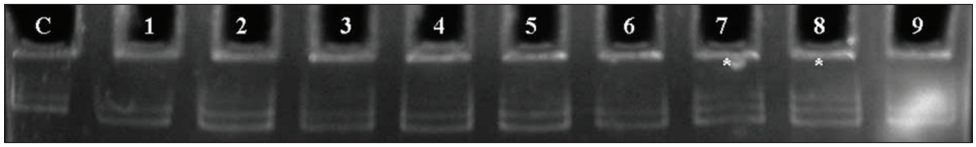

Fig. 2: A 9% polyacrylamide gel showing polymerase chain reaction - single-strand conformation polymorphism for P53 exon 5. Lanes C represents negative control group; lane 1, Atrazine (Atra) group; lane 2, Malathion (Mal) group; lane 3, pomegranate peel extract (PPE) group; lane 4, Atr + PPE group; lane 5, Atr + pomegranate juice (PJ) group; lane 6, Mal + PJ group; lane 7, Mal + PPE group; lane 8, Atr + PPE + PJ group and lane 9, Mal + PPE + PJ group. In which lanes 7 and 8 show extra band in comparison with the negative control group

with the negative control group. While, there is not any difference in the PCR-SSCP pattern of the other treated groups relative to the negative control group. Mutated PCR samples were subjected to sequencing to detect the point of mutation. Direct sequencing didn't show any change in DNA sequence for mutated samples in comparison with the negative control group using forward (Fig. 3) and reverse P53 exon 5 primers.

The genotoxic effect for different treated groups was evaluated by using comet assay. Fig. 4a shows a typical nuclei of undamaged cells for the negative control group; while Fig. 4b is a representative photomicrograph for the various degree of DNA damage observed as comets that were seen in all different treated groups. Fifty isolated comets were randomly selected for all groups and measured for comet tail length, %DNA in tail and tail moment using COMETSCORE software. The selected data (mean ± SE) for all treated groups were compared using Student's t-test (significant difference $p<0.05$). The results show a significant increase in tail length, % DNA of tail and tail moment for all treated groups in comparison with the negative control group, except for PPE group at tail length (Fig. 5).

In order to report the effect of PPE or/and PJ on Atra treatment, we compared the combined Atra groups with Atra group. Fig. 5 shows a non-significance decrease in all parameters of DNA damage for Atra + PJ, Atra + PPE and Atra + PPE + PJ combined groups in comparison to Atra group, except at tail length of Atra + PPE group, it shows a non-significance increase.

Moreover, Fig. 5 reports the effect of PPE or/and PJ on Maltreatment. It shows a decrease in %DNA in tail and tail moment for all combined Mal groups in comparison to Mal group, except for Mal + PJ group. This decrease is significant in %DNA in the tail for combined Mal + PPE and Mal + PPE + PJ groups. In addition, there is a non-significance increase in tail length of all combined Mal groups in comparison to Mal group.

Significant difference ($p<0.05$) using Student's t-test, in which: *Statistically compared all different groups with the negative control group and $ Statistically compared combined Mal groups with Mal group.

DISCUSSION

The PCR-SSCP profile of PPE, Mal and Atra single treated groups did not show any change of band patterns indicating their antimutagenic effect. In addition, Atra/Mal + PJ, Atr + PPE and Mal + PPE + PJ combined groups have the same band pattern as the negative control group. In contrast, Mal + PPE and Atr + PPE + PJ combined groups show a change in bands pattern from the negative control group represented by the third extra band as shown in Fig. 2. Our results were in agreement with Zahin *et al*. [30], they reported the antimutagenic activity of PPE *via* ames Salmonella/microsome assay against sodium azide (NaN_3), methyl methane sulfonate, 2-aminofluorene and benzo(a)pyrene-induced mutagenicity in *Salmonella typhimurium* tester strains. While there was a contradictory in the mutagenic effect of Atra [31,32].

However, PPE combined groups of Mal + PPE and Atr + PPE + PJ show somatic mutation, represented by an extra third band. While direct sequencing did not show the point of mutation. Our results were in agreement with other previous studies, in which SSCP is more sensitive

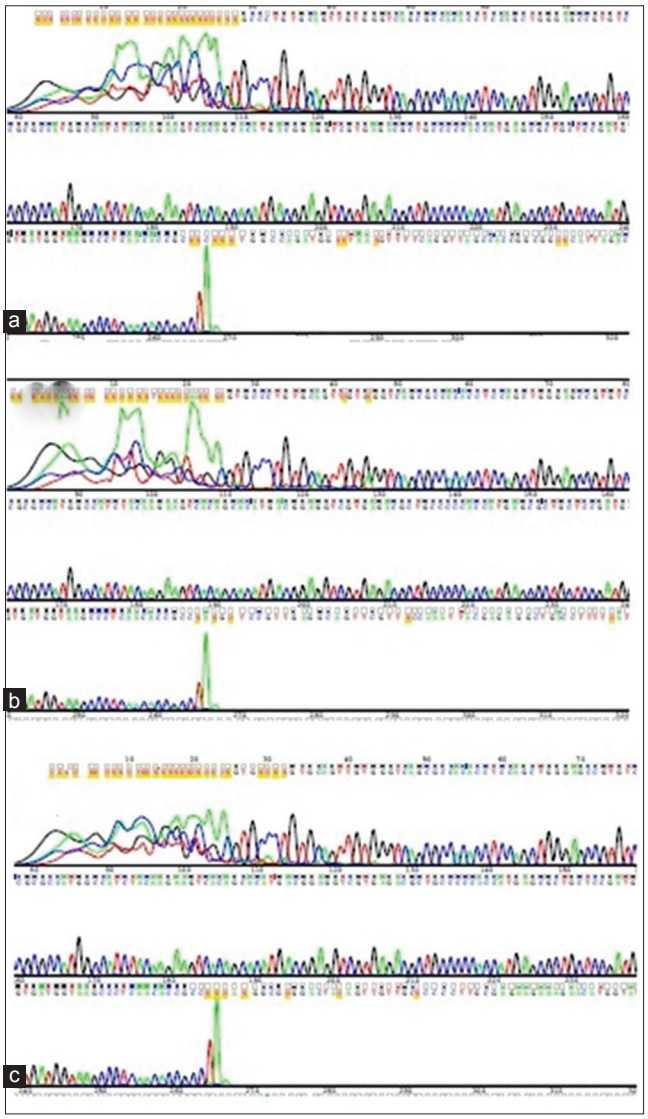

Fig. 3: DNA sequence chromatogram of (a) negative control, (b) Malathion + pomegranate peel extract (PPE) and (c) Atrazine + PPE + pomegranate juice groups, using P53 exon 5 forward primer. In which treated groups have the same sequence of the negative control group

than direct sequencing, allowing the detection of as little as 5-10% mutant alleles in a DNA sample, while sequencing requires at least 25-30% of mutated DNA [33,34]. These results indicate the mutagenic effect of the combined PPE with the pesticides Mal and Atra but in low frequency that is detected by SSCP and failed to be detected by direct sequencing.

The present results show the genotoxic effect of the pesticides Mal and Atra represented by the comet assay. Maltreatment shows a highly

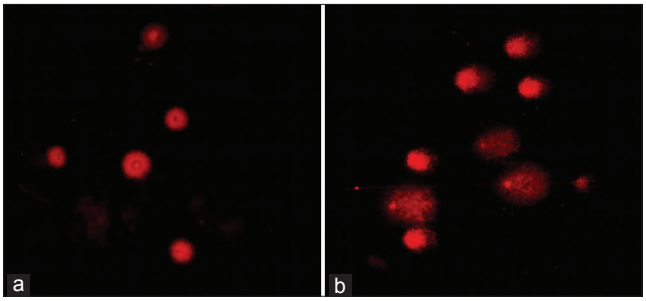

Fig. 4: Representative photomicrograph showing (a): Typical nuclei of undamaged cells of negative control group and (b): Various degree of DNA damage observed as comets that were seen in all different treated groups

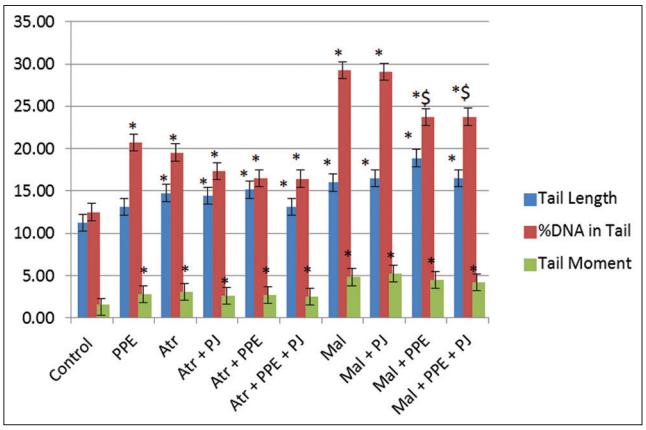

Fig. 5: Effect of pomegranate peel extract and pomegranate juice on the DNA damage (represented by Comet assay) induced by Atrazine and Malathion pesticides treatment in mice liver cells

significant increase in tail length, %DNA in tail and tail moment in comparison with the negative control group, which indicates the higher genotoxic potential of Mal. Our results are in agreement with Moore *et al.* [35], they reported Mal genotoxic potential in bone marrow cells and peripheral blood obtained from Sprague-Dawley rats; represented by significant increase in the number of structural chromosomal aberrations, decrease in mitotic index, and increase in percentage of DNA damage (using alkaline single-cell gel electrophoresis (comet assay)) as toxicological endpoints in comparison with the negative control group. Similar results have been observed in the peripheral blood and hippocampus following both chronic and acute Mal treatments [36], human liver carcinoma cells [37], as well as in lymphocytes, gill cells, liver and brain cells due to organophosphate exposure [38,39]. Our previous study reported that Mal was responsible for the onset of oxidative stress that might lead to the alterations in DNA molecules of treated mice [40].

Treatment with Atra (120 mg/kg) leads to increase in DNA damage, represented by a significant increase in tail length, %DNA in tail and tail moment in comparison to the negative control group. Our results are in agreement with Nwani *et al.* [41], they reported the genotoxic and mutagenic effect of Atra-based herbicide in erythrocyte and gill tissues of *Channa punctatus* fish using micronucleus (MN) and Comet assays. They used three different sub-lethal concentrations of Atra according to LC50 and samples collected at different sampling time. They reported significant induction ($p<0.01$) of MN and increase in DNA damage measured as % tail DNA in fish specimens tissues at different concentrations and durations due to Atra exposure compared to the control group. Induction of MN and DNA damage in both tissues was found to be dose and time dependent. Moreover, Sharma and Vig [42] reported the gentotoxic potential of Atra due to induction of different chromosomal aberrations.

It was noticed that Mal showed more genotoxic potential than Atra. Our results were in agreement with Foster *et al*. [43], they reported that insecticides chlorpyrifos and Mal were the most toxic pesticides investigated over other several pesticides including Atra using *Ceriodaphnia dubia* as a test organism.

In this study, we used the peel extract to evaluate its genotoxic and mutagenic effect alone and combined with pesticides. While we did not use pomegranate juice alone because it must be the safe part of the whole fruit although it is the edible part. The present study reports the genotoxic effect of PPE represented by a significant increase in %DNA in tail and tail moment in comparison to the negative control group. Our results were in agreements with Sanchez-Lamar *et al.* [44], they reported a significant genotoxic activity of the whole fruit *P. granatum* including the peel represented by MN and head sperm malformation *in vivo* assays. They explained that the genotoxic effects induced by the extracts could be detected both in the presence and in the absence of metabolic activation and at different DNA damage expression levels, suggesting that the plant mixture contains one or more directly acting genotoxic components.

As shown in Fig. 5, the juice extract of pomegranate shows slight non-significant decrease in the genotoxic effect occurred as a result of Atra treatment; while it failed to decrease the genotoxic effect induced by Mal. This was in agreement with Farkas *et al*. [45], they reported that pomegranate juice does not alter the clearance of oral or intravenous Midazolam drug "a probe for cytochrome p450-3a (CYP3A) activity" in humans. On the other hand, treatment with PPE for Atra/Mal groups showed a non-significant decrease in genotoxic parameters in comparison to Atra/Maltreated groups, respectively. Except for Mal + PPE group; it shows a significant decrease in % of DNA in the tail in comparison to Mal group. These findings were in agreement with Abdel Motaal and Shaker [46], they reported that the peel extract of pomegranate showed the highest antioxidant activity compared to whole fruit and pulp extracts, therefore they used the standardized peel extract to be formulated into capsules and they reported the possible use of pomegranate peels, a biological waste product, as a natural pharmaceutical preparations. Therefore the antioxidant potential of PPE decreased the genotoxic parameters induced by Atra and/or Mal as a result of their oxidative stress induction.

The combined group Mal/Atra + PPE + PJ groups showed a non-significant decrease in the genotoxic parameters in comparison to Mal/Atra treated groups, except for % of DNA in tail in Mal combined group, as shown in Fig. 5. These results seem to be as Mal/Atra + PPE treated groups, reporting that the decrease of genotoxic parameters is returned to PPE treatment and not PJ. This was agreed according to previous studies, in which Singh *et al*. [47] reported that the methanol extract of peels (50 ppm) showed a higher antioxidant activity 83 and 81% than that of seeds (100 ppm) 22.6 and 23.2% at using the beta-carotene-linoleate and DPPH model systems, respectively.

CONCLUSION

The present study is a supplemental study to our previous work. In which, we report previously the antioxidant, hepatoprotective and nephroprotective potential of of Al-Taif Pomegranate (*P. granatum L*) extract against toxicity induced by Atra and Mal pesticides in male albino mice. However, hereby we report the genotoxic potential of Al-Taif Pomegranate (*P. granatum L*) peel extract PPE alone and its failure to significantly decrease the genotoxic effect of the pesticides Mal and Atra. Moreover, when PPE combined with Mal and Atra, it shows a low-frequency DNA mutation, that is detected by the highly sensitive and accurate assay, SSCP. We recommend for further study to detect the hotspot point of mutation.

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