**ORIGINAL ARTICLE**

# TOXICITY INVESTIGATION OF AN EXTRACT OF *AMARANTHUS RETROFLEXUS* L. (*AMARANTHACEAE*) LEAVES

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**Abstract**

The dry hydro-ethanolic extract obtained from leaves of *Amaranthus retroflexus* L. (*Amaranthaceae*) with an yield of 22.53% was assessed with respect to its acute toxicity in NMRI mice, cytotoxicity on the crustacean *Daphnia magna* and phytotoxicity on *Triticum aestivum* L. The lack of lethality and of any change in the indicators followed in mice (2000 mg/kg b.w. po) was demonstrated, the extract being classified into the 5th category of the Global Harmonized System (GHS). The *Daphnia magna* test results indicated a low toxicity (LC50 of 1053.0 µg/mL), in agreement with the results above, and the cytotoxicity and genotoxicity on plant cell, investigated by the *Triticum* bioassay manifest at high concentrations only (1.00% - 0.100%), for which changes in the mitotic film are also observed: mitoinhibition, anaphases with retarded chromosomes, metaphases with "sticky" chromosomes.

**Rezumat**

Extractul hidroetanolic, uscat, obţinut din frunze de *Amaranthus retroflexus* L. (*Amaranthaceae*) cu un randament de 22,53%, a fost supus evaluării privind toxicitatea acută la şoareci NMRI, citotoxicitatea la crustaceul *Daphnia magna* şi fitotoxicitatea la *Triticum aestivum* L. S-a demonstrat lipsa letalităţii şi a vreunei modificări a indicatorilor urmăriţi la şoareci (doza 2000 mg/kg corp po), extractul încadrându-se în categoria a 5-a a Sistemului Armonizat Global (GHS). Rezultatele testului *Daphnia magna* au indicat o toxicitate scăzută (LC50 de 1053,0 µg/mL), în acord cu rezultatul anterior, iar citotoxicitatea şi genotoxicitatea asupra celulei vegetale, investigate prin testul *Triticum*, se manifestă doar la concentraţii mari (1,00% - 0,100%), la care se observă şi modificări ale filmului mitotic: mitoinhibiţie, anafaze cu cromozomi întârziaţi, metafaze cu cromozomi „lipicioşi".

**Keywords**: *Amaranthus*, citotoxicity, genotoxicity, phytotoxicity, *Daphnia magna*, *Triticum* test

## Introduction

*Amaranthus retroflexus* L. *Amaranthaceae*, known by vernacular names such as common amaranth, pigweed amaranth or common tumbleweed, is a species native to North America, spread on all continents, especially between 30º and 60º North Latitude. In Romania it may be found across all the country in steppe areas, in the beech layer, especially on productive soils, fertilized with nitrogen. It is included on the list of weed species causing losses to agricultural production [8, 24].

In the last years, *Amaranthus* species have generated an increasingly larger interest, due to their nutritional importance and the potential pharmacological relevance. From a methanol extract of *Amaranthus retroflexus* L. leaves, several polyhydroxylated terpenes with nerolidole skeleton were isolated, evaluated for their antioxidant effects and phytotoxicity on *Lactuca sativa* L. [9, 21].

Ethanol 70% extracts of *A. retroflexus* leaves and inflorescences showed inhibitory effects against *Klebsiella pneumoniae, Bacillus subtilis* and *Candida famata* (but not on *Staphylococcus aureus*) [19].

*A. retroflexus* may be a toxic plant for cattle, causing extensive degeneration and necrosis of proximal and distal tubules, with interstitial fibrosis and tubular proteinosis [7, 16, 30]. Its toxic potential has been attributed to nitrates and oxalates, but there is no supporting evidence that these compounds are responsible for the nephrotoxic action and other compounds might also be involved [4, 6]. Toxicity seems to be species-related, because feeding adult and weanling rabbits with redroot pigweed did not cause any apparent nephrotoxicity [27]. The pollen

of *A. retroflexus* may be a cause of IgE-mediated respiratory allergies, especially in semi-desert countries such as Iran, Kuwait, Saudi Arabia [28, 29, 31].

Taking into consideration that species of genus *Amaranthus* have been investigated for a variety of potential pharmacological benefits such as hepato-protective, cardioprotective or antidiabetic effects [13, 23, 32, 33] and that *A. retroflexus* has been little investigated in this direction thus far, we are interested in obtaining a leaf extract in order to evaluate it for the potential use in therapy. For a start we have assessed the toxicity of this extract on plant and animal cells.

## Materials and Methods

*Plant material and extract preparation*

The plant material consisted in leaves (*folium*) of *Amaranthus retroflexus* L. collected in April - May 2014, Teleorman County, Romania. A specimen has been preserved in the collection of the Pharmaceutical Botany department, "Carol Davila" University of Medicine and Pharmacy, Bucharest. The species identity was established on the basis of macroscopic and microscopic examination. For the latter, surface preparations clarified with chloral hydrate [23] and cross-sections stained with iodine green and carmine red - alum were analysed.

The hydro-alcoholic extract was obtained from 10 g of dried herbal product ground and refluxed for 30 minutes with ethanol 50%. This step was repeated three times. The extractive solution was concentrated with a rotary evaporator and subjected to lyophilisation for 24 hours at -55ºC (ScanVac CoolSafe 55 Freeze Dryer, LaboGene, Denmark). The dry extract obtained (yield 22.531%) was conditioned in brown glass vials, in a desiccator.

*Mouse acute oral toxicity*

The acute oral toxicity of the dry hydroethanolic leaf extract of *A. retroflexus* L. was evaluated in mice using the limit test according to the recommendations of the Organization for Economic Cooperation and Development (OECD 420) [3].

All procedures were carried out in accordance with the directive 2010/63/EU of 22 September 2010, regarding the protection of animals used for experimental and other scientific purposes [1], including approval of the local ethics committee.

Adult male NMRI mice (23.0 ± 2.92 g, n = 5) were housed in plexiglass cages. Drinking water and food were provided *ad libitum* throughout the experiment, except for the short fasting period where the drinking water was still in free access but no food supply was provided 4 h prior to treatment and 2 h after. All animals were habituated for 5 days prior to the experiment to the testing environment and maintained on a 12 h light/dark cycle. The temperature and relative humidity were continuously monitored using an electronic hygro-thermometer. The temperature was between 21ºC and 24ºC and the relative humidity was generally maintained at 35 - 45%.

A single dose of 2.000 mg/kg b.w. of dry extract (as water suspension 20%, 1mL/100g b.w., no suspending agent being added) was administered to a single mouse *per os*. After 48 h, the other 4 mice received the same treatment.

The mice were observed in detail for any indications of toxicity effect within the first four hours after the treatment period, and daily for a further period of 14 days. The animals were weighed initially, at 7 days and 14 days after the start of the experiment. Visual observations for mortality, behavioural pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.

*Daphnia magna* bioassay

Young daphids were selected according to their size from a culture maintained parthenogenetically at "Carol Davila" University (Department of Pharmaceutical Botany and Cell Biology) since 2012. The bioassay was performed according to the method described in the literature [12, 20, 27]. Ten daphnids were inserted in test tubes with serial dilutions of the extract obtained by dissolving the extract in synthetic media with 1.0% DMSO (Sigma-Aldrich). The concentrations of the extract ranged from 50 to 1500 µg extract/mL. 1.0% DMSO in synthetic media was used as control. Each sample contained 10 daphnids and was performed in duplicate. After 24 h, the number of survivors was counted and the lethality (L%) calculated. The experiment was performed under controlled conditions of temperature and humidity (25°C, 75% RH) using a climatic chamber (Sanyo MLR-351H, USA).

*Triticum bioassay*

The cytotoxicity and genotoxicity of the extract were evaluated by the Constantinescu method (*Triticum* test) as described elsewhere [5], for the 1.00%, 0.50%, 0.10%, 0.05%, 0.01% and 0.001% concentrations (water solutions termed A1-A6).

*Statistical analysis*

For the *Daphnia* test, lethality was calculated for each sample and LC50s were determined by interpolating on the lethality - logarithm of concentration curve. 95% confidence interval of LC50 (CI95%) and the determination coefficient ($r^2$) were also calculated. All calculations were performed using GraphPad Prism version 5.0 software (USA).

For the *Triticum* bioassay, the statistical analysis was performed on the 48 hour measurements ($2^{nd}$ day) using the R statistics and computing environment, version 3.2.0 [22]. The normality of residuals was assessed visually (using quantile-quantile plots and histograms) and using the d'Agostino and Shapiro-Wilk tests (as implemented in the "car" package [15]). The homogeneity of variance was examined

visually (boxplots, histograms, spread-and-level plots) and with the help of the non-parametric Levene test ("car" package). Because both normality and homoscedasticity were marginally acceptable, for sensitivity analysis purposes we used heteroscedastic one-way ANOVA procedures (Whelch and White adjustments), a non-parametric Kruskal-Wallis test and a heteroscedastic one-way ANOVA based on a generalization of Welch's method using trimmed means at a level of 0.2 (R package WRS2 [18]), with non-parametric relative effects estimated based on global rankings and simultaneous confidence intervals as proposed by F. Konietschke and implemented within the *mctp* function of the "nparcomp" package [17]. The inhibition index was computed in MS Excell 2007, based on the median values.

## Results and Discussion

*Plant material identification*

The consistency of the morphological features of the specimens with those described in the literature allowed the identification of the species as *Amaranthus retroflexus* L. *Amaranthaceae* [25]. The microscopic examination indicated characters supporting the species identity: leaf with Krantz-type anatomical structure typical for C4 leaves [15], multicellular trichomes on the leafstalk and middle vein, cells with large druses and oxalate sand, and anomocytic stomata (Figures 1 – 4).

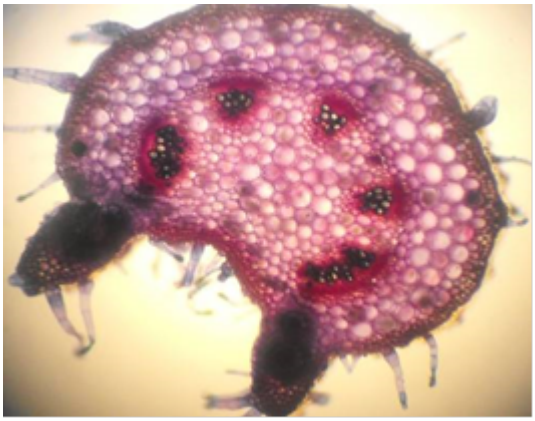

**Figure 1.**
Leafstalk cross-section (ob. 4x)

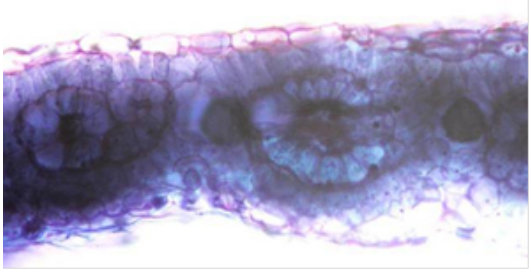

**Figure 2.**
Leaf - bundle sheaths (ob.10x)

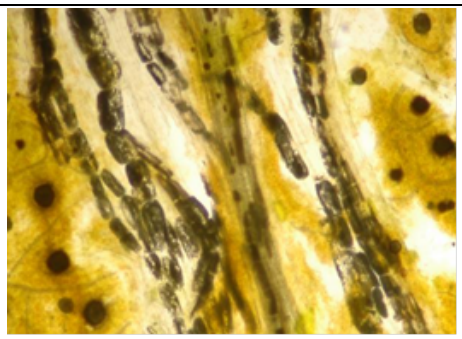

**Figure 3.**
Leaf- cells with druses and oxalate sand (ob. 4x)

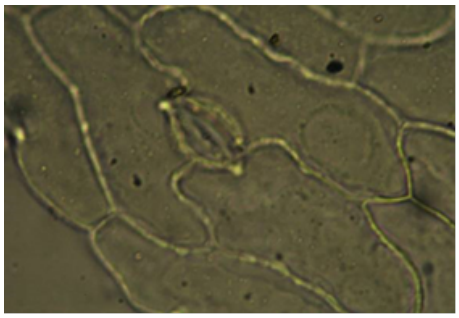

**Figure 4.**
Anomocytic stoma (ob. 40x)

*Mouse oral toxicity*

No toxic symptoms or mortality were observed in any animals, which were monitored up to 14 days after the administration of the dry hydroethanolic leaf extract of *A. retroflexus* L at single dose level of 2000 mg/kg body weight. Skin, fur, eyes, mucous membrane, behavioural pattern, salivation and sleep pattern parameters of the treated animals were found to be normal. The body weight of all the assessed mice increased after the administration of the dry hydroethanolic leaf extract of *A. retroflexus* L ($p = 0.001$, paired t test) (Table I).

**Table I**

Effect of the dry hydroethanolic leaf extract of *A. retroflexus* L on the body weight of mice at 2000 mg/kg b.w. dose

| Day | 1 | 7 | 14 |
|---|---|---|---|
| Mouse | Weight (g) | | |
| 1 | 19 | 22 | 24 |
| 2 | 21 | 27 | 30 |
| 3 | 26 | 30 | 31 |
| 4 | 24 | 31 | 33 |
| 5 | 25 | 31 | 34 |
| Mean(g) | 23 | 28.2 | 30.4 |
| Standard Deviation (g) | 2.92 | 3.83 | 3.91 |
| Relative Standard Deviation (%) | 12.68 | 13.60 | 12.87 |

This is in contrast with the known toxicity of the *A. retroflexus* for cattle, as discussed in the introductory section. This might be due to the species difference, because in the case of cattle, their rumen serves as a specialized bioreactor where cellulases and a

variety of enzymes of bacterial, archaeal, fungal and protozoal origin allow the full hydrolysis of the cell wall polysaccharides, thus potentially releasing increased amounts of oxalates or other aggressive compounds [10], unlike rodents (such as mice or rabbits) and unlike humans.

*Daphnia magna bioassay*

The LC50 value was 1053.0 µg extract/mL, indicating a low toxicity induced by the extract. The result is supported by the 95% CI of LC50 which is relatively narrow, ranging from 863.3 to 1285.0 µg extract/mL (Figure 5).

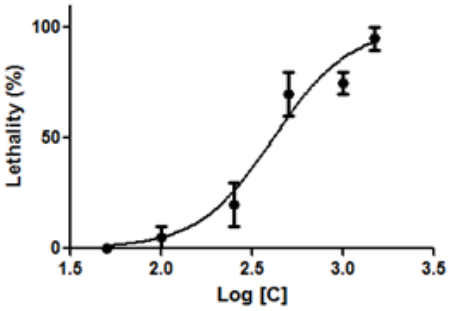


**Figure 5.**
The lethality - logarithm of concentration curve

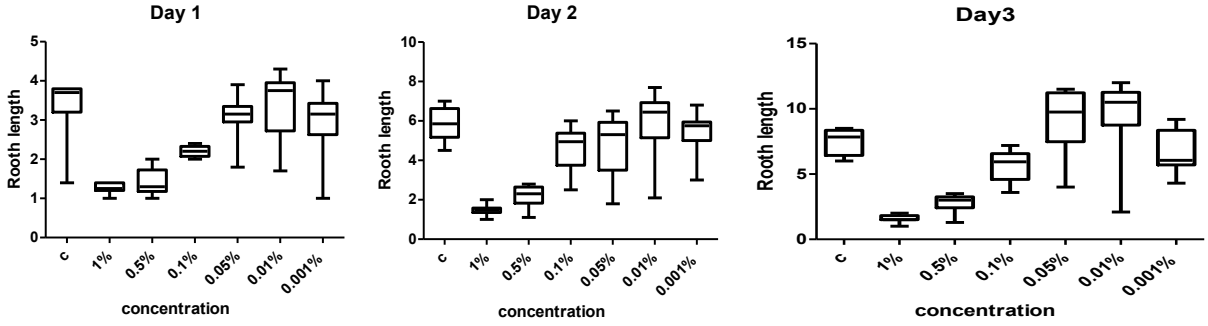


**Figure 6.**
Variation of root length under the influence of *Amaranthus retroflexus* L. leaf extract (c = control)

The analysis of the phytotoxicity testing at 48 hours has shown that the root length was strongly influenced by the extract concentration ($\omega^2 = 0.85$, ANOVA with White adjustment). Samples A1 and A2 (1% and 0.5% concentrations) have a statistically significant inhibitory effect ($p < 0.001$), but the two concentrations were different in their effects over time: the inhibition index for the highest (1%) tended to remain constant along the 72 hours of measurement, while for the second concentration (0.5%) the inhibitory effect tended to decrease slightly in time (from 82.28% to 71.91%). At the other concentrations, no statistically significant inhibitory effect was recorded in comparison with the negative control group ($p > 0.100$).

The analysis of the photomicrograph of the root tips treated with various concentrations of *Amaranthus retroflexus* L. extract was consistent with the data obtained in the root length measurements: under the first three concentrations of the extract an important inhibitory effect occurs, the photomicrograph showing mitosis inhibition, anaphases with delayed chromosomes having elongated arms, metaphases with "sticky chromosomes" or in tropokinesis (Figure. 6 and 7); at low concentrations, for which the calculated inhibitory effect was low, normal divisions or rare modifications of division phases (e.g. telophases with delayed chromosomes) were observed.

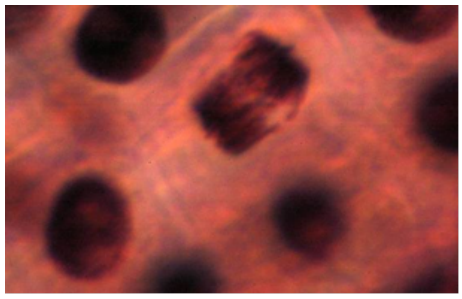

**Figure 7.**
Anaphase with delayed chromosomes and elongated arms, seen under treatment with 0.1%

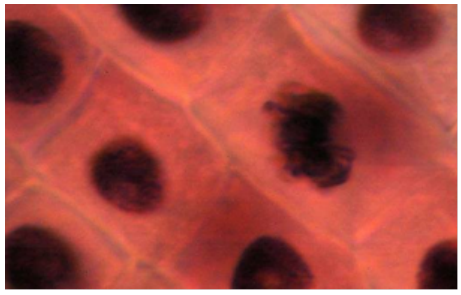

**Figure 8.**
Metaphase with "sticky chromosomes", induced by the 0.1% *Amaranthus retroflexus* L. leaf extract

The three tests performed (on rodents, crustaceans and *Triticum*) converge in indicating a low level of toxicity of the extract. This may be related to the composition of the extract and the dosage used, but it might also reflect the fact that (as supported by a

relatively large body of evidence) *Amaranthus* toxicity is not uniform across animal species: cattle, pigs and sheep seem to be particularly susceptible to toxic events, the evidence for toxicity on horses is very rare, whereas experimental attempts of producing the toxic symptoms in rabbits failed [6]. In experiments where rats were fed leaf concentrate led to poor feed intake and problems related to weight gain were reported; they were attributed to phenolics and saponins from leaves, but in our mice experiment such problems were not detected. Besides the difference in chemical composition between the leaf concentrate and the extract, the absence of toxic effects in our experiment may also be related to the short-term ("acute") character of our experiment; even in cattle, it has been shown that most often toxicity is reported in animals consuming fresh plants in large amounts, for 5 - 10 days [6].

## Conclusions

According to OECD 420 guideline (Acute Oral Toxicity – Fixed Dose Procedure) the extract may be placed in the Globally Harmonized System (GHS) category 5 (LD50 > 5000 mg/kg b.w., po) [2]. The results of the *Daphnia magna* bioassay were largely in agreement with this, indicating a low toxicity, with an LC50 of 1053.0 µg/mL. Finally, the extract was cytotoxic and genotoxic on plant cells only at a relatively high, 1.00% - 0.50% concentration range.

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