**ARTÍCULO ORIGINAL**

# Evaluation of the mutagenic effects of *Passiflora edulis* Sims (maracujá) tincture on the *Allium cepa* test system

## Evaluación de efectos mutagénicos de tintura de *Passiflora edulis* Sims (maracuyá) en sistema-test *Allium cepa*

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**ABSTRACT**

**Introduction:** ever since prehistoric times, plants have been used by the population to treat all sorts of diseases. One of the most popular medicinal species in Brazil and other Latin American countries is *Passiflora edulis* Sims, of the family Passifloraceae and known as *maracujá*, which is widely used to treat nervous disorders. However, in order to validate its safety it is necessary to determine whether the plant has the capacity to change the cell cycle and cause toxic effects on the genetic material.
**Objective:** evaluate the toxic, aneugenic and clastogenic effect of *Passiflora edulis* Sims tincture on the initial development and mitotic index of meristematic onion root cells using the *Allium cepa* test system.
**Methods:** *Allium cepa* seeds were subjected to continuous and discontinuous treatments (acute - 20 h and chronic - 72 h) with distilled water (witness), 20 % alcohol and three concentrations of *Passiflora edulis* tincture (6, 12 and 24 drops).
**Results:** the mitotic index showed a significant reduction at concentrations of 6 drops (continuous treatment), 12 and 24 drops (acute discontinuous treatment - 20 h), and all concentrations in the chronic discontinuous treatment - 72 h. The aneugenic effect occurred at concentrations of 24 drops in both discontinuous treatments, whereas a clastogenic effect was observed at concentrations of 12 and 24 drops in the two discontinuous treatments.
**Conclusions:** the tincture showed great mutagenic potential, and was responsible for inducing damage to the cell cycle. Validation of its use in human beings requires further testing.

**Key words:** *Allium cepa* test system, mutagenicity, *Passiflora edulis* Sims, medicinal plants, allelopathy.

**RESUMEN**

**Introducción:** desde tiempos prehistóricos las plantas han sido utilizadas por la población como un recurso terapéutico para todos los tipos de enfermedades. De las especies medicinales más populares en Brasil y otros países de Latinoamérica, *Passiflora edulis* Sims, de la familia Passifloraceae, conocido como maracuyá, se distingue por su uso en el tratamiento de disturbios nerviosos. Sin embargo, para validar su seguridad, es necesario evaluar si la planta posee la capacidad de cambiar el ciclo celular y causar efectos tóxicos sobre el material genético.
**Objetivo:** evaluar el potencial tóxico, aneugénico y clastogénico de la tintura de *Passiflora edulis* Sims en el desarrollo inicial e índice mitótico de células meristemáticas de raíces de cebolla a través del sistema-test *Allium cepa.*
**Métodos**: semillas de *Allium cepa* fueron sometidos a tratamientos continuos y discontinuos (agudo - 20 h y crónico - 72 h) de agua destilada (testigo), 20 % alcohol y tres concentraciones de tintura de *Passiflora edulis* (6, 12 y 24 gotas).
**Resultados:** el índice mitótico mostró una reducción significativa en las concentraciones de 6 gotas (tratamiento contínuo), 12 y 24 gotas (tratamiento discontínuo agudo – 20 h) y en todas las concentraciones del tratamiento discontínuo crónico – 72 h. El efecto aneugénico ocurrió a concentraciones de 24 gotas en ambos de los tratamientos discontínuos, mientras que se observó un efecto clastogénico en las concentraciones de 12 y 24 gotas de los dos tratamientos discontínuos.
**Conclusiones:** la tintura demostró gran potencial mutagénico y fue responsable de inducir daño en el ciclo celular. Para validar su uso por los seres humanos, más pruebas necesitan realizarse.

**Palabras clave:** sistema-test *Allium cepa*, mutagenicidad, *Passiflora edulis* Sims, plantas medicinales, alelopatía.

## INTRODUCTION

The use of plants for the treatment of diseases has occurred since pre-history and did not require scientific knowledge "at first" for humans to use the abundant herbal resources. The discovery of the healing properties of plants was merely empirical and accomplished through observation.[1] Since then, plants have occupied an important role in the history of humanity, especially in the treatment of diseases.[2,3]

Throughout history, this practice has experienced increases and decrease in popularity. However, in recent decades, phytotherapy has become an increasingly common type of medical therapy,[4] primarily by providing the population a cheap alternative treatment and reducing the exclusion of the most disadvantaged portion of society from the public health systems. In addition, phytotherapy provides new remedies for the pharmaceutical industry (which cannot be obtained synthetically),

especially when coupled with the ability to manipulate compounds obtained from plants to make them more effective or less toxic.[5]

The first reference to Brazilian medical plants and their medicinal properties was made by Pero Vaz de Caminha in his letter to the King of Portugal;[1] this interest increased with the Jesuits, slaves, and European colonists.[6] However, the first normative act of medicinal plants in Brazil occurred with the publication of the first edition of the *Brazilian Pharmacopoeia* in 1929, in which over 280 monographs, based on popular reports, were published concerning the use of Brazilian medical plants.[1]

About half of the extant species of flowering plants are found in tropical forests; therefore, these forests are considered by scientists to be potential reserves for new drugs.[3] Thus, preservation of these forests is necessary and urgent. Brazil is a privileged country for being a true vegetable empire, and several native species have been widely utilized by the population. In some cases, chemical and/or toxicological studies that support their use have been performed, and other plants have been used based solely on traditional knowledge.[7]

Of these plants, *Passiflora edulis* Sims, popularly known as passion fruit a member of the family *Passifloraceae*, is extensively used commercially in Brazil, and its use became popular in the 17$^{th}$ century. The leaves and fruits are indicated for nervous system disorders, such as disquietude and anxiety, in addition to its antioxidant effects. Its seeds show anthelmintic activity, and its oil is indicated for the treatment of acne.[1]

The major chemical constituents are fatty acids, flavonoids, steroids, tannins, alkaloids, polyphenols (antioxidants), carotenoids and coumarins (responsible for its antibacterial activity).[8] In pharmacological tests, the active compounds were harmano (known as passiflorin, a natural sedative found in leaves and fruits), maracujina and cardioespermin (a cianogenic glycoside).[2]

The leaf-based treatment should be used only for short periods and only after lengthy boiling to remove the excess of hydrocianidric acid, a toxic substance can cause respiratory depression and other problems in high doses as a result of its ability to bind to metals and the functional groups of most enzymes.[9]

In addition to its medical importance, *P.edulis* has been investigated for possessing inhibitory substances (generically called allelochemicals), whose primary function is to ensure that the seeds to not do germinate inside the fruit (local optimum germination conditions), but it also exhibits considerable allelopathic action on the germination of other species.[10] These substances are in greater concentration in the aril, which involves the whole seed.[11]

The use of plants by society receives much media attention and is often justified by the idea that everything that comes from nature is not hazardous to human health; therefore, there is no need to control their use.[12] However, the use of medicinal herbs does have potential risks. Because of this, the use of herbal remedies and herbal products should only occur after physiological and genetic testing that proves their effectiveness, quality and safety, similar to the testing of any manufactured drug before it enters the market.[7]

These experiments use test organisms that are susceptible to cytotoxic agents; of these organisms, higher plants are important for the genetic testing of environmental pollutants. This use of higher plants is common in the scientific community because they provide greater knowledge of genotoxicity and especially

mitotic aberrations in eukaryotes.[13] *Allium cepa* (onion) is an exceptionally efficient test-system for the identification of dangerous substances, especially concerning the effects of different exposure times and the determination of their influence on organisms. The use of *Allium cepa* was originally introduced by Levan in 1949.

*Allium cepa* is globally recognized as a model system by the scientific community because of its convenience and low cost. In addition, it facilitates the study of damage in chromosomes or perturbations in cell division as well as the risks of aneuploidy. Although the *Allium cepa* system being largely used in monitoring studies to assess environmental pollutants, it is can be used as assay to evaluate the effects of the plants use as medicine in humans because it has shown good correlation with other bioassays, as mammals test.[13] Add to that, many researchers support that results obtained with higher plants bioassays, as *Allium cepa*, should not be discarded because the substance able to induce DNA damages in plants can also offer risk to other organisms whereas the genetic material is common to all creatures.[13] Therefore, positive results in an *Allium cepa* test should be considered an indication that the tested chemical is potentially hazardous to human health.[14] Besides, for a more comprehensive assessment of the different aspects of an substance action, is essential to use distinct test systems in order to obtain a complete overview of its hazard.[13]

Because passion fruit is widely used in folk medicine and does not have any study published about its effect in the genetic material, the present study aimed to analyze its toxic, cytotoxic, aneugenic and clastogenic potentials on the initial development and mitotic index of meristematic root cells of onions using the *Allium cepa* system.

## METHODS

### Vegetable tincture

A 30 mL *P. edulis* leaves tincture (lot nº 040601), with 20 % of alcohol, was obtained through the pharmaceutical assistance program of the city of Vitória, ES, Brazil through the laboratory of Phytotherapy of the Municipal Health Office of the municipality.

### Evaluation of the cytotoxic, aneugenic and clastogenic activities of the tincture on the *Allium cepa* test system

Seeds of *Allium cepa* obtained from a commercial source and from the same lot were germinated in three concentrations of *P. edulis* tincture (6, 12 and 24 drops), in 20 % alcohol and in distilled water (negative control-CN).

Were carried out two types of treatment:

1) Continuous treatment – the seeds were germinated directly in three different ways: in distilled water (CN), 20 % alcohol and three different tincture concentrations (6, 12 and 24 drops).

2) Discontinuous treatment – the seeds were first germinated in distilled water until they reached a length of 1 cm; they were then transferred to their respective treatments (CN, alcohol and three dosages of tincture). After 20 hours (acute treatment), half of the roots were collected randomly, and the rest remained to receive the appropriate treatment until complete at 72 h (chronic treatment).

Subsequently, the roots were fixed in Carnoy 3:1 (three parts ethanol to one part acetic acid), kept at ambient temperature for 24 hours, and then wrapped in the refrigerator.

**Preparation and analysis of slides**

For the cytological analyses, all slides were prepared by maceration, in which the slides were subjected to hydrolysis in 1 N hydrochloric acid at 60 ºC for 5 minutes and then washed with distilled water. The slides were treated with Schiff Reagent for two hours in the dark for staining. Maceration of the tips of the roots was carried out with 1 drop of 1 % acetic orcein and 1 drop of 45 % acetic acid; the cells were immersed in liquid nitrogen, dried at ambient temperature and fixed.

Five slides of each treatment were used, and 1000 cells in each slide were counted under an optical microscope at 40x; the total number of cells analyzed per treatment was 5000.

**Analysis of the cytotoxic, aneugenic and clastogenic effects**

For the toxicity analysis took into consideration the germination index obtained from the ratio between the number of *Allium cepa* seeds submitted to germination in the continuous treatment and the number of seeds that effectively germinated.

For analysis of the cytotoxic effect, the mitotic index was calculated as below:

$$IM = \frac{\text{Number of cells in division}}{\text{Total numbers of cells analyzed}} \times 100$$

For analysis of the genotoxic effect, the aberration index was estimated as below:

$$IA = \frac{\text{Number of aberrant cells}}{\text{Total number of cells analyzed}} \times 100$$

To evaluate the aneugenic effects (IEA), were analyzed cells in division with irregular metaphase (c-metaphase), changes in anaphase (multipolar, with delay, etc.), changes in telophase (delays), and/or binucleated and multinucleated cells and chromosomal losses.

To evaluate the clastogenic effects (IEC), the frequencies of cells with micronuclei, grip, bridges and chromosomal breaks and cell death were determined. The mitotic index (IM) and aberration index were analyzed for the acute discontinuous treatments, and the germination index was determined for the continuous treatment (in addition to the mitotic indices and aberration).

### Statistical Analysis

The data were statistically analyzed using the Chi-squared method, with significance levels fixed at < p 0.05.

## RESULTS

### Germination index and radicle growth

The seeds subjected to continuous treatment suffered total inhibition of germination when treated with alcohol (20 % ethanol) and with 12 and 24 drops of tincture; after treatment with 6 drops, there was a significant decline in germination relative to the control treatment (CN) (Fig. 1).

To evaluate the possible relationship between the decrease in the mitotic and germination indices with the growth and development of the radicle, was measured the radicle length treated with the negative control (CN) and with 6 drops of tincture; although the rate of germination and the amount of roots germinated in 6 drops, there was no significant difference in the average radicle length between them, at the end of four days of observation.

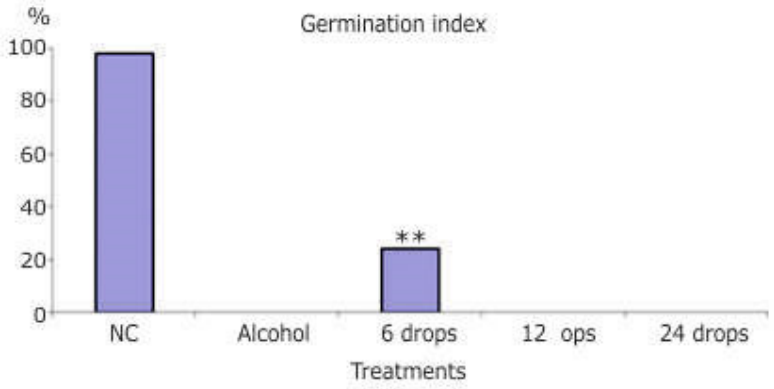


**Fig. 1.** Germination index (GI) of seeds of *A cepa* in response to continuous treatment with the negative control (NC), alcohol (20 %) and three concentrations of *Passiflora edulis tincture* (6, 12 and 24 drops). (** significant to p < 0,01)

### Mitotic Index

In the continuous treatment (TC), changes in the mitotic index (IM) could only be evaluated at the concentration of 6 drops (Fig. 2) because there was no germination with other treatments (alcohol and 12 and 24 drops of the tincture). Compared to the control (CN), seeds treated with 6 drops of tincture of *P. edulis* showed a significant reduction in the mitotic index.

With the acute discontinuous treatment (TD 20 h), there was a significant reduction in the mitotic index with 12 and 24 drops compared to the control. However, when analyzing the chronic discontinuous treatment (72 h), in which the radicles were

exposed to the tincture and alcohol for a longer time, a reduction occurred in all treatments.

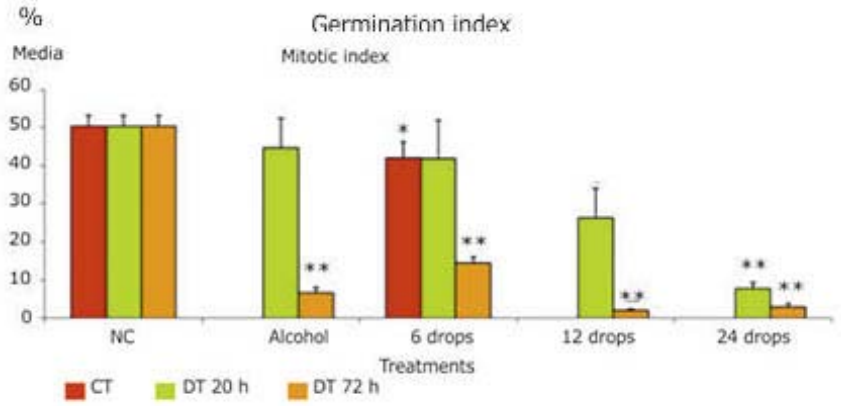


**Fig. 2.** Mitotic index (MI) of cells of *A. cepa* in response to continuos treatment and acute (DT 20 h) and chronic (DT 72 h) discontinous treatment with the negative control (NC), alcohol and three concentrations of *Passiflora edulis* tincture (6, 12 and 24 drops). (* Significant to $p < 0,05$; ** significant to $p < 0,01$)).

**Aneugenic effect index**

Figure 3 shows that at the 24-drop concentration with discontinuous acute treatment (20 h) and chronic treatment (72 h) showed a tendency to increase in the aneugenic effect index (IEA). With the other concentrations (alcohol and 6 and 12 drops), the values of the IEA decreased; however, this result cannot be regarded as a potential protective effect. Because the data did not show a consistent trend, it is not appropriate to assess this parameter.

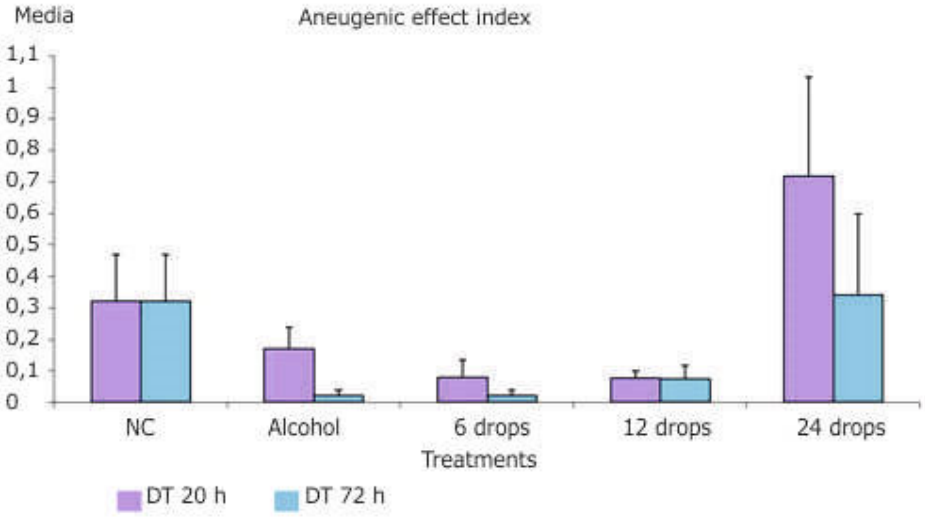


**Fig. 3.** Aneugenic effect index (AEI) of cells of *A. cepa* in response to acute (DT 20 h) and crhonic (DT 72 h) discontinuous treatment with the negative control (NC) and the concentartions of *Passiflora edulis* tincture (6, 12 and 24 drops).

### Clastogenic effect index

The clastogenic effect index (IEC) increased significantly with the acute discontinuous treatment when the seeds were exposed to concentrations of 24 drops (figure 4). However, this increase was also observed at the 12-drop concentration in the chronic discontinuous treatment (figure 5).

Notably, the few cells that were able to enter the cell cycle showed many morphological changes in their chromosomes and presented smaller nuclei after treatment with 12 drops.

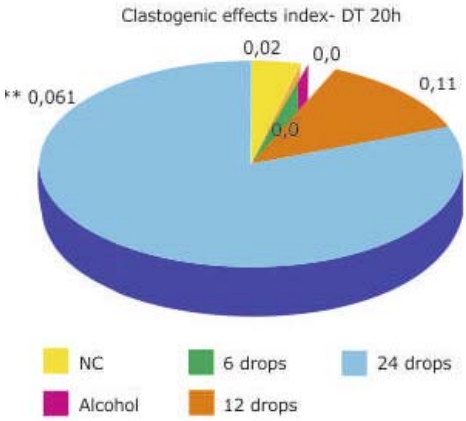


**Fig. 4.** Clastogenic effects index (CEI) of *A. cepa* cells in response to the acute discontinuous trestment (DT 20 h) with the negative control (NC) and three concen-trations of *Passiflora edulis* tincture (6, 12 and 24 drops). (** Significant to p < 0,01).

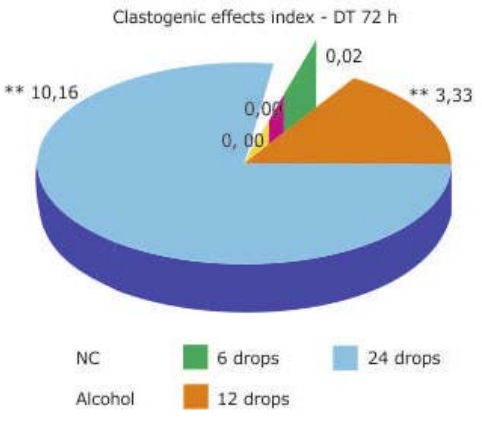


**Fig. 5.** Clastogenic effects index (CEI) of *A. cepa* cells in response to the acute discontinuous trestment (DT 72 h) with the negative control (NC) and three concentrations of *Passiflora edulis* tincture (6, 12 and 24 drops). (** Significant to p < 0,01).

## DISCUSSION

The decline in the germination rate with the 6-drop treatment was significant when compared with the control, but it was not sufficient to cause total inhibition, as observed with the 12- and 24-drop treatments. By adding water until the volume reached 1 mL, the concentration of 6 drops became more diluted, potentially reducing its toxic effects on germination and causing changes in the mitotic cells. Thus, a significant reduction in the mitotic index occurred, corroborating the toxicity of the tincture. With the chronic discontinuous treatment (TD 72 h), almost all of the analyzed cells were in interphase, indicating that greater exposure to the tincture of *P.edulis* stopped the cell cycle and blocked the entry to cell division, which was reflected in the reduction in the mitotic index.

The negative effects of lengthy exposure of the seeds to a higher concentration of tincture are reinforced by the IEA, which indicates that longer exposure of seeds to a higher concentration of tincture affects the functioning of the cell cycle. This results demonstrate that exist a correlation between exposure time and increase of tincture concentration. A likely explanation is that when exposure at higher concentrations by a higher time, the root cells die or their mitotic cycle suffers significant changes; one factor that explain this fact is the increase in the

concentration of the phytochemicals in higher dosage used, make their effects over the cellular cycle more powerfull. The decrease in the IEA with the other treatments (alcohol and 6 and 12 drops) cannot be understood to be a potential protective effect. As the data did not show a consistent trend, it was not appropriate to assess this parameter.

With respect to the IEC, a high number of dead cells were observed (data not shown), corroborating the interference of the treatment with the cell cycle, culminating with the decline in mitotic index and consequent inhibition of germination at these concentrations under continuous treatment. As reported, Balsalobre *et al.* related that *P.edulis* can exhibit allelopathic action.[10] The behavior observed in the present study, with respect to the inhibition of germination and changes in the mitotic index of *Allium cepa*, may be related to that feature.

The observed effects may be partially due to the alcohol present in the formulation because even at low concentrations (20 %), alcohol inhibited germination, contributed to the increase in IEA and affected the chromosome structure (IEC).

The tincture of *Passiflora edulis* showed great mutagenic potential and was responsible for inducing damage in the cellular cycle. However, to validate its safety use by humans, more tests need to be performed. The results of this study are the first step to understanding the effects of the use of passion fruit and its formulation, but they were not conclusive regarding the action of the tincture with respect to its toxicity in humans. Experiments with animals have not yet been carried out, which will provide more accurate information.

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