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Genotoxicity study of *Cannabis sativa* L. extract

Alana Costa [a], Arquimedes Gasparotto Jr. [b], Cícero Pereira [a], Emerson Lourenço [c], Alana Garcia [c], Helena Joaquim [a,*]

[a] GreenCare Pharma, Vinhedo, SP, Brazil
[b] Laboratory of Electrophysiology and Cardiovascular Pharmacology, Faculty of Health Sciences, Federal University of Grande Dourados, Dourados, MS, Brazil
[c] Laboratory of Pharmacology and Toxicology of Natural Products, Universidade Paranaense, Umuarama, PR, Brazil

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ABSTRACT

Cannabis sativa L., a member of the Cannabaceae family, has been thoroughly investigated for its diverse therapeutic properties, primarily attributed to cannabinoids such as delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Secondary, metabolites like terpenes also exhibit pharmacological effects. This study examined the genotoxicity of a whole Cannabis sativa flower extract 160.32 mg/mL using three OECD-recommended protocols: the Ames test, micronucleus test, and comet assay. Five groups of six Wistar rats were used. Three doses of the extract (500, 1000, and 2000 mg/kgbw) or negative control (placebo) were administered orally, while cyclophosphamide monohydrate (20 mg/kgbw) was used as a positive control via intraperitoneal injection. Blood was collected for the comet test, and the animals were euthanized for bone marrow collection for the micronucleus test. The Cannabis extract did not increase the number of revertant bacterial colonies at (375, 250, 125, and 62.5 µg/plate) in TA100 or TA98, nor did it affect the number of micronucleated polychromatic erythrocytes (MNPCEs) or the ratio of polychromatic to normochromatic erythrocytes (PCEs/NCEs). It also did not alter the index or frequency of DNA damage in hematopoietic cells. These results suggest no genotoxic effects, supporting its potential therapeutic use.

1. Introduction

Cannabis sativa L., a member of the Cannabaceae family, is a plant that has been the subject of intense scientific research due to its various therapeutic properties [1,2]. Cannabinoids, a class of oxygen-containing aromatic hydrocarbons, represent one of the most extensively researched phytochemical groups in *C. sativa*, encompassing over 150 distinct compounds, of which delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are some of the most well-known [3]. These molecules interact with the endocannabinoid system, especially with the cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), playing a role in homeostatic regulation, and their action has been described in some medical conditions, including pain, mood, appetite, and memory [4].

In addition to cannabinoids, other secondary metabolites of *Cannabis*, such as alkaloids, terpenes, flavonoids, phenolic acids, lignans, cinnamates, and saponins, have shown pharmacological effects [5]. Among them, terpenes stand out as an important class of compounds produced by the species, contributing to its characteristic aroma [6]. Each *Cannabis* strain bears a typical terpenoid profile, differing from other strains both qualitatively and quantitatively according to their relative amounts and the assemblage of the given terpenes present [7]. Importantly, terpenes were suggested not only to convey the smell of the different *Cannabis* flowers but also to have some therapeutic effect either by themselves or as co-activating agents [8], enhancing the beneficial activity of phytocannabinoids on humans [9].

Despite all these therapeutic benefits that are observed from the use of medical cannabis, there are worrying concerns that must be evaluated such as the genotoxicity of cannabis, which can also lead to an increased risk of development of cancer and congenital anomalies [10].

Genotoxic agents are substances that interact with DNA, causing changes in its structure or function [11]. When these changes are permanently fixed and can be passed on to future generations, they are known as mutations. Mutations are responsible for genetic diversity within a population and are vital for species survival [12].

Several studies have found damage to various chromosomal associated with cannabinoid use, as well as chromosomal mis-segregation errors [13], horizontal transversions, and gene amplifications on

* Correspondence to: GreenCare Pharma, Division of Medical & Scientific Affairs, 255 Edgar Marchiori st. ZIP 13288-006, Vinhedo, Brazil.
E-mail address: helena.joaquim@greencarepharma.com.br (H. Joaquim).

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chromosome 12 in testicular cancer [14] and lung cancer [15].

Considering numerous studies demonstrating the genotoxicity of cannabis, it is noteworthy that many of these investigations have focused on individuals who consume cannabis through smoking or in cigarette form, normally rich in THC. Consequently, there is a lack of control over the specific compositions of these substances and their respective concentrations.

It is essential to assess the genotoxicity of cannabis extract oil to understand the potential damage it may cause to cells or DNA. Given the growing interest in the use of cannabinoid-containing products in humans and considering that most cannabis treatments utilize whole inflorescences rather than isolated compounds, the aim was to conduct a genotoxicity study on a whole Cannabis sativa extract rich in CBD, derived from a Chemotype III variant and formulated in MCT oil.

2. Material and methods

2.1. Drugs and reagents

Cyclophosphamide monohydrate, ethidium bromide, arocloror 1254, 4-nitro-o-phenylenediamine, sodium azide, 2-anthramine, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Cannabis sativa flower extract

The *C. sativa* extract used in this study was derived from a Chemotype III variant, characterized by high cannabidiol content and a THC concentration below 0.3 % [16]. The oil extraction process involves using dried and ground flowers with a moisture level of less than 15 %. The ground plant material is weighed, put in an oven for decarboxylation, and then loaded into the supercritical CO_2 extraction equipment. From this extraction process, a crude extract containing cannabinoids, terpenes, and waxes is obtained. The waxes are removed by dissolving the extract in ethanol and putting it through the winterization process in ultra-freezers to separate the waxes, which are then filtered at low temperatures to prevent them from re-dissolving. After filtration, the ethanol is evaporated to obtain the crude extract. By diluting in medium-chain triglyceride (MCT), a concentration of 160.32 mg/mL of *C. sativa* extract was achieved, consisting of 96 mg/mL of CBD and 2.4 mg/mL of THC. The utilized product's fingerprint (Fig. 1) is obtained by ultra-high efficiency chromatography (CORTECS® UPLC® Shield RP18 column) with PDA detector (220 nm).

2.3. Genotoxicity assays

2.3.1. In vitro assay

2.3.1.1. Ames test. For the AMES test, the TA 100 and TA 98 strains of Salmonella typhimurium were used. The TA 100 strain, which detects mutagens that cause base-pair substitutions in DNA, contains a mutation in the hisG gene (hisG46), which encodes the first enzyme in histidine biosynthesis, with the CG pair as the preferred point for reversion. The TA 98 strain has a mutation in the hisD gene for the reversion of eight repetitive GC residues and detects mutagenic compounds that cause a shift in the DNA reading frame. According to the methodology of direct incorporation into plates, developed by Maron and Ames [17], different concentrations of *Cannabis* extract (375, 250, 125, and 62.5 µg/plate) were mixed with 0.1 mL of bacterial culture and 2 mL of top agar, supplemented with traces of histidine and biotin. For assays with metabolic activation, 0.5 mL of homogenized S9 microsomal fraction from the livers of rats treated with Aroclor 1254 was also added. Distilled water (vehicle) was used as a negative control [17]. The positive control for strain TA98 was 4-nitro-o-phenylenediamine dissolved in DMSO at a 10 µg/plate concentration. For strain TA100, in assays without metabolic activation, sodium azide was dissolved in distilled water at a concentration of 1.25 µg/plate. In assays with metabolic activation, 2-anthramine was used as a mutagen at a 3 µg/plate concentration for both TA100 and TA98. The contents of each tube were gently homogenized and poured onto the surface of a plate containing minimal glycosylated agar. After the top agar solidified, the plates were incubated for 48 hours at 37 °C. After this period, the number of revertant colonies per plate was counted. The assay was performed in triplicate. Distilled water (vehicle) was used as a negative control [17]. The positive control for strain TA98 was 4-nitro-o-phenylenediamine dissolved in DMSO at a 10 µg/plate concentration. For strain TA100, in assays without metabolic activation, sodium azide was dissolved in distilled water at a concentration of 1.25 µg/plate. In assays with metabolic activation, 2-anthramine was used as a mutagen at a 3 µg/plate concentration for both TA100 and TA98. The contents of each tube were gently homogenized and poured onto the surface of a plate containing minimal glycosylated agar. After the top agar solidified, the plates were incubated for 48 hours at 37 °C. After this period, the number of revertant colonies per plate was counted. The assay was performed in triplicate.

2.3.2. In vivo testing

2.3.2.1. Animals. Male Wistar rats, aged 3 months and weighing between 300 and 350 g, were acquired from the Animal Facility of the Federal University of Grande Dourados (UFGD). The rats were housed in a controlled environment with regulated light and temperature

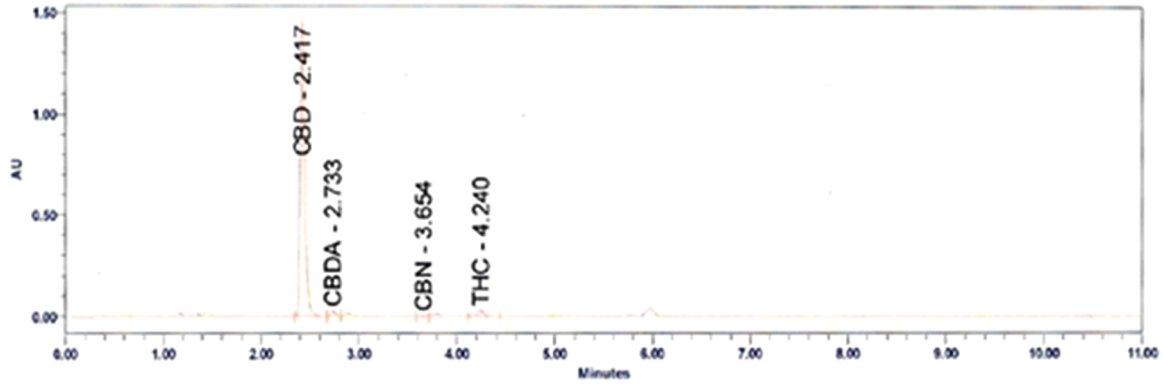

Fig. 1. Chromatogram of 160,3,2 mg/mL Cannabis sativa extract used in genotoxicity tests. Legends: AU absorbance unit; CBD Cannabidiol; CBDA Cannabidiolic acid; CBN Cannabinol; THC Delta9-tetrahydrocannabinol.

conditions (12-hour light/dark cycle; 22 ± 2°C) and provided with food and water ad libitum. All procedures conducted were reviewed and approved by the Ethical Committee for Animal Use of UFGD (Authorization No. 35/2022) prior to implementation.

2.4. Micronucleus test

The micronucleus test was conducted following the guidelines outlined by the Organization for Economic Co-operation and Development (OECD) number 474 [18]. Initially, the animals were randomly assigned and divided into groups of six male rats. The negative control groups were given only the vehicle. The animals were administered oral solutions containing *Cannabis* extract (500, 1000, and 2000 mg/kg bw; in an average volume of 0.17, 1.5, and 3 mL, respectively) via gavage once a day for 2 days. The positive control group received cyclophosphamide monohydrate (20 mg/kg bw) intraperitoneally. A group of rodents known as naïve were not treated and served as the species' control. After the treatments, the animals were euthanized by decapitation. The femurs were extracted, and the proximal epiphysis was removed to expose the medullary canal. 3 mL of NaCl solution (150 mM) was used to wash out the marrow using a 13 × 4.5 mm needle. The material was then centrifuged at 1000 rpm for 5 minutes. After centrifugation, the supernatant was discarded, and the pellet was resuspended in a 4 % formaldehyde solution and slightly homogenized using a Pasteur pipette. A drop of this cell suspension was placed on a clean dry slide, and a smear was performed. The slides were air-dried at room temperature and stained with Leishman eosin-methylene blue after 24 hours for microscopic analysis. To determine the number of micronuclei, 2000 polychromatic erythrocytes were counted per animal (1000 on each slide) at a magnification of 400x using an optical microscope. The assessment of the slides was done by a single evaluator, who was blinded to the analysis.

2.5. Comet assay

The comet assay was conducted following OECD guideline number 489 [19]. Initially, the animals were randomly assigned and divided into groups of six male rats. The negative control groups received only the vehicle. The animals were given oral solutions containing *Cannabis* extract at doses of 500, 1000, and 2000mg/kg bw via gavage once a day for 2 days. The positive control group was administered cyclophosphamide monohydrate (20 mg/kg bw) intraperitoneally. A group of rodents known as naïve were not treated and served as the species' control. After completing the treatments, whole blood samples were collected from the retro-orbital plexus in tubes with EDTA, and the animals were euthanized. Whole blood was used to create slides containing cells which were then run on an electrophoresis gel for DNA damage assessment. A cell suspension was obtained by mixing with 5 % low melting point agarose at 37°C and then deposited on slides that were precoated with normal agarose (1.5 %) and refrigerated at 4°C for 10 minutes (covered with coverslips) to harden the agarose. To avoid exposure to direct light (irradiation) and prevent further DNA damage, the coverslips were carefully removed, and the slides were stored in a lysis solution in a refrigerator at 4°C for 1 hour. After the lysis period, the slides were placed in a horizontal electrophoresis tank, covered with alkaline electrophoresis buffer, and submerged in a container filled with ice at a temperature of 4°C for 25 minutes to denature the DNA. The electrophoresis was conducted at 25 V and 300 mA for approximately 25 minutes. After the electrophoresis, the slides were neutralized with a TAE Buffer (Tris Acetate-EDTA) solution, the process was repeated two more times, dried, fixed with ethanol, and then stored in the refrigerator until analysis. For analysis, the slides were stained with ethidium bromide, covered with coverslips, and examined after approximately 5 minutes. To observe DNA damage, the slides were viewed at 400 times magnification using a fluorescence microscope equipped with a 515–560-nanometer excitation filter and a 590-nanometer barrier filter. A total of 100 cells from each animal were analyzed. In the visual analysis, the slides were observed under an optical microscope at 200x magnification. The comet assays were classified into five damage classes (DNA fragment intensity), labeled Class 0 to Class 4. Class 0 corresponds to comets considered intact, with no damage from exposure; Class 1 corresponds to comets with minimal damage; Class 2 corresponds to comets with moderate damage; Class 3 corresponds to comets with intense damage; and Class 4 corresponds to comets with maximum damage. For the interpretation of the results, two indices of visual analysis were used, namely the Damage Index and the Damage Frequency. The Damage Index (DI) was calculated as the total of the products of the multiplication between the number of comets in each class and the denominator digit of the class (0, 1, 2, 3, and 4). The Damage Frequency was calculated as the percentage of all damaged comets (classes 1–4) in relation to the total number of counted comets (n total).

2.6. Statistical analysis

The statistical analysis was conducted using the analysis of variance (ANOVA), followed by Bonferroni-adjusted significance tests for pairwise comparisons ($p < 0.05$). The results are presented as mean ± standard deviation (SD).

3. Results

3.1. AMES test

Table 1 shows the mean and standard deviation of the number of revertants (his+/plate) for the control groups (C+ and C-) or exposed to different concentrations of *Cannabis sativa* extract (62.5, 125, 250 and 375 µg/plate) against *Salmonella typhimurium* strains TA98 and TA100, in the presence and absence of metabolic activation. None of the tested concentrations elicited a significant mutagenic response when compared to the negative control group.

3.2. Micronucleus test

Fig. 2**A** shows the average number of micronucleated polychromatic erythrocytes (MNPCE) among the different experimental groups, while Fig. 2**B** shows the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE). All animals that received *C. sativa* extract presented the MNPCE number that was found for animals that were naive or treated only with a vehicle. In contrast, animals treated with cyclophosphamide (C+) showed a significant increase in the average number

Table 1
Mutagenicity of the C. sativa extract with (+S9) and without (-S9) metabolic activation for two strains of Salmonella typhimurium (TA98 and TA100).

Treatment (µg/plate)	TA98		TA100	
	-S_9	+S_9	-S_9	+S_9
C-	22 ± 2,3	21 ± 2,9	152 ± 7,1	211 ± 9,7
C. sativa 62,5 µg/plate	20 ± 2,1	21 ± 4,1	151 ± 7,7	202 ± 8,2
C. sativa 125 µg/plate	19 ± 3,2	19 ± 4,2	147 ± 8,8	205 ± 9,9
C. sativa 250 µg/plate	19 ± 3,6	20 ± 4,6	148 ± 7,7	200 ± 7,1
C. sativa 375 µg/plate	18 ± 3,1	20 ± 4,3	150 ± 8,1	203 ± 6,8
C+	2123 ± 313*	2055 ± 355*	1771 ± 203*	2033 ± 203*

Values are presented as mean ± standard deviation. Negative control (C-) = distilled water; positive control (C+) for TA98 (-S9) = 4-nitro-o-phenylenediamine; TA98 (+S9) = 2-anthramine; TA100 (-S9) = sodium azide; TA100 (+S9) = 2-anthramine. *p < 0.001 (one-way ANOVA followed by Bonferroni test).

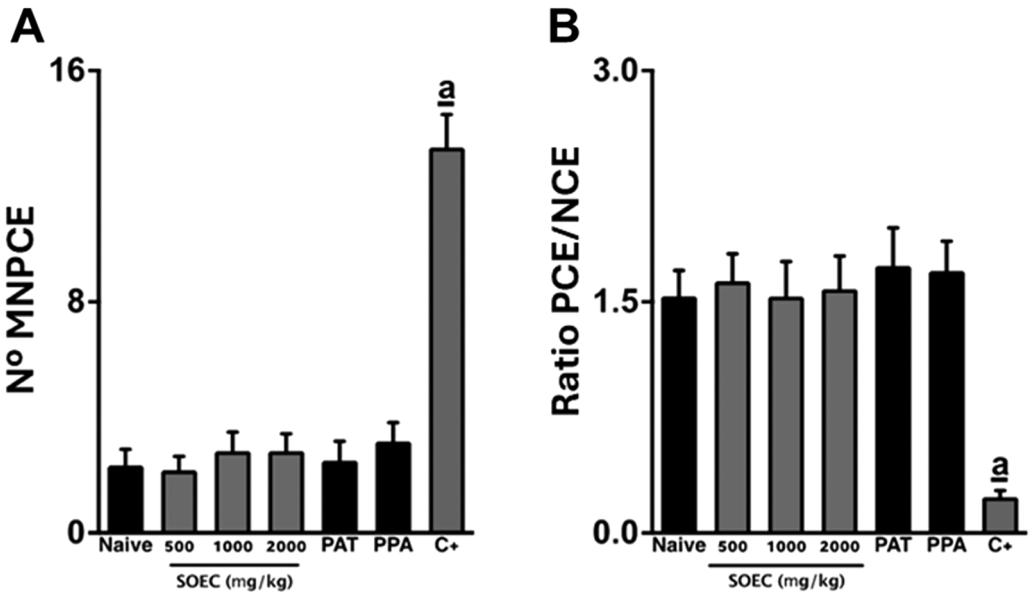

Fig. 2. Effects of prolonged treatment with *C. sativa* extract and cyclophosphamide on micronucleated polychromatic erythrocytes (MNPCE) and on the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in male rats. Each bar represents the mean ± standard deviation of six animals. The asterisk (*) denotes the level of significance compared with naïve animals, *C. sativa* extract (500, 1000 and 2000 mg), and vehicle (one-way ANOVA followed by Bonferroni's test) (*$p < 0.001$).

of MNPCE. Similarly, the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE) was significantly altered only by cyclophosphamide treatment.

3.3. Comet assay

Fig. 3 displays the impact of acute treatment with *C. sativa* extract and cyclophosphamide on the damage index (**A**) and damage frequency (**B**) to the DNA of male rats. None of the tested doses caused any significant alteration in DNA fragment intensity compared to animals that were either naïve rats or treated only with the vehicle. However, cyclophosphamide caused a 100 % increase in the index and frequency of damage in all treated rats.

4. Discussion

Products derived from *Cannabis sativa* provide a new and complementary pharmacotherapeutic approach for clinical conditions not fully controlled by conventional medications, justifying their increasingly widespread use worldwide. Therefore, considering the phytochemical complexity of different *Cannabis* preparations, we investigated the genotoxicity of a Cannabis sativa flower extract with a concentration of 160.32 mg/mL, including 96 mg/mL of CBD and 2.4 mg/mL of THC. For this purpose, we used three different experimental protocols recommended by the OECD to identify genotoxic substances, including the Ames test, the micronucleus test, and the comet assay.

The most relevant aspect is whether there is scientific data to support

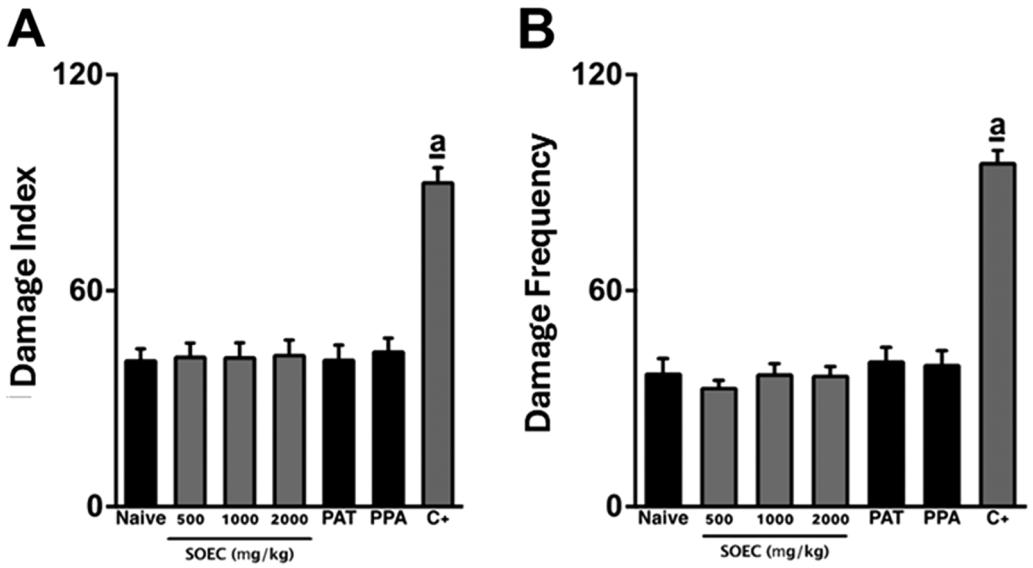

Fig. 3. Effects of acute treatment with *C. sativa* extract and cyclophosphamide on the damage index (type of damage multiplied by the number of cells with the damage) (A) and damage frequency (number of cells with the damage, in 100 cells evaluated) (B) on the DNA of male rats. Each bar represents the mean ± standard deviation of six animals. The asterisk (*) denotes the level of significance compared with naïve animals, *C. sativa* extract (500, 1000 and 2000 mg), and vehicle (one-way ANOVA followed by Bonferroni's test) (*$p < 0.001$).

the use of different *Cannabis* preparations. However, before questioning their effectiveness, it is essential to have information on the toxicity and safety of the different preparations obtained from the plant species [20]. Additionally, other critical factors to consider include dosage, route, and frequency of administration, as well as concurrent use with other drugs [21,22].

To date, significant toxicity has not been described with *Cannabis* products during preclinical studies. However, the available data is inconclusive due to the great diversity of secondary metabolites present in the species. Additionally, crude extracts of *Cannabis sativa* are complex in their phytochemistry, and studies with isolated phytochemicals do not accurately reflect the safety of a full spectrum formulation [23]. More specifically, crude extracts tend to contain molecules with synergistic effects, including CBD and THC. Studies in humans and animals suggest a high potential of CBD in attenuating the effects of THC [24], particularly in terms of cognition and memory [25,26]. Furthermore, some research has shown that experimental conditions can significantly influence the interaction between CBD and THC, such as the route of administration, dosage, timing, and clinical outcomes [27].

The Ames test is a method for detecting mutagenic chemical agents, where special strains of *Salmonella typhimurium* are used [17]. The bacterial strain has a mutation that inactivates the histidine biosynthesis pathway. Since the bacteria are unable to produce their own histidine, the colonies do not grow in media lacking the amino acid, unless there is a reverse mutation. In our results showed that the different concentrations of *Cannabis sativa* extract tested in the Ames test were not mutagenic when compared with the negative controls, in both Salmonella typhimurium strains TA98 and TA100, about to induce a mutagenic response compared to the negative control group. In line with our findings, Tesfatsion and colleagues did not observe mutagenic properties in the Ames test using CBD [28]. This result may indicate the absence of genotoxic effects of CBD, as they used the same bacterial strains and did not observe an increase in mutation frequency [29]. Additionally, another study using cannabis oil extract, which contains CBD and other phytocannabinoids, did not find mutagenic results in the Ames test [30]. This suggests that the cannabis extract does not induce mutagenic DNA damage based on gene mutations of base pair substitutions or frameshifts.

In our micronucleus test, the number of MNPCEs was consistent across all treated animals, including the vehicle control, showing no significant difference between the *Cannabis sativa* extract and the control group. The micronucleus test can detect a substance's capacity to induce mutations in living organisms by changing the DNA sequence [31]. This leads to a heritable change in the affected gene's function. If these mutations occur in germ cells, they can impact reproductive function and result in genetic diseases in future generations [32]. In somatic cells, mutations are linked to developing degenerative diseases like cancer [33,34]. In the literature, the findings of the micronucleus test are similar with no significant difference in rats treated with CBD compared to vehicles [29], although the dose of 1000 mg/kg used is lower than the one we used in our study of 2000mg/kgbw. Moreover, animals treated with cyclophosphamide, an agent known as genotoxic [35], showed a significant increase in the average number of MNPCE, including the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE).

The comet assay detects genomic lesions that can lead to mutations or undergo a repair process [36]. Its methodology allows for detecting damage in a single cell or different tissues, which is extremely important because DNA damage is typically cell- and tissue-specific [37]. Our findings revealed no significant differences in the animals treated with any dose of *Cannabis sativa* flower extract compared to the vehicle control, indicating the absence of genotoxic effects at the tested doses. In contrast to our study, Carvalho and colleagues found a significant increase in DNA damage in sperm, although they did not observe any difference in leukocytes in the comet assay, which aligns with our results. Their study involved treating animals with CBD via gavage for 34 consecutive days at doses of 15 or 30 mg/kg [38]. Our comet assay results are consistent with findings from [39], which reported no DNA damage in both tests involving isolated CBD and CBD extract. As expected, the positive control group—animals treated with cyclophosphamide—showed a 100 % increase in the index and frequency of DNA damage compared to other groups.

Although the current literature on *Cannabis sativa* extracts remains inconsistent, most evidence suggests that these extracts are safe for cells and DNA under both acute and chronic experimental conditions, even at high doses, in studies involving both male and female animals. However, additional studies are needed to fully understand the potential toxicity of this plant, especially for therapeutic applications.

Our findings are particularly significant given that we used a full-plant extract containing CBD, THC, and other cannabinoids in combination, and the doses tested in this study were higher than those typically used in long-term clinical treatments. These results suggest that *Cannabis sativa* extract does not exhibit mutagenic or genotoxic potential at doses compatible with those commonly required for the clinical management of conditions such as anxiety, chronic pain, and epilepsy, among others.

5. Conclusion

The Cannabis sativa whole flower extract did not result in an increased number of revertant bacterial colonies in the Ames test using TA 100 (base-pair substitution mutation in DNA), nor TA 98 (frameshift mutation in the DNA), nor did it affect the MNPCE count, PCEs/NCEs ratio, or DNA damage indices in hematopoietic cells, indicating the absence of genotoxic effects in the experimental models used.

CRediT authorship contribution statement

Helena Joaquim: Writing – review & editing, Writing – original draft, Project administration, Data curation. **Alana Garcia:** Methodology, Formal analysis, Conceptualization. **Arquimedes Gasparotto:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Alana C Costa:** Writing – original draft, Project administration. **Emerson Lourenço:** Methodology, Formal analysis, Conceptualization. **Cícero A C Pereira:** Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Helena P G Joaquim reports financial support was provided by GreenCare Pharma. Alana C Costa reports financial support was provided by GreenCare Pharma. Cicero A Pereira reports financial support was provided by GreenCare Pharma. Helena P G Joaquim reports a relationship with GreenCare Pharma that includes: employment. Alana C Costa reports a relationship with GreenCare Pharma that includes: employment. Cicero A Pereira reports a relationship with GreenCare Pharma that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's STATeMENT

E.L.L.B., A.K.G., and A.G.J. created the project, conceived, and

planned the experiments. A.G.J. performed experiments. E.L.L.B., A.K. G., and A.G.J. contributed to data analysis. A.C.C., CACP and H.P.G.J. contributed to the discussion and writing of the article. A.G.J. and H.P. G.J. made critical reading and editing of the article. All authors read and approved the final article.

Data availability

Data will be made available on request.

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