

Cytogenotoxic and forced degradation studies of pendimethalin using root growth, comet assay and LC-MS/MS

Cytogenotoxic and forced degradation studies of pendimethalin

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Abstract

Aim: This study aimed to assess the effects of pendimethalin herbicide, which has been used for controlling broadleaf weeds and annual grasses for approximately 30 years, on growth and DNA damage in *Allium cepa* root tips using root growth and the comet assays. Total pendimethalin in *A. cepa* root tips and the stability of pendimethalin under stress conditions were also determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods: The effective concentration (EC_{50}) value of pendimethalin (50% reduction in root length compared to the negative control group) was found to be approximately 10 mg/L by the *Allium* root growth inhibition test. The roots were exposed to three different concentrations of pendimethalin (5, 10 and 20 mg/L), distilled water, and 10 mg/L methyl methanesulfonate (MMS) to determine DNA damage by the comet assay and the amount of pendimethalin by LC-MS/MS for different treatment periods (24, 48, 72 and 96 h). The stability profile of pendimethalin under various stress conditions of UV light, acid and base hydrolysis, oxidation, and thermal conditions was also assessed via LC-MS/MS.

Results: All tested concentrations of pendimethalin statistically increased DNA damage and the amount of pendimethalin in *A. cepa* roots compared to the control group, not only dose-dependently but also time-dependently. Pendimethalin was extremely degraded under thermal, oxidative and basic conditions, and relatively degraded under acidic and UV light conditions.

Discussion: Pendimethalin should be stored and used carefully. Further studies are needed to identify the cytogenotoxic mechanism of pendimethalin at molecular levels and under different forced conditions.

Keywords

Pendimethalin; DNA damage; LC-MS/MS; Forced conditions; *Allium cepa*

DOI: 10.4328/ACAM.20343 Received: 2020-09-11 Accepted: 2020-10-12 Published Online: 2020-11-02 Printed: 2021-05-15 Ann Clin Anal Med 2021;12(Suppl 1): S96-100

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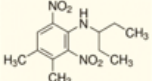
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Introduction

Amid® 330 EC is a liquid emulsified dinitroaniline-type herbicide, whose active ingredient is pendimethalin (330 g/L). Pendimethalin [N-(1-ethylpropyl)-2,6-dinitro-3,4-xylylidine] is a pre-emergence herbicide of dinitroaniline used in large field crops including cotton, rice, onion, garlic, soybean, cabbage, peanut and tobacco etc. to control broadleaf weeds and annual grasses [1]. Pendimethalin has been available on the market for about 30 years and ranked third in all of the herbicides behind glyphosate and parquat. Pendimethalin halts cell division and cell elongation in plant cells by inhibiting the microtubule assembly, like other dinitroaniline herbicides, hence preventing the growth of roots and shoots of weeds [2]. It was classified as a persistent bioaccumulative toxic herbicide and a Group C carcinogen (possible human carcinogen) that contaminates the soil and aquatic environments as a consequence of drainage and leaching [3,4]. Herbicides provide effective weed control in agriculture, yet their continuous and excessive use may cause toxicological problems in non-target organisms. Some of the properties of pendimethalin are given in Table 1.

Table 1. Some of the properties of pendimethalin

Chemical name	IUPAC Name	Chemical structure	Molecular Weight	Water solubility (mg/L)
Pendimethalin	3,4-Dimethyl-2,6-dinitro-N-pentan-3-yl-aniline		281.312 g/mol	0.275

A. cepa root tips have been used for determining the DNA damage of herbicides by the comet assay (single-cell gel electrophoresis) because onions are easy to obtain and store. Root tips can be used independently of mitosis, and few cells are needed for the examination. In addition to these, the comet assay is a relatively inexpensive, fast, reliable, and highly reproducible method [5-7].

LC-MS/MS is extensively used for the qualitative and quantitative analysis of herbicides in environmental samples because it is a fast, simple, effective, and safe method [8].

Stress testing offers detailed information on the pathways for degradation and potential degradation products. The findings of stress tests can then be used to improve production strategies or to choose suitable packaging. Tests carried out under forced conditions show under which conditions a pesticide remains stable and under which conditions it degrades [9]. No previous method was reported regarding the degradation behavior of pendimethalin.

This research aimed to assess the effects of pendimethalin on growth and DNA damage of *A. cepa* root tips using root growth and the comet assays. LC-MS/MS analysis was also performed for the estimation of total pendimethalin in *A. cepa* root tips and the stability of pendimethalin under stress conditions.

Material and Methods

Materials

Equal-sized (25–30 mm diameter) and healthy *A. cepa* bulbs and Amid® 330 EC (330 g/L pendimethalin) were obtained from the local market in Uşak and Astranova (Turkey), respectively. MMS,

sodium chloride, ethidium bromide (EtBr), potassium phosphate monobasic, glacial acetic acid, low melting point agarose (LMPA), sodium hydroxide, hydrochloric acid, disodium hydrogen phosphate, Triton X-100, potassium chloride, methanol, formic acid, magnesium chloride hexahydrate, normal melting point agarose (NMPA), trizma hydrochloride, trizma base and EDTA were bought from Sigma Aldrich (Munich, Germany).

Allium root growth inhibition test

The Allium root growth inhibition test was carried out as described by Küçük and Liman [6] with minor modifications. The outer dry brown layers and roots of the onions were eliminated without destroying the root primordia. Onions bulbs were treated with pendimethalin at increasing concentrations (0.5, 1, 2.5, 5, 10, 25, 50 and 100 mg/L) and distilled water (negative control) in dark at room temperature for 96 hours. Solutions were renewed every 24 hours. The average length of 50 roots from 5 onions per each application was calculated after 96 hours to determine EC₅₀.

Comet assay

Onions were germinated in distilled water for 48 hours until their root lengths reached 2-3 cm. Distilled water (negative control), 5 (½xEC₅₀), 10 (EC₅₀) and 20 (2xEC₅₀) mg/L of pendimethalin, and 10 mg/L of MMS (positive control) were exposed to Allium roots for 24-96 hours in dark at room temperature. The effect of pendimethalin on DNA damage was studied with minor modifications, as defined by Küçük and Liman [6]. Nuclei were isolated by gently slicing 7 root tips (~1 cm) with a new razor blade in pre-cooled Tris-MgCl₂ buffer (0.5% w/v Triton X-100, 0.2 M Tris, 4 mM MgCl₂·6H₂O, pH 7.5) into a Petri dish kept over an ice base. After filtration, the samples were centrifuged for 7 min at 1200 rpm. Pelleted cells were resuspended for comet analysis, while the supernatant was used with LC-MS / MS for quantitative experiments. Then a (50:50 µL) mixture of 1.5 % LMPA and nuclear suspension was smeared on the pre-coated slides with 1% NMPA at 40 °C with a cover slip. After the samples were left in the ice cassette for 5 min, the coverslips were carefully removed. The slides were incubated at 4 °C for 20 min in a cold electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH>13), and then electrophoresed under the same conditions at 25 V (0.8V/cm). The slides were neutralized three times with 0.4 M Tris (pH 7.5) and stained with 70 µL EtBr (20 µg/mL) for 5 min. Randomly 50 comets per each slide were analyzed for determining DNA damage expressed as arbitrary unit (AU) into five categories (0-4) using a TAM-F fluorescence microscope (BAB, Ankara, Turkey) according to Özkan and Liman [7]. Three slides were evaluated for each application.

Determination of pendimethalin in A. cepa root meristem cells

The supernatants (described above) were used for quantitative analysis of pendimethalin in *A. cepa* root meristem cells by LC-MS/MS. Agilent 6460 triple quad mass spectrometer was used for the LC-MS/MS analysis. This instrument was equipped with electrospray ionization, operating in positive mode and configuration scan mode monitoring, and multi reaction mode. First, optimized chromatographic conditions (flow rate, column temperature, and mobile phase composition) were found using Zorbax Eclipse Plus C18 (2.1×5 mm, 1.8 µ) column 1 mL/min, 40 °C and methanol-H₂O with 0.1% formic acid (75-25, V/V), respectively. Then mass spectrometric detections were

optimized (Table 2). Pendimethalin molecular weight was 282 g/mol. Each application was repeated three times. The constructed calibration curve was found to be linear over the concentration range of pendimethalin (0.00125-0.1 mg/L). The linearity of the calibration curve was confirmed by the high value of the correlation coefficient for pendimethalin ($r^2=0.9998$). The limit of detection, the limit of quantitation, and relative standard deviation % values were calculated as 0.05595, 0.0965, and 1.6375 $\mu\text{g/L}$, respectively.

Table 2. LC-MS/MS conditions of pendimethalin

Instrument Parameters	Conditions	Product Parameters	Values
Gas Temperature	260 °C	[M+H]	282 g/mol
Gas Flow	11 L/min	Daughter Ions	282-211,9 g/mol 282-193,8 g/mol
Nebulizer	45 psi	Polarity	Positive
Sheat Gas Heater	400 °C	Fragmentor Voltage	110 eV
Sheat Gas Flow	12 L/min	Collision Energy	5 V
Capillary	3500 V	Retention Time	1,249 min

Forced Degradation Studies

The experimental conditions were analyzed according to the guidelines for stability testing of new drug substances and products of the International Council for Harmonization of Technical Criteria for Pharmaceuticals for Human Use with minor modifications. Forced degradation studies were performed under stress conditions of UV light, thermal, oxidation, acid and base hydrolysis to evaluate the stability of pendimethalin using the optimized method as described above for LC-MS/MS. The pendimethalin stock solution (100 mg/L, 1 mL) was transferred to a 10 mL flask and 9 mL of 0.1 and 1 M HCl or 0.1 and 1 M NaOH or 3% and 30% of H_2O_2 (V/V) was added for 30 min at 80 °C. Pendimethalin stock solution was diluted to 10 mg/L with distilled water for the thermal and UV studies. The diluted solution was exposed to UV light (254 nm) in a UV cabinet at room temperature for 6 and 24 hours and heat for 6 and 24 hours in an incubator (100 °C) for UV and thermal degradations, respectively. Each application was repeated three times.

Statistical analysis

The results (mean \pm standard deviation) were analyzed with One-Way Analysis of Variance (ANOVA) and Duncan multiple range tests at $p \leq 0.05$ using IBM SPSS Statistics for Windows (version 23). Dose-response and dose-time relationships of pendimethalin were also determined with Pearson correlation analysis at $p=0.01$ significance level.

Results

Pendimethalin decreased root growth within the range from 22.1% (0.5 mg/L) to 91.2% (100 mg/L) dose-dependently ($r=-0.965$) compared to the control group. The EC_{50} of pendimethalin was found to be approximately 10 mg/L (49.57 %) by Allium root growth inhibition test (Figure 1).

The effect of pendimethalin on DNA damage using the comet assay on *A. cepa* roots is shown in Figure 2. All concentrations of pendimethalin statistically increased DNA damage between

68.67 \pm 5.03 and 136.33 \pm 2.52 Arbitrary Unit compared to the control group, not only dose-dependent (for 24 hours $r=0.984$, for 48 hours $r=0.979$, for 72 hours $r=0.969$, and for 96 hours $r=0.924$) but also time-dependent (for 5 mg/L $r=0.986$, for 10 mg/L $r=0.964$, and for 20 mg/L $r=0.937$). LC-MS/MS, which enables analysis in $\mu\text{g/L}$ degree is samples, is suitable for both trace residue analysis in the samples and the analysis of the products obtained as a result of the degradation study. Figure 3a shows quantitative analysis of pendimethalin in *A. cepa* roots by LC-MS/MS. The amount of pendimethalin increased from 0.13 \pm 0.04 to 4.9 \pm 0.42 $\mu\text{g/L}$ dose (for 24 hours, $r=0.926$, for 48 hours, $r=0.916$, for 72 hours, $r=0.804$, and for 96 hours, $r=0.949$) and time (for 5 mg/L $r=0.895$, for 10 mg/L $r=0.884$, and for 20 mg/L $r=0.838$) dependently.

The stability of pendimethalin under stress conditions (UV light, acidic and base hydrolysis, oxidation and thermal) was investigated by LC-MS/MS. Pendimethalin was chemically unstable under all stress conditions tested (Figure 3b). The extent of degradation strictly depended on the different experimental conditions. The percentage of pendimethalin degradation for UV light reached 35.98% and 17.37% for 24 and 6 hours, respectively. When treated thermally at 100 °C in an incubator, pendimethalin was most unstable during 6 and 24 hours, recording 67.31% and 70.3% degradation percentages, respectively. After 30 min exposure to H_2O_2 , pendimethalin showed 43.61 and 46.33% degradation for 3% and 30% H_2O_2 . Under basic stress conditions, degradation occurs more rapidly than under acidic stress conditions.

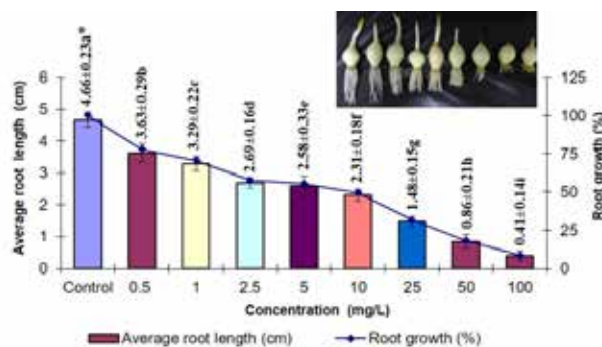


Figure 1. Average root length and root growth (%) of *A. cepa* exposed to pendimethalin after 96 h. * Different letters are significantly different at $p \leq 0.05$.

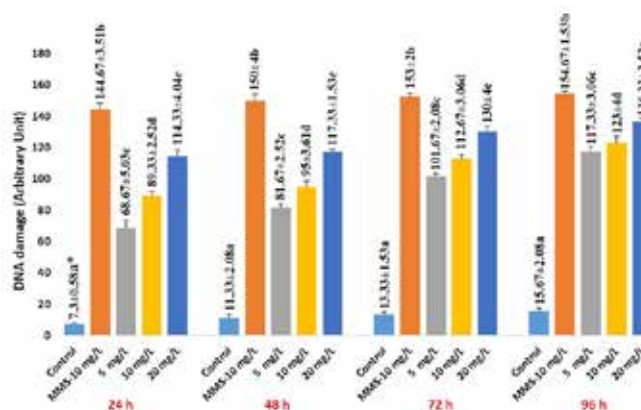


Figure 2. Pendimethalin induced DNA damage in *Allium* roots. * Different letters for each treatment time are significantly different at $p \leq 0.05$.

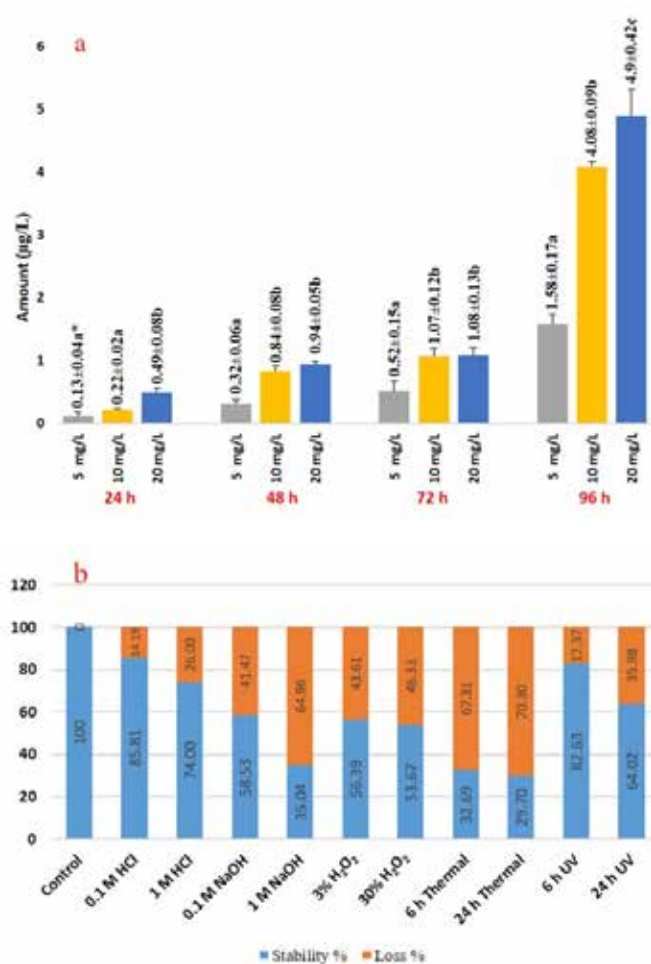


Figure 3(a). Quantitative analysis of pendimethalin in *A. cepa* root meristem cells by LC-MS/MS. **(b)** The stability of pendimethalin under stress conditions. * Different letters for each treatment time are significantly different at $p \leq 0.05$.

Discussion

The percentage of the recommended dose (7.5 mL/L) of pendimethalin at EC_{50} was found 0.16% for *A. cepa* after 96 hours [10]. Inhibition of root length was also observed on *A. cepa* treated with pendimethalin at 0.033, 0.044, 0.055 and 0.066 g/kg soil for 7 to 45 days [11] and at 0.033, 0.066, 0.099, 0.132 and 0.264 g/L for 48 hours [12] compared to control group. Reduction of root growth was also observed in *Tradition azalea* exposed to pendimethalin for 4 and 8 weeks [13]. Yield in *Oryza sativa* seedlings was decreased with pendimethalin rate increased from 800 to 1600 g ai ha⁻¹ [14].

Similar to our results, pendimethalin induced DNA damage observed by the comet assay on Chinese hamster ovary cells [15], on *Channa punctatus* blood, liver, and gill cells [16], on Chinese hamster lung fibroblast cells and on human peripheral lymphocytes [17], on *Oreochromis niloticus* liver and gills tissues [18], on human lymphocytes, rat bone-marrow and rat peripheral blood mononuclear cells [19] and on *Biomphalaria alexandrina* snails for 48 hours at a concentration of 1.299 mg/L [20]. The DNA damage could be attributed to oxidative stress generated by pendimethalin exposure via the generation of reactive oxygen species leading to inhibition of antioxidant enzymes, as well as causing lipid peroxidation and carbonylation of proteins,

etc. [21,22]. Pendimethalin was also observed in honeybees at 75 µg/L [23] and in eggs at 88.1 µg/L with LC-MS/MS [24]. The results of the present study confirmed cytogenotoxic effects of pendimethalin by reducing root growth and DNA damage in *A. cepa* roots. The amount of pendimethalin in *A. cepa* increased dose and time dependently. Forced degradation studies of pendimethalin have not previously been described. Pendimethalin was extremely degraded under thermal, oxidative and basic conditions, and relatively degraded under acidic and UV light conditions. Therefore, forced conditions should be taken into consideration during the use and storage of pendimethalin. In addition, more research should be performed to establish pendimethalin's cytogenotoxic function at molecular levels and different forced conditions.

Acknowledgment

The authors thank Usak University, Scientific Analysis Technological Application and Research Center (UBATAM) for LC-MS/MS analysis.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: This work was partially funded by the Scientific and Technological Research Council of Turkey (TUBITAK) 2209-A-Research Project Support Programme for Undergraduate Students (1919B01100255).

Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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How to cite this article:

Büşra Taşdemir, Recep Liman, Süleyman Gökçe, Eslem Amaç, İbrahim Hakkı Cığerci, Safiye Elif Korcan. Cytogenotoxic and forced degradation studies of pendimethalin using root growth, comet assay and LC-MS/MS. *Ann Clin Anal Med* 2021;12(Suppl 1): 596-100