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Antimutagenic effects of copaiba oils (*Copaifera langsdorffii* Desf.) and flaxseed (*Linum usitatissimum* L.) against the clastogen – cyclophosphamide, in Wistar rat

Efeito antimutagênico dos óleos de copaíba (*Copaifera langsdorffii* Desf.) e linhaça (*Linum usitatissimum* L.) contra o clastogênico – ciclofosfamida, em ratos Wistar

Efecto antimutagénico de los aceites de copaiba (*Copaifera langsdorffii* Desf.) y linaza (*Linum usitatissimum* L.) frente a la clastogénica – ciclofosfamida, en ratas Wistar

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Abstract
Copaiba oil extracted from the trunk of *Copaifera langsdorffii* Desf. and flaxseed oil extracted from the seeds of *Linum usitatissimum* L., are widely used for the prevention and treatment of many diseases, including cancer. The aim of this study was to assess the antimutagenic effect of these oils against the damage-inducing agent cyclophosphamide, in Rattus norvegicus treated in vivo, by gavage, using cytotoxicity and chromosomal alteration test. The animals were submitted to a single dose of copaiba oil and of flaxseed oil in the simultaneous treatment, pre-treatment and pos-treatment for 24 hours. None of these treatments presented cytotoxic effect. Treatments both oils and cyclophosphamide have significantly reduced the chromosomal damage caused by the clastogen. In groups treated with copaiba oil the reduction percentage was 77% for the simultaneous treatment, 83% for the pre-treatment and 75% for the pos-treatment, whereas in those treated with flaxseed oil was 94% for the pre-treatment and 96% for the simultaneous and pos-treatment. These results indicate that the both oils are not mutagenic, in contrast, they present antimutagenic activity against this clastogenic agent, which represents a protective effect on the cells against the drug that has damaged the genetic material.
Keywords: Medicinal plants; Functional food; Cytotoxicity; Chromosomal alteration.

Resumo
O óleo de copaíba extraído do tronco de *Copaifera langsdorffii* Desf. e o óleo de linhaça extraído das sementes de *Linum usitatissimum* L., são amplamente utilizados para a prevenção e tratamento de diversas doenças, incluindo o câncer. O objetivo deste estudo foi avaliar o efeito antimutagênico desses óleos contra o agente indutor de danos ciclofosfamida, em Rattus norvegicus tratado in vivo, por gavagem, por meio dos ensaios de citotoxicidade e de alteração cromossômica. Os animais foram submetidos a uma única dose de óleo de copaíba e de óleo de linhaça no tratamento simultâneo, pré-tratamento e pós-tratamento por 24 horas. Nenhum desses tratamentos apresentou efeito citotóxico. Os tratamentos com óleos associados com a ciclofosfamida reduziram significativamente os danos cromossômicos causados pelo clastógeno. Nos grupos tratados com óleo de copaíba o percentual de redução foi de 77% para o tratamento simultâneo, 83% para o pré-tratamento e 75% para o pós-tratamento, enquanto nos tratados com óleo de linhaça foi de 94% para o pré-tratamento e 96 % para o tratamento simultâneo e pós-tratamento. Esses

resultados sugerem que ambos os óleos não são mutagênicos, ao contrário, apresentam atividade antimutagênica contra este agente clastogênico, o que representa um efeito protetor sobre as células frente o fármaco que danificou o material genético.
Palavras-chave: Plantas medicinais; Alimento funcional; Citotoxicidade; Alteração cromossômica.

Resumen
Aceite de copaiba extraído del tronco de *Copaifera langsdorffii* Desf. y el aceite de linaza extraído de las semillas de *Linum usitatissimum* L., son ampliamente utilizados para la prevención y tratamiento de diversas enfermedades, entre ellas el cáncer. El objetivo de este estudio fue evaluar el efecto antimutagénico de estos aceites contra el agente inductor de daño ciclofosfamida, en Rattus norvegicus tratados in vivo por sonda, mediante ensayos de citotoxicidad y alteración cromosómica. Los animales fueron sometidos a una dosis única de aceite de copaiba y aceite de linaza en el tratamiento simultáneo, pretratamiento y postratamiento durante 24 horas. Ninguno de estos tratamientos mostró un efecto citotóxico. Los tratamientos con aceites asociados a ciclofosfamida redujeron significativamente el daño cromosómico causado por el clastógeno. En los grupos tratados con aceite de copaiba, el porcentaje de reducción fue del 77% para el tratamiento simultáneo, del 83% para el pretratamiento y del 75% para el postratamiento, mientras que en los tratados con aceite de linaza fue del 94% para el pretratamiento y 96% para tratamiento simultáneo y post-tratamiento. Estos resultados indican que ambos aceites no son mutagénicos, por el contrario, tienen actividad antimutagénica frente a este agente clastogénico, lo que representa un efecto protector de las células frente al fármaco que dañó el material genético.
Palabras clave: Plantas medicinales; Comida funcional; Citotoxicidad; Alteración cromosómica.

1. Introduction

Plants with medicinal potential have been used throughout history by many different peoples (Pei et al., 2020). The presence of phytochemical constituents supports the pharmacological effectiveness of some of them, so for more than a decade there has been an increasing interest in their use for the treatment and prevention of various diseases, and among them, several types of cancer (Park & Pezzuto, 2002; Meeran & Kativar, 2008; Hamedi et al., 2022). The discovery and investigation of compounds that possess antimutagenic properties, have been of great importance, since it is estimated that 75-85% of all chronic diseases linked to lifestyle, and these compounds can reduce the rate of mutation, and consequently, the incidence of cancer and other diseases resulting from changes in the genetic material (De Flora & Ramel, 1998; Wong et al., 2005; Durnev, 2018; Malik et al., 2021). Studies show that plant-derived compounds such as vitamins, carotenoids, flavonoids, polyphenols, sesquiterpenoids, α-linolenic acid, coumarins, among others, may act as mutagenesis inhibitors (Weisburger, 2001; Veiga Junior & Pinto, 2002; Ferguson & Philpott, 2004; Majolo et al., 2019; Azevedo et al., 2019).

Copaiba oil, obtained from the trunk of *Copaifera langsdorffii* Desf. (Leguminosae Juss.), is a transparent liquid, which color varies from brown to yellow, has a strong smell, acrid and bitter taste and is composed mainly of sesquiterpenes, sometimes, these account for more than 90% of their composition and diterpenes (Veiga Junior & Pinto, 2002; Leandro et al., 2012; Pasquel-Reátegui et al., 2022; Santos et al., 2022). It is widely used in folk medicine, mainly in the Brazilian Amazon. Several studies have shown its effectiveness in the treatment of bronchitis, skin diseases, as anti-inflammatory, healing, antiseptic, antitumor, analgesic and gastroprotective (Veiga Junior & Pinto, 2002; Paiva et al., 2004; Carvalho et al., 2005; Gomes et al., 2007; Santo Junior et al., 2010; Kobayashi et al., 2011; Gelmini et al., 2013; Toboutia et al., 2017; Arruda et al., 2019; Becker et al., 2020; Menezes et al., 2022).

Linum usitatissimum L., popularly called flaxseed, is a herbaceous annual belonging to the family Linaceae. There are records showing that the ancient Egyptians, used its fibers for making fabrics and its seeds for nutritional and medical purposes. The seed, from which is extracted flaxseed oil, contains 30-40% fatty acids, 20-25% protein and 20-28% fibers, phenolic compounds, as lignans (Bernacchia et al., 2014). Among the polyunsaturated fatty acids (PUFAs), it has mainly α-linolenic acid and less linoleic acid (Cunnane et al., 1993; Oomah, 2001). Studies suggest that the fatty α-linolenic acid found in the oil composition and lignans present in flaxseed and oil traits, have antioxidant activity and important role in the prevention and treatment of neurological and visual conditions, hemorrhagic dermatitis, folliculitis, growth retardation,

rheumatoid arthritis, ulcers, osteoporosis, hypoglycemia, and also acts as antithrombotic, antiinflammatory, antitumor, among others (Cunnane et al., 1993; Roynette et al., 2004; Weiler et al., 2007; Williams et a., 2007; Berquin et al., 2008; Dugani et al., 2008; Mason et al., 2010; Toure & Xu, 2010; Ezza et al., 2018; Parikh et al., 2018).

As medicinal plants or their derivatives have an important role in disease prevention and are widely used by the population, the objective of this study was to evaluate the antimutagenic activity at chromosome level and cytotoxic in the cell division cycle, of *Copaifera langsdorffii* Desf. (Copaiba) and *Linum usitatissimum* L. (Flaxseed) oils, against cyclophosphamide clastogens in bone marrow cells of Wistar rats treated *in vivo*.

2. Methodology

2.1 Test System

Were used bone marrow cells of *Rattus norvegicus*, Wistar strain, treated *in vivo*. The animals, males and females with 35 days of age and approximately 100g of body weight (bw) each, were obtained from the Central Vivarium of State University of Maringá (UEM). These animals were kept during the experimentation period in the Sectorial Vivarium of the Department of Biotechnology, Genetics and Cell Biology, under controlled temperature ± 25ºC, humidity ± 50%, photoperiod of 12 hours light/dark, with food and water *ad libitum*, according to the standards set by the Guide for the Care and Use of Laboratory Animals (2011) and Ethics Committee on Experimentation with Laboratory Animals/UEM (process number: PRO 520/2003).

2.2 Oils and Treatments

The oils used in this study, *Copaifera langsdorffii* Desf. and *Linum usitatissimum* L were purchased from commercial sources (pharmacies) and produced by laboratories authorized by Anvisa. The tested concentrations were defined through preliminary studies.

Wistar rats were divided into 10 groups. Each group of six animals, three males and three females, received an acute treatment in a single dose for 24 hours and were divided as follows: 1) Control (Co): 1mL filtered water/100g bw by gavage, 2) Treatment (CP): 1.5mg cyclophosphamide/1mL distilled water/100g bw, intraperitoneally, 3) Copaiba oil (Cl): 0.5mL/100g bw by gavage, and 4) Flaxseed oil (Lu): 1mL/100g bw by gavage; 5 and 06) Simultaneous Treatment: each oil administered by gavage simultaneously with cyclophosphamide intraperitoneally; 7 and 8) Pre-treatment: each oil administered by gavage and after 2 hours, cyclophosphamide intraperitoneally; 9 and 10) Pos-treatment: cyclophosphamide intraperitoneally and after 2 hours, each oil administered by gavage.

2.3 Test for Chromosomal Alteration

In order to evaluate chromosomal alterations, mitotic cells were interrupted in metaphase through intraperitoneal administration of colchicine (0.16%), 0.5mL/100g bw, one hour and thirty minutes before the euthanasia. The euthanasia was performed through the intraperitoneally injection of 0.5mL of Thionembutal (1g sodic thiopental/25mL distilled water). Euthanasia has accomplished in accordance with AVMA (American Veterinary Medical Association) guidelines on euthanasia (AVMA, 2007).

The technique used for obtaining bone marrow cells of rats was described by Ford and Hamerton (1956), with modifications. The cytological analysis was performed under light microscope, 100 metaphases were analyzed per animal, totaling 600 per group, which assessed the onset of alterations such as breaks, gaps, fragments and others.

2.4 Test Cytotoxicity

The mitotic index was calculated to evaluate cytotoxicity to this, 5,000 cells were analyzed by sex, totaling 10,000 cells per group. The calculation was made with the number of dividing cells divided by the total number of cells present in the fields, as a percentage.

2.5 Statistical Analysis

The frequency of chromosomal alterations and the percentage of mitotic index were evaluated using the Chi-square test with p=0.05 using the statistical software GraphPad Prism 5.0.

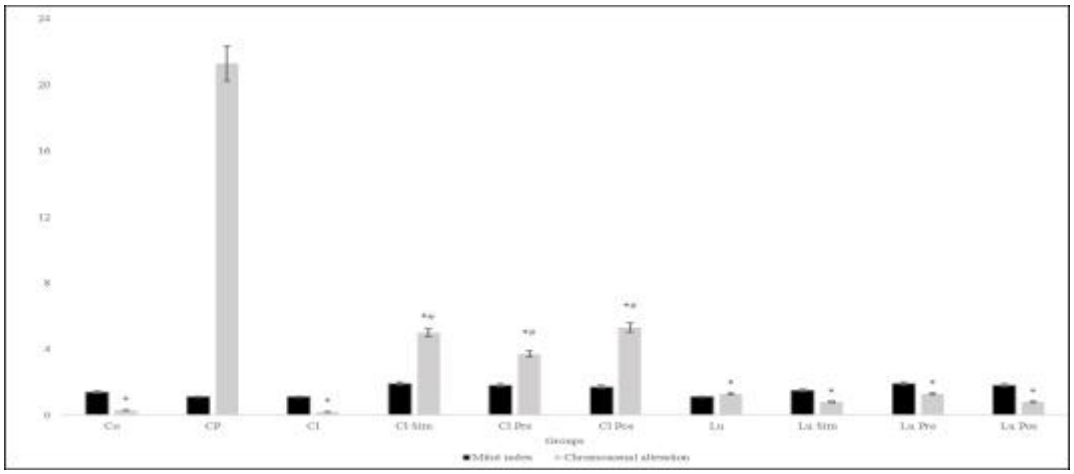
3. Results

Figure 1 shows the data obtained for the mitotic index, in percentages, and the percentage of chromosomal abnormalities found in the control groups and treatment groups with cyclophosphamide and oils from *Copaifera langsdorffii* Desf. and *Linum usitatissimum* L., and the different treatments with oils together with cyclophosphamide. According to the results obtained by the chi-square test, there were no statistically significant differences when comparing the results among themselves or with the control group for the mitotic index.

The statistical analysis showed there was a statistical difference, for the test for chromosomal alteration, between the results of the control groups and the ones treated with copaiba oil and flaxseed oil, as well as with their respective antimutagenicity treatments, pre-treatment, simultaneous treatment and pos-treatment with the two oils in relation to the results of the group treated with cyclophosphamide.

Only the results of the three treatments with copaiba oil, simultaneous, pre-treatment and pos-treatment were statistically different from the results of the control made with water and the treatment only with oil. In the comparison between the results of three different treatments with each of the oils, there were no statistically significant differences among them.

Figure 1. Mean percentage and standard deviation of mitotic index (MI) and chromosomal alteration (CA) obtained for the different groups: Control (Co) and treatments: cyclophosphamide (CP), *Copaifera langsdorffii* Desf. oil (Cl) and *Linum usitatissimum* L. oil (Lu), in simultaneous treatments (Cl Sim, Lu Sim), pre-treatments (Cl Pre, Lu Pre), and post-treatments (Cl Pos; Lu Pos) in Wistar rats. * Statistically significant results, in relation to cyclophosphamide. # Statistically significant results compared to control and *Copaifera langsdorffii* Desf. oil.



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4. Discussion

The study of the antimutagenic potential of plants is a very important field of research, because chemoprevention by phytochemical components, such as those present in copaiba and flaxseed oils, is considered both an adoptable focus, easily applicable, acceptable and accessible for the prevention and control of cancer and other diseases related to mutational events.

In the present study, for the cytotoxicity evaluation using the mitotic index, the results of all treatments did not differ significantly from the control ones (Figure 1). Therefore, *Copaifera langsdorffii* Desf., Copaiba, and *Linum usitatissimum* L. Flaxseed oils, administered alone or together with cyclophosphamide in the treatment of antimutagenicity showed no cytotoxicity at the concentrations used in this test system, Wistar rats, as well as the treatment with the clastogen. Different of the results obtained in this study, Maistro et al. (2005) found out that the oil of *Copaifera duckei* Dwyer has shown cytotoxicity at high doses in Wistar rats *in vivo*, however, the animals were treated for three consecutive days via dorsal dermal injection. This may explain the different results, since in the present study, the animals were treated in single dose gavage via or oral, which occurs throughout the digestive system and for a short period of 24 hours. Stomach pH, and lipid solubility of oils are factors that may interfere with its absorption in the gastrointestinal tract, therefore, limiting its bioavailability.

Regarding chromosomal changes in bone marrow cells of Wistar rats treated *in vivo*, there was no significant induction of chromosomal alterations when the oils were administered alone, in other words, it showed no mutagenic action when compared to the control and with cyclophosphamide, a damaging agent. When cyclophosphamide was administered together with copaiba oil or flaxseed oil in different types of protocols, simultaneous treatment, pre-treatment and pos-treatment for both oils it was observed a decrease in the percentage of chromosomal damage. Thus, the results indicate that all three types of treatments made with both oils had a protective effect, antimutagenic, in relation to the clastogenic effects of cyclophosphamide, under the conditions of the present study

Cyclophosphamide is an alkylating agent and has a clastogenic effect against DNA. As a result, has been used as a damage inductor in studies that evaluate the protective effect of compounds (Leite-Legatti et al., 2012).

The treatment with copaiba oil has significantly reduced the levels of chromosomal alterations induced by cyclophosphamide and the reduction rate was 77% in the simultaneous treatment, 83% pre-treatment and 75% post-treatment. Similar results were found by Gomes-Carneiro et al. (2005) in test with microsome in *Salmonella*, which found no mutagenic action of α-bisabolol, sesquiterpene present in copaiba oil, but it did find antimutagenic activity of this compound when inhibiting the effects of aflatoxin B1, possibly by its inhibitory effect on activators of pro-mutagens. In another study, the oil of *Copaifera duckei* Dwyer also had no mutagenic potential *in vivo* chromosomal alteration test in bone marrow cells and micronuclei in peripheral reticulocytes of Wistar rats (Maistro et al., 2005).

Different mechanisms have been postulated as involved in protection process (De Flora & Ramel, 1998; Koklesova et al., 2020), as the work of Di Sotto et al. (2010) which evaluated the anticlastogenic effect in lymphocyte cultures, of β-caryophyllene compound, which is also found in copaiba oil, and found that this was effective in the simultaneous treatment and pre-treatment. The hypothesis is that an antioxidant effect (Álvarez-González et al., 2014) or an interaction with the chemical mutagen, or even an effect of destabilization of the membrane, may have contributed to the activity of β-caryophyllene anticlastogenicity against ethyl methanesulfonate (Di Sotto et al., 2010). Second Sarpietro et al. (2015) this compound can affect membrane permeability and transporter function, interfering with mutagen uptake into cells. Furthermore, β-caryophyllene promotes cell cycle checkpoints, that can to activate DNA repair systems or signalings to reparation (Di Sotto et al., 2020).

This hypothesis can also be used in this study, and allied to it, the presence of other compounds in addition to β-caryophyllene may be related to the antimutagenic effect of copaiba oil. Studies indicate that a combination of chemopreventive agents may result in more meaningful activities than that of an isolated phytochemical (Kok et al., 2008).

This is because some compounds present in this oil may have synergistic effects, which could also explain the results obtained in this study, with the copaiba oil and too with flaxseed oil.

The antimutagenic treatments evaluated in this study, simultaneous, pre-treatment and pos-treatment with flaxseed oil were the most effective, because they significantly reduced the percentage of chromosomal alterations induced by cyclophosphamide clastogens by 94% in pre-treatment and 96% in simultaneous and pos-treatments, showing its great potential presenting an antimutagenic effect above 90%.

Similar effect was found on the administration of α-linolenic acid, compost present in flaxseed oil, this reduced the DNA damage promoted by methyl mercury exposure by, about 94%, in Comet assay of blood lymphocytes of male albino rats. It's possible that in *in vivo* conditions this linolenic fatty acid reduced the formation of hydro-peroxides by scavenging the free radicals and peroxidation of PUFAs occurs in lipids (Pal & Ghosh, 2012). Trentin et al. (2004) combined clastogenic agents with flaxseed supplementation administered in mice, and also found the same protective effect on induced chromosomal damage. This effect can be caused by the antioxidant properties of lignans, with traits present in the flaxseed oil, which have reduced the percentage of micronuclei. Besides them, the presence of phenolic compounds, fiber and Omega-3 may have contributed to this result. Tülüce et al. (2012) observed that flaxseed oil may be useful for preventing photoreactive damage by activating the DNA repair systems or scavenging ROS. Wang et al. (2017) found that the phytochemical, phenolic and flavonoid content, between fiber and oil flaxseeds were not significantly different, as also the antioxidant activity. This assumption about the action of the compounds present in flaxseed, may be considered for the non clastogenic effect of this plant's oil, observed in this study.

Glutathione peroxidase (GSH-Px) is an antioxidant enzyme that reduces the formation of hydroxyl radicals, and the ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) can be a sensitive indicator of oxidative stress. Bhatia et al. (2006) found in mice pretreated with flaxseed oil orally, that the oxidative stress induced by cyclophosphamide was reduced, because the levels of GSH-Px and GSH/GSSG ratio were maintained close to normal. Furthermore, flaxseed oil protected cell membrane permeability by protecting the activity of alkaline phosphatase, which is reduced due to cyclophosphamide action. By protecting the cell membrane permeability, the administration of flaxseed oil in this study may have affected the functional state of the bone marrow cell plasmatic membranes and thereby prevented the entry of clastogenes in the cell in pre-treatment.

The protection granted by the flaxseed oil can be attributed to its constituents, which includes omega-3 essential fatty acids and lignans. They also acts as free radical scavengers, inhibiting DNA oxidation. It seems that flaxseed oil plays an important role in the modulation of cellular responses on oxidative stress and in the reduction of the chromosomal damage observed in this study. The action against cyclophosphamide may be related to those responses.

As the DNA chain breaks induced by cyclophosphamide are related to their metabolism it is likely that both copaiba and flaxseed oils simultaneous treatments and pre-treatment may have acted through chemical or enzymatic inactivation, neutralizing its action. Furthermore, prevention by oils, the formation of active species, either by inactivation of metabolizing enzymes, such as by direct connection with cyclophosphamide, may have prevented its metabolic activation *in vivo*, which also prevented the production of active metabolites that could alkylate nucleophilic sites in DNA, RNA and protein. As both oils possess antioxidant compounds, a likely explanation for their protective effect would be the scavenging of free radicals, preventing them to establish contact with the genetic material. Finally, the oils can act on DNA repair system level, promoting damage reversal, resulting in a decrease in mutation frequency, thus explaining the results found for pos-treatments in this test system of mammals treated *in vivo*.

Therefore, copaiba oil and flaxseed oil, presented antimutagenic effects on bone marrow cells of Wistar rats, since their efficiency in the reduction of the damage caused by cyclophosphamide was statistically significant in simultaneous, pre-treatment and post-treatment, without being cytotoxic nor mutagenic. The constituents of copaiba oil, such as sesquiterpenes,

α-bisabolol, β-caryophyllene, bisabolene and γ-caryophyllene, and flaxseed oil, such as Omega-3 fatty acids, α-linolenic acid and lignans, may have been responsible for these protective effects, probably by a combinatory and/or synergistic action. The present work has demonstrated that both oils possess antimutagenic protective effect against DNA damage induced by cyclophosphamide in these types, times and forms of treatments. This can be a safer indication of the use of these oils, due to its protective effect on drugs that damage genetic material.

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