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Original Research Article

Effect of *Ambrosia tenuifolia* Spreng on *Danio rerio* Embryo and Human Cells *In Vitro*

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ABSTRACT

Ambrosia tenuifolia is a perennial herbaceous plant of the Asteraceae family, commonly used in Paraguay for multiple purposes as an abortive, antipyretic, digestive, and against headaches commonly in the form of an infusion of aerial parts. However, the lack of information on adverse effects raises questions about its safety and potential side effects. This study was undertaken to establish the potential health risks of *A. tenuifolia* for human health by evaluation of the aqueous and ethanolic extracts on *Danio rerio* embryos and to determine the potential toxicity or teratogenicity of the aqueous extract on human cells using human peripheral blood lymphocytes (PBL). For the test with *D. rerio*, observations were made 24 to 96 hours after fertilization. Coagulation, somite formation, heart rate, blood flow, pigmentation, edema formation, hatching, and spinal deviation were evaluated. As a result, the extract has lethal, sublethal, and teratogenic effects on the embryonic development of *D. rerio*, whose effects were dose-dependent. In the PBL, cells with and without bioactivation with the solution containing the S9 fraction were found to have genotoxic effects. This study provides the first report of toxicity and genotoxic effects of extracts of *A. tenuifolia* using *D. rerio* embryos and PBL after *in vitro* exposure.

***Keywords*:** zebrafish, medicinal plants, teratogenesis, genotoxicity, *in vitro* culture

Introduction

Plants have been used for the treatment of human health problems since ancient times until today [1] and their use is increasing worldwide. [2] The use of medicinal plants has a long history, as at some point all medicines were derived from them, this fact established a close and productive relationship between humans and the plant environment. [3]

Nowadays, the utilization of plants as agents for health is widely known in many cultures around the world and passed down through generations as traditional knowledge. This knowledge has been continuously improved over time with the aid of scientific rigor, applying chemical, pharmacological, toxicological, and clinical assays in order to rationally explain the therapeutic use of a plant, thus ensuring its relevance. [4]
In Paraguay, the use of plants for preventive and curative purposes is a cultural practice, it can be stated that approximately 90% of the population uses medicinal plants. [5]
Ambrosia tenuifolia Spreng, commonly known as *Altamisarã*, is a perennial herb native belongs to the Asteraceae Family, in its composition a large number of secondary metabolites such flavonoids, essential oil, and sesquiterpene lactones. [6] In Paraguay, *A. tenuifolia* was reported as an abortive, antipyretic, digestive aid, and headache relief; and the part used for medicinal purposes is the aerial parts. [6]

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Fish possess specific characteristics and specialized functions also found in higher vertebrates, and many species are amenable to both field and laboratory studies, allowing for controlled experiments. [7] Embryo-larval toxicity assays conducted in the zebrafish, *Danio rerio* [8] is used for the study of embryonic development in vertebrates [9] bridging the gap between more extensively studied animal models such as *Drosophila melanogaster* and *Mus musculus*. [10] Zebrafish embryos are a suitable model for predicting teratogenicity in mammals, including humans, due to their shared signaling pathways involved in development and their low incidence of false-positive and false-negative results. [11,12,13] Zebrafish has been used as a model in numerous studies in the fields of molecular genetics, vertebrate biology and development, neurobiology, toxicology, and ecotoxicology, making it one of the most important models in vertebrate developmental biology. [14, 15]

Analyzing extracts from medicinal plants, Xia *et al.* [16] demonstrated lethal, sub-lethal, and teratogenic effects produced by *Carthamus tinctorius* extract, Ponpornpisit *et al.* [17] observed similar effects with *Physalis minima* in *D. rerio* and Gence *et al.* [18] evaluated the pharmacological potential of *H. lanceolatum* as an antioxidant or pre- and post-regenerative agent.
On the other hand, genotoxicity is the toxic effect of a substance on DNA or other genetic material. [19] Another assay used to determine the side effects of chemical or physical agents is the *in vitro* Chromosomal Aberration assay. [20,21] Genotoxicity assays in peripheral blood lymphocytes are highly useful for an initial evaluation of the side effects of chemical, physical, or biological agents. [22, 23]
Medicinal plants, generally lack scientific studies that determine their possible side effects. Plant species with abortive properties may pose a dual health risk due to their toxicity, potentially causing harm to both the mother and the embryo, and resulting in permanent damage. [24] Taking into account that the aqueous extract of this plant species is used as an abortive, it leads us to hypothesize that a toxic effect on the human embryo may pose a risk. Therefore, the aims of this study were: 1) to

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establish the potential health risks of *A. tenuifolia* for human health by evaluating the aqueous and ethanolic extracts on *Danio rerio* embryos, 2) to determine the toxicity potential of the aqueous extract on human cells.

Materials and Methods

Chemicals and reagents

Fetal Bovine Serum (PAA), Heparin, colchicine (Sigma), RPMI culture medium, antibiotics (penicillin/streptomycin) and Phytohemagglutinin (GIBCO), KCl 4% hypotonic solution, methanol, acetic acid, Caffeine, Mitomycin C and Cyclophosphamide (Sigma), mix S9 (S9: Code: S2067, Sigma), in a solution with 2.9 mM NADP, 3.3 mM glucose-6-phosphate, 24 mM KCl and $MgCl_2$ 5.9 mM.

Plant collection and identification

Ambrosia tenuifolia was collected from medicinal plant plots at the *Jardín Botánico de Asunción* (Botanical Garden of Asunción, Asunción-Py), in June 2018, the coordinates are 25°15'13.7"S 57°34'03.5"W and was identified by the MSc. Maria Vera, from the *Laboratorio de Recursos Vegetales*-LAREV, of the *Facultad de Ciencias Exactas y Naturales*. The voucher number was named 01-Kristha Paredes-Branda and has been deposited at the LAREV.

Plant extraction

The part selected for the study was the aerial part, which was washed with potable water to remove any residue and dried at room 26 °C. To obtain the aqueous extract, 35 grams of dried leaves were mixed with 300 ml of distilled water at a temperature of 100ºC, and then filtered through a Whatman paper. To obtain the ethanol extracts, 35 grams of dried leaves were pulverized using a mill. The volume of ethanol was 1/3 (V/V). The contents were vacuum-filtered with Whatman filter paper, concentrated with a rotary evaporator, and dried in an oven at a temperature of 30 ºC. The extracts remained at -20°C until use.

Fish embryo acute toxicity

This study was carried out according to the OECD, [25] Nagel [13] and Benitez [26] guidelines, with some modifications. The modifications to the original protocol were that we used 30 embryos *per test* instead of 20. Another modification to consider was the use of caffeine as a positive control and 96-well plates were used instead of 24 wells (for convenience).

Embryo collection: samples of wild-type *Danio rerio* with high genetic homogeneity were used, obtained and kept in the Hydrobiology laboratory of the Faculty of Natural and Exact Sciences (FACEN). The colony remained at a constant temperature of 26ºC, under a light-dark cycle of 14-10h. Viable eggs were washed with water and placed in 96-well plates, one embryo *per* well, and kept in Embryo Medium. [27]

Thirty eggs were used *per* treatment. All treatments were performed in triplicate for each concentration, with a final volume of 200µL/well of embryo medium. Repetitions were performed on different days, with their respective controls. The doses for the aqueous extract were 0.63%, 1.25%, 2.50% and 5% (vol/vol) prepared in serial dilutions. For ethanol extract, a stock solution of 0.1 g of the ethanol extract in 10mL of embryo medium and the concentrations were 0.02%, 0.03% and 0.06% (w/vol) in serial dilutions. The negative control consisted of embryo medium, and the positive control treated the embryos with 2.4 mM caffeine. Each embryo was examined at different time points: 24, 48, 72, and 96 hours *per* fecundation, using a microscope. Observations were recorded and evaluated through photographs.

Mammalian Chromosomal Aberration Test (CA)

For cytogenetic analysis the following procedure was applied, five milliliters of peripheral blood were extracted using a heparinized syringe from a healthy, non-smoking donor under 35 years of age. Ten drops of whole blood were seeded in plates containing 8mL of RPMI medium (GIBCO), supplemented with 10% fetal bovine serum, 1% antibiotic (penicillin/streptomycin), and 2% Phytohemagglutinin. The plates were incubated for 48 hours at a temperature of 37ºC and a 5% CO_2 atmosphere. The cells were exposed to the aqueous extract at different concentrations, namely 1.25%, 2.5%, and 3.75% (100µl, 200µl, and 300µl) for 24 hours, without S9 mix. Mitomycin C (0.2µg/ml) was used as the positive control, and RPMI medium was used as the negative control.

To determine whether the aqueous extract contains metabolites that need to be bioactivated to act as genotoxic agents, the cells were treated with a concentration of 3.75% (300µL) of the extract and the S9 mixture was added to the plate. Negative control (culture medium with S9 mix), and positive control (culture medium with 28µg/mL of cyclophosphamide (CP)), were included. The treatment period was 24 hours until to obtain metaphase cells. After 24 hours of incubation, colchicine (0.6µg/ml) was added to each plate, 3 hours before performing the CA assay. The samples were then centrifuged. The precipitate was re-suspended and incubated in hypotonic solution (0.4% KCl) for 15 minutes at 37°C, followed by another centrifugation step to discard the supernatant. The precipitate was re-suspended in a fixative solution (methanol-acetic acid 3:1) three times. The samples were prepared on slides and stained with 4% Giemsa. A total of one hundred cells were counted *per treatment.* For the determination of cytotoxic effects, the Mitotic Index was determined, and thousand cells *per* treatment were counted.

Statistical analysis

For the evaluation of the assay with *Danio rerio*, ANOVA statistical analysis was applied using the SPSS 21 program. The data obtained at 96 *hpf* were used since all the proposed parameters were observed at that time. For the evaluation of chromosomal aberrations and MI (mitotic index), the data were processed using the statistical program SPSS 21 program, applying ANOVA and Tukey tests to assess significant differences between the extract used and the control group.

Results and Discussion

To evaluate the toxic effects of *A. tenuifolia* extracts, *D. rerio* embryos were treated, considering the high homology of their genomes. [28] Thirty eggs *per* concentration were used, in three replicates of 10 eggs each, for each concentration evaluated [29] and two replicates were performed on different days.

The embryos cultivated in the medium for embryo (CTL-) did not present any abnormality in their development in the evaluation from 24 to 96 hours post fertilization (hpf) (Figure 1). Morphological changes caused by the different concentrations of the both extracts (aqueous and ethanolic) were evaluated as mentioned before following theses parameters; coagulation and somite formation from 24 hpf, heartbeat and blood circulation at 48 hpf, delayed development, pigmentation, formation of edemas, hatching, and spinal deformation from 72 hpf onwards. [13]

For the evaluation of the toxic effects of the aqueous and ethanolic extracts of *A. tenuifolia*, we considered the cut-off of 15 viable (non-coagulated) embryos for statistical analysis, representing 50% of the treated eggs. A dilution was prepared to obtain a concentration of 5%, and from this, serial dilutions of 2.5%, 1.25%, and 0.625% were prepared. All dilutions were carried out in a final volume of 200 µL, which is consistent with the methodologies described in the literature. [30,31] Results observed on *aqueous* extracts of *A. tenuifolia* on *Danio rerio* embryos at four decrescent concentrations at 96 *hpf* were presented at Table 1 and Figure 2. At 0.60% concentration of extract any developmental delay, but 42% of embryos did not hatch (Figure 2A), at 1.25%; 31% formation of edema, 16.6% developmental delay, 56.6% lack of hatching ($p<0.05$), 16.6% spinal deformity (Figure 2B) were observed, at 2.5% 63 % of coagulated eggs were observed (Figure 2C), of the remaining 11 embryos, 100% showed developmental delay, where 90% failed to hatch, and 50% exhibited edema formation (Figure 2D). Embryos exposed to a concentration of 5% were coagulated at 100% (Figure 2E). The results obtained indicate that the aqueous extract of *Ambrosia tenuifolia* exhibits lethal, sub-lethal, and teratogenic effects on the embryonic development of *D. rerio*, with 100% lethality observed at the highest concentration evaluated (5%). Coagulation in embryos was directly proportional to the concentrations used, reaching 100% lethality at the highest concentration of the aqueous extract. The results observed in this study coincide with those of Álvarez *et al*., [32] who evaluating the *M. officinalis* extract, mention

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that mortality is a dose-dependent effect. Malformations are more noticeable than other abnormalities because of their visible nature and because they do not affect the mortality of fish embryos. Although the number of malformations was not significant, their occurrence is indicative of the teratogenic potential of the extract.

To determine the potential toxicity of the medicinal plant *A. tenuifolia*, the ethanol extract of the plant was also evaluated in *Danio rerio* embryos. The concentrations used were different from those used in the aqueous extract assay, as in the pilot assay, concentrations above 0.06% (mg/mL) resulted in the coagulation of all embryos (non-viable).

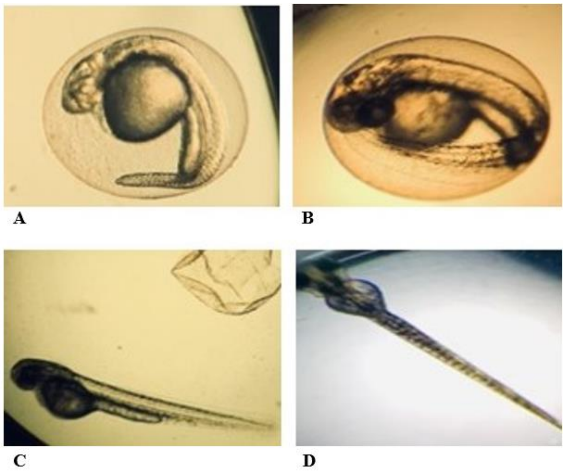

Figure 1: Normal embryonic development of *Danio rerio* in Medium for embryo (CTL-). A, 24hpf; B, 42hpf; C, 72 hpf; D, 96 hpf.

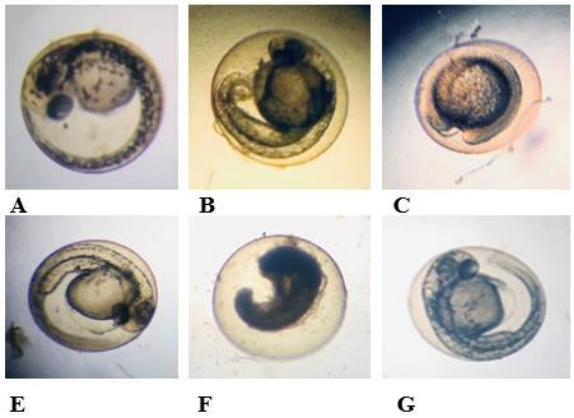

Figure 2: Embryos treated with different concentrations of the aqueous extract of *A. teunifolia*, evaluated at 96 hpf. A- Embryo treated with 0.6% aqueous extract. B- Embryo with lack of hatching, treated, 1.5% aqueous extract. C and D-: Embryos coagulated and with lack of hatching, treated with 2.5% aqueous extract. E- Coagulated embryo, treated with 5% of the extract. F- Embryo exposed to caffeine, with edema formation.

The results of treatment carried out with the ethanol extract of *A. tenuifolia* on *D. rerio* embryos were presented at (Table 2). Embryos treated with 0.02% of the ethanol extract exhibited the same developmental characteristics as the embryos in the negative control group at 96 hpf until the time of hatching. At the time of hatching, 22% (6/22) failed to hatch (Figure 3. A). Embryos exposed to 0.03%, 55% (16/29) showed edema formation ($p<0.05$), 30% (10/29) failed to hatch, and 17% (5/29) exhibited spinal deformation (Figure 3B), finally at a concentration of 0.06%, the 60% of the treated eggs coagulated ($p<0.05$), out of the remaining 12 non-coagulated (viable) embryos, 100% exhibited edema formation, 90% failed to hatch, and 58% of the embryos showed spinal deformation (Figure 3C). Similar studies with different extracts, where the same parameters were evaluated, suggested that most of the responses are reactions to toxicants in the early stages of development [32] such as scoliosis, kyphosis, and lordosis. [33] In our case both, the aqueous and ethanolic extracts showed deformations in the spinal column, with deviations and curvatures.

In this study, the aqueous extract and the ethanolic extract were evaluated to determine if the extracts had the same or different toxic potential, as observed by Fouche *et al*., [34] that the hydro-ethanolic extract of the plant species *S. pinnata* had greater acaricidal activity than the aqueous extract. Other authors, such as Grzegorczyk-Karolak & Kiss, [35] observed that hydroalcoholic and aqueous extraction of *Salvia viridis* were equally efficient for the objective evaluated in their research. Tan *et al*. [36] showed that aqueous extraction was more efficient for the extraction of phenolic compounds associated with the antioxidant activity of *Momordica charantia* L. than the organic solvents used for the same effect.

Our objective was to see if both extracts were toxic to embryos (genotoxic effect and teratogen), the first hypotheses of this work were based on the traditional use of this plant species, which is used by humans in an aqueous extraction. On the other hand, to determine the toxic potential, the aqueous and ethanolic extract was evaluated. Our result show that the ethanolic extract was more toxic (Table 2) and (Figure 3), this could be because ethanol is less polar than water and could have carried away apolar metabolites or a greater amount of polar metabolites [37,38] or as supported by Debella *et al*.,[39] analyzing the analgesic effect of aqueous and ethanolic extracts of medicinal plants, they observed that the ethanolic extract was better proprieties and that this could be due to the fact that the active components evaluated (genotoxic and/or cytotoxic) are more soluble in ethanol than in water.

Controls were performed independently for each *A. tenuifolia* extract. In positive control, 2.4 mM caffeine was used for the aqueous and ethanolic extract, due to the toxic effects described in the literature [40] and the availability of the reagent. Caffeine is teratogenic in chick embryos. [41] In this investigation, was observed to show edema formation absence of circulation and a lack of hatching in both evaluations (Figure 3D). In the positive control group of the aqueous extract treatment, coagulation and absence of heartbeat were also observed by Rana, [42] who analyzed the physiological effects of caffeine in zebrafish embryos, where they found that, depending on the concentration tested, the heartbeat slowed [43] or stopped.

In the control group for the ethanolic extract, coagulation wasn't observed. Embryos treated with caffeine showed edema formation in the heart, which coincides with the work of Cruces, [44] who describes the formation of pericardial edema from 48 hpf in embryos treated with 2.4 mM caffeine, also observed the total absence of blood circulation and hatching of embryos, this effect may be caused due to the constriction of blood vessels produced by caffeine and its general effect to the neurological system. [40] In our analysis, no spinal deformation was observed, while in Rodriguez *et al*.,[40] alterations were found in embryos treated with caffeine. As far as we know, no previous studies on toxicity or teratogenesis have been reported with *Ambrosia tenuifolia*, that why results cannot be directly compared.

In Vitro Mammalian Chromosomal Aberration Test of A. tenuifolia Aqueous Extract

To evaluate the genotoxic effects of the aqueous extract of *A. tenuifolia*, human peripheral blood cells (PBL) were treated *in vitro* for 24 hours, and then the chromosome aberration assay was applied. All treatments were performed in duplicate. Negative control was found to be free of aberrations. In the assay conducted without metabolic bioactivation, three concentrations of 1.25%, 2.5%, and 3.75% (100µl, 200µl, and 300µl, respectively) were analyzed, and diluted in a final volume of 8 mL of supplemented RPMI. A total of 100 metaphase cells were counted *per* treatment. Cells exposed to different doses of *A. tenuifolia* extract showed chromatin and fragment damage, which increased with the dose. The positive control showed ring formations and a greater number of affected cells compared to the other treatments (Table 3).

Treatments with the aqueous extract of *A. tenuifolia* showed dose-dependent effects on cells, while the lower treatment had a significant effect with observed metaphases containing fragments. The highest treatment exhibited genotoxic effects on cells, with recorded fragments,

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chromatin breaks and rings. Positive control treatment with Mitomycin C (MMC) was also significant, validating the trial.

Cells in culture do not have a metabolic bioactivation system, so a bioactivation system must be added to the medium. [45] For evaluation with metabolic bioactivation, cells are treated with the S9 mix, an exogenous metabolic activation system with the ability to metabolize chemicals *in vitro* [46,47,48] and the genotoxic effect was observed at the concentration tested. Comparing the treatment with 3.75% (300µl) plus the S9 mix to the negative control with S9 mix, resulting in a significant difference but lower than that found in the positive group with S9 mix. No significant difference was observed in the treatments of 300 µl with or without bioactivation. There was no increase in aberrations in cells treated with the S9 mix, indicating that potential secondary metabolites with genotoxic activity may not require bioactivation to exert their effects.

Regarding genotoxicity, there are no previous records related to this plant species. The chromosome aberration assay in peripheral blood lymphocytes is used to determine the genotoxic potential of substances. [49] Using the chromosomal aberrations assay, several genotoxicity studies were carried out to determine the toxic potential of plants commonly used by humans. Santa Cruz, C. [50] demonstrated the cytotoxic and genotoxic effect of the aqueous extract of *Physalis peruviana* on meristematic cells of *Allium cepa* and on human lymphocytes using the chromosome aberration test. Landeros, J. [51] evaluated the genotoxic effect of *Psittacanthus calyculatus* by culturing human lymphocytes and bone marrow in rats, demonstrating its null genotoxic effect and concluding that its ingestion is healthy and desirable for humans as long as it is not consumed in high quantities.

The results demonstrated that the aqueous extract of *A. tenuifolia* induces the formation of chromosomal aberrations in human lymphocytes treated *in vitro* and these effects were independent of bioactivation. For the assessment of cytotoxic effects, one thousand cells, including metaphases were analyzed *per* treatment, and it was observed that all mitotic indices were significant when compared to the negative control.

Conclusion

The effect of aqueous and ethanolic extracts of *Ambrosia tenuifolia* on the embryonic development of *Danio rerio* was evaluated. The aqueous extract showed 100% lethality at the highest concentration evaluated. Both aqueous and ethanolic extracts exhibited lethal and sub-lethal effects (statistically significant), were dose-dependent, but as not have statistically not significant teratogenic effects. In the evaluation using human cells, the aqueous extract demonstrated genotoxic and cytotoxic effects. It is recommended to conduct a phytochemical analysis of the evaluated plant species to identify the phytochemical components that may cause the mentioned effects and to use different concentrations to observe if there is variation in these effects.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

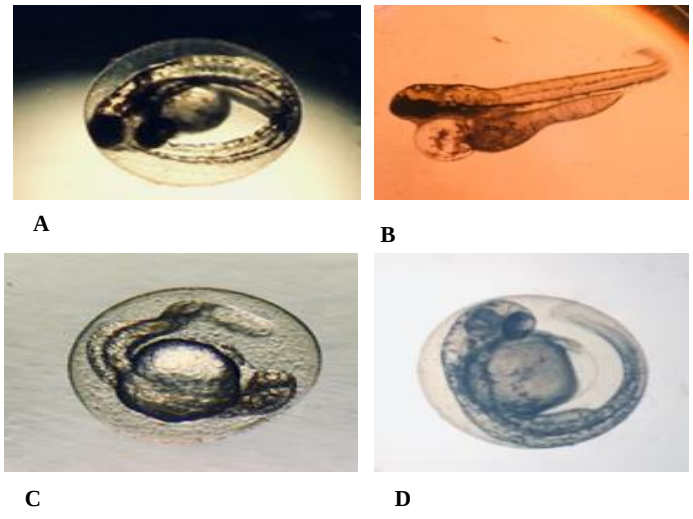

Figure 3: 96 hpf embryos, treated with different concentrations of the ethanolic extract. A- Embryo with lack of hatching, treated with 0.02% ethanolic extract. B- Embryo with edema of the heart and deformation in the terminal part of the tail, treated with 0.03% of the ethanolic extract. C- Embryo edema of the heart, deformation of the column and lack of hatching, treated with 0.06% of the ethanolic extract. D- Embryo with lack of hatching, and blood circulation and with edema formation, treated with caffeine.

Table 1: Treatment carried out with the aqueous extract of *A. teunifolia* on *Danio rerio* embryos. Statistical evaluations were performed at 96 hours post fertilization (hpf)

Parameter	CTL-	0.60%	1.25%	2.50%	5%	CTL-
Coagulation	07/30	06/30	04/30	19/30*(63%)	30/30*(100%)	15/30*(50%)
Absence of somites	00/23	00/24	00/26	00/11	-	00/15
Edema formation	00/23	00/24	08/26	05/11	-	15/15*(100%)
Developmental delay	00/23	00/24	05/26	11/11	-	00/15
No heartbeat	00/23	00/24	00/26	00/11	-	08/15
No circulation	00/23	00/24	01/26	00/11	-	15/15*(100%)
Absence of pigmentation	00/23	00/24	00/26	04/11	-	00/15
Lack of hatching	00/23	10/24*(42%)	17/26*(57%)	09/11	-	15/15*(100%)
Column deformation	00/23	00/24	05/26	00/11	-	00/15

CTL-: negative control; CTL+: positive control. %: percentage (µL) of the aqueous extract in 200 µL of volume per well. "/": the effect was calculated on the total (in the case of coagulation) or on the survivors. *Statistically significant $p \leq 0.05$.

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Table 2: Treatment carried out with the ethanolic extract of *A. teunifolia* on embryos of *D. rerio*. The evaluations were made at 96 hours post fertilization (hpf)

Parameter	CTL-	0,02%	0,03%	0,06%	CTL+
Coagulation	05/30	03/30	01/30	18/30*(60%)	00/30
Absence of somites	00/25	00/27	00/29	00/12	00/30
Edema	00/25	00/27	16/29*(55%)	12/12**	30/30*(100%)
Developmental delay	00/25	00/27	00/29	04/12	00/30
No heartbeat	00/25	00/27	01/29	00/12	00/30
No circulation	00/25	00/27	01/29	00/12	10/30*(30%)
Absence of pigmentation	00/25	00/27	01/29	00/12	00/30
Lack of hatching	00/25	06/27	10/29*(30%)	11/12**	30/30*(100%)
Column deformation	00/25	00/27	05/29	07/12**	00/30

CTL-: negative control; CTL+: positive control. %: percentage (mg) of the ethanolic extract in 200µL of volume per well. "/": the effect is calculated on the total (in the case of coagulation) or on the survivors. *Statistically significant p≤0.05. **: Not statistically evaluated.

Table 3: *Aqueous extract of A. teunifolia genotoxic evaluation*. The types of Chromosomal Aberrations found in the different treatment groups are observed.

Treatment	Fragments	Breaks	Rings	Abnormal cells	Metaphases
CTL-	0	0	0	0	100
CTL- + S9	0	0	0	0	100
A. t 100 µL	4	4	0	6*	100
A. t 200 µL	10*	3	0	13*	100
A. t 300 µL	9*	3	1	9*	100
A. t 300 µL + S9	6*	3	0	10*	100
MTC	22*	11*	2	22*	100
CP + S9	12*	7	1	15*	100

CTL-: negative control. At: *A. teunifolia*. MTC: Mitomycin C. CP: Cyclophosphamide. S9: cytochrome S9 fraction. *: Statistically significant (p<0.05).

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