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In vivo antimutagenic activity of the medicinal plants *Pfaffia glomerata* (Brazilian ginseng) and *Ginkgo biloba*

I.V. Almeida[1], E. Düsman[2], G.I. Mattge[1], F. Toledo[1], A.F. Reusing[1] and V.E.P. Vicentini[1]

[1]Laboratório de Mutagênese e Monitoramento Ambiental, Departmento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá, Paraná, Brasil.
[2]Departamento Acadêmico de Química e Biologia, Universidade Tecnológica Federal do Paraná, Campus Francisco Beltrão, Brasil.

Corresponding author: I.V. Almeida
E-mail: igoralmeida.bio@gmail.com

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ABSTRACT. Complementary and alternative therapies, including the use of medicinal plants, have become almost standard among the world's population. *Pfaffia glomerata* (PG), popularly known as Brazilian ginseng, is widely used as a restorer of vital functions, increasing mental balance, and is used for the treatment of diabetes and rheumatism. *Ginkgo biloba* (GB) is one of the oldest known gymnosperms, whose leaves are widely used for its potentiating action on the nervous system. The biological activities of these plants were determined on bone marrow cells of Wistar rats treated *in vivo*. For cytotoxic and mutagenic acute analysis, plant extracts were administered by gavage at concentrations of 0.15, 1.5, and 15 mg PG/mL water and 1, 2, and 3 mg GB/mL water. For antimutagenic analysis, plant extracts aqueous solution (PG,

1.5 mg/mL or GB, 2 mg/mL) were administered by gavage before (pretreatment), simultaneous to (simultaneous treatment), or after (post-treatment) the administration of cyclophosphamide (1.5 mg/mL, intraperitoneally). Both plant extracts have no cytotoxic or mutagenic potential, and they significantly reduce the percentage of chromosomal aberrations induced by the cyclophosphamide given simultaneously (PG, 87%; GB, 75%), pretreatment (PG, 98%, GB, 78%) and post-treatment (PG, 99%, GB, 75%). This beneficial antimutagenic property of the medicinal plants *P. glomerata* and *G. biloba* presented here, with no cytotoxic or mutagenic activity, can efficiently contribute to improvements in quality of life and recovery for people undergoing chemotherapeutic treatment, or those looking for health and preventive habits.

Key words: Alternative therapies; Chromosome aberration; Cytotoxicity; Herbal extract; Mutagenicity

INTRODUCTION

Plants have been used for medicinal purposes since the earliest historical registers. China, India, and Egypt were most likely the pioneers in the use of medicinal herbs, and phytotherapy was already widely practiced in Europe in Medieval times. Since then, a wide variety of plants has been used for medical treatments (Licata et al., 2013). In Brazil, folk medicine is derived from a mixture of Brazilian indigenous cultures as well as European and African influences from the period of colonization, forming the basis for the traditional use of medicinal plants (Santos et al., 2012b).

The *Pfaffia* genus occurs in Guyana, Bolivia, Argentina, and in Brazil, mainly in the States of São Paulo, Paraná, Mato Grosso do Sul, and Goiás. *Pfaffia glomerata* (Spreng.) Pedersen (Amaranthaceae) is a plant whose tuberous roots are widely used in Brazilian folk medicine; it is a perennial herb that is traditionally known as Brazilian ginseng due to its morphological and chemical similarity with Korean ginseng (*Panax* spp) (Festucci-Buselli et al., 2008; Santos et al., 2012a). The Brazilian ginseng is used for restoring vital functions, increasing physical strength and mental equilibrium, and protecting the gastric mucosa from injury. It is also used for the treatment of diabetes and rheumatism, and it possesses antioxidant, analgesic, anti-inflammatory, trypanocidal, antileishmanial, and aphrodisiac actions (De Oliveira, 1986; Neto et al., 2004; Queiroz et al., 2014).

Several important compounds have been isolated and identified from the roots of *P. glomerata*, such as pfaffic acid, some saponins named pfaffosides A-F, glomeric acid (a triterpenoid), and pfameric acid (a nortriterpenoid), together with ecdysterone (β-ecdysone), rubrosterone, oleanolic acid, β-glucopyranosyl oleanolate, glycosides, and sterols (stigmasterol and sitosterol). Different studies have shown that these elements have antineoplastic activities. Saponins of different origins have been shown to have antineoplastic activities by reducing cellular proliferation, inducing apoptosis and increasing immunological response (Felipe et al., 2014). The ginsenosides (triterpene saponins) are the main active components responsible for the multiple effects of Korean and Brazilian ginseng. The older the plant, the higher the total amount of ginsenosides in roots (Radad et al., 2011).

Ginkgo biloba L. is considered a "living fossil" because it is the oldest gymnosperm, able to exceed 1000 years of life. It is the only representative of Ginkgoaceae family, originally from China, Japan, and Korea (Jacobs and Browner, 2000). The leaves, fruits, and seeds of *G. biloba* are widely used in popular medicine for the treatment of a variety of diseases, including Alzheimer's disease, dementia, low cerebral and ocular blood flow, hypertension, fatigue, anxiety, depression, and premenstrual discomfort. In addition to its anticoagulant activity, *G. biloba* is also a modulator of apoptosis and is an antioxidant (Qiao et al., 2014). In recent decades, *G. biloba* has become important for horticulture as it is widely cultivated around the world due to its tolerance to heat and cold, desiccation and salinity, as well as being very resistant to diseases (Wang et al., 2013).

Various chemical constituents of *G. biloba* have been isolated and identified in several publications, including organic and inorganic compounds, simple carbohydrates, organic acids, and secondary metabolites, such as terpene trilactones (ginkgolide and bilobalide) and flavonoids (ginkgoflavonoids). Terpene trilactones and flavonoids, together, are considered the constituents with the greatest bioactive potential from this plant (Sabater-Jara et al., 2013). The standardized *G. biloba* EGb761 extract (which is among the most studied and best-selling herbal medications worldwide) is obtained from dried leaves and contains 6% terpene trilactones (3.1% ginkgolide and 2.9% bilobalide) and 24% flavonoids (van Beek and Montoro, 2009).

The world's population is increasing the use of natural products to have a better quality of life or is using them as an adjuvant therapy to conventional treatments, mainly by consumption of dehydrated vegetable pieces, pills, infusions (tea), extracts, or other preparations. Therefore, the aim of this study was to evaluate the cytotoxic, mutagenic, and antimutagenic/protective potential of two commercial presentations of the medicinal plants *P. glomerata* and *G. biloba*, which are recognized as revitalizing and improvers of brain capacity in humans, against the mutagenic/clastogenic/pro-oxidant actions of the chemotherapeutic agent cyclophosphamide in bone marrow cells of Wistar rats *in vivo*.

MATERIAL AND METHODS

Treatment solutions

Commercial formulations of the two medicinal plants were acquired from Herbarium® Laboratory (Curitiba - Brazil). According to the manufacturer, Ginseng Brasileiro® is presented in the form of capsules containing 300 mg *P. glomerata* (PG) root dry extract, with the standardized extract containing 0.96% β-ecdysone (2.88 mg). The content of the capsule was diluted in water at concentrations of 0.15, 1.5, and 15 mg/mL. *G. biloba*® is presented in the form of capsules containing 20 mg *G. biloba* (GB) leaves dry extract and starch as the excipient. According to the manufacturer, the extract is standardized to 24% ginkgoflavonoids (4.8 mg) and 6% terpene lactones (1.2 mg). The content of the capsule was diluted in water at concentrations of 1, 2, and 3 mg/mL. The concentrations were obtained by extrapolating the use and body weight of the human to rats and ten times higher and lower.

Wistar rats

Six Wistar rats (*Rattus norvegicus*), three males and three females for each group were

obtained from the Central Vivarium of the State University of Maringá. Experiments were carried out using 35-day-old rats weighing approximately 100 g body weight (bw).

Treatments

For cytotoxic and mutagenic acute analysis, plant extracts were administered by gavage (1 mL/100 g bw). For mutagenic analysis, plant extract aqueous solutions (PG: 0.15, 1.5, and 15 mg/mL or GB: 1, 2, and 3 mg/mL) were administered by gavage (1 mL/100 g bw). For antimutagenic analysis, plant extract aqueous solutions (PG 1.5 mg/mL or GB 2 mg/mL) were administered by gavage (1 mL/100 g bw) prior (2 h pretreatment - PRE), simultaneous to (simultaneous treatment - SIM), or after (2 h post-treatment - POST) the application of an intraperitoneal injection of 1.5 mg/mL cyclophosphamide (CP). The control group (CO) was administered water by gavage (1 mL/100 g bw) and the CP group (positive group) received an intraperitoneal injection of the drug (1.5 mg/mL 100 g bw). The animals were euthanized 24 h after the treatments with an intraperitoneal dose of 0.5mL Thionembutal (1 g sodium thiopental/25 mL distilled water).

Chromosomal aberration test

The chromosomal aberration test was performed on bone marrow cells of Wistar rats using the Ford and Hamerton (1956) method, with some modifications. Mitotic cells were interrupted in metaphase with the intraperitoneal administration of 0.5 mL/100 g bw colchicine (0.16%), 90 min before euthanasia.

Analysis of the slides was performed by a light microscope, analyzing 100 metaphases per animal, totaling 600 cells each for control and treatment groups. Cells were assessed for the appearance of alterations, such as gaps, breaks, fragments, and others.

Mitotic index (MI) for the cytotoxicity evaluation was calculated from 5000 cells from each sex, totaling 10000 cells per group. The MI was calculated as a percentage as follows: the number of proliferating cells divided by the total number of cells present in the fields.

Ethics statement

During the experimentation period, the animals remained under controlled temperatures of ±25°C, with humidity at ±50% and with a photoperiod of a 12-h light/dark cycle. Furthermore, all ethical principles, protocols, and regulations on experimentation with laboratory animals were used according to the standards established internationally and by the approved project by the Ethics Committee on Animal Use in Experimentation/State University of Maringá, following the Ethical Principles for Animal Experimentation established by the Brazilian College of Animal Experimentation (COBEA), as well as the specific treatment and collection protocols made to chromosomal aberration test.

Statistical analyses

Statistical analyses was performed using the chi-square test (χ^2) (N = 6, $\alpha = 0.05$).

RESULTS AND DISCUSSION

A large and growing use of medicinal plants and their extracts, especially *P. glomerata* and *G. biloba*, for the treatment of various diseases and the search for improved quality of life directed the present study to investigate the cytotoxicity of aqueous solutions of these plants on Wistar rats' bone marrow cells *in vivo*. The results indicate that the mutagenicity test of Brazilian ginseng showed no cytotoxic effects at any of the tested concentrations when compared to the control, as measured by the MI (PG: [0.15] $\chi^2 = 0.07$; [1.5] $\chi^2 = 0.16$; [15] $\chi^2 = 0.01$). The same effect was observed for treatment with *G. biloba* (GB: [1] $\chi^2 = 0.01$; [2] $\chi^2 = 0.01$; [3] $\chi^2 = 0.09$) (Table 1). With respect to the cytotoxicity analyses in the antimutagenicity test, no changes in the MI for treatment with different concentrations of the two plants were observed compared to the control (CP $\chi^2 = 0.08$; PG [1.5] $\chi^2 = 0.06$; PG: SIM $\chi^2 = 0.22$, PRE $\chi^2 = 0.25$, POST $\chi^2 = 0.19$; GB [2] $\chi^2 = 0.004$; GB: SIM $\chi^2 = 0.09$, PRE $\chi^2 = 0.29$, POST $\chi^2 = 0.13$) (Table 1).

Table 1. Mitotic index (MI) for the mutagenicity and antimutagenicity tests with *Pfaffia glomerata* (PG) and *Ginkgo biloba* (GB) aqueous solutions in Wistar rat bone marrow cells.

Treatments	*Pfaffia glomerata*		*Ginkgo biloba*	
	Groups	MI (%) ± SD	Groups	MI (%) ± SD
Mutagenicity	CO	1.43 ± 0.17	CO	1.43 ± 0.17
	CP	1.13 ± 0.13	CP	1.13 ± 0.13
	PG [0.15]	1.11 ± 0.14	GB [1]	1.56 ± 0.19
	PG [1.5]	1.92 ± 0.23	GB [2]	1.33 ± 0.16
	PG [15]	1.29 ± 0.15	GB [3]	1.08 ± 0.13
Antimutagenicity	CO	1.41 ± 0.17	CO	1.41 ± 0.19
	CP	1.08 ± 0.13	CP	1.08 ± 0.13
	PG [1.5]	1.12 ± 0.14	GB [2]	1.33 ± 0.16
	PG SIM	0.85 ± 0.10	GB SIM	1.06 ± 0.13
	PG PRE	0.82 ± 0.12	GB PRE	0.77 ± 0.09
	PG POST	0.89 ± 0.11	GB POST	0.99 ± 0.12

Mutagenicity test - CO: control group (1 mL water/100 g bw); CP: cyclophosphamide 1.5 mg/mL; PG: 0.15, 1.5, or 15 mg/mL per 100 g bw; GB: 1, 2, or 3 mg/mL per 100 g bw. Antimutagenicity test - CO: control group (1 mL water/100 g bw); CP: cyclophosphamide 1.5 mg/mL; PG: 1.5 mg/mL per 100 g bw; GB: 2 mg/mL per 100 g bw; treatments: simultaneous (SIM), pretreatment (PRE), and post-treatment (POST) to cyclophosphamide.

Studies with *P. glomerata* are scarce in the literature. However, studies with *Pfaffia paniculata*, which is morphologically and chemically similar to Brazilian ginseng, have revealed that this plant possesses chemotherapeutic effects, reducing cell proliferation and increasing the rate of apoptosis in induced hepatocellular carcinoma in BALB/c mice, indicating a cytotoxic effect of the root (Silva et al., 2010). Nagamine et al. (2009) also observed cytotoxic effects of butanolic extract from *P. paniculata* in human breast carcinoma (MCF-7) cells and demonstrated the degradation of cytoplasmic components, the disappearance of mitochondria, rupture of the plasma membrane, and profound changes in the nuclear morphology. In this study, it is noteworthy that normal bone marrow cells were evaluated, which may explain the absence of cytotoxic effects of Brazilian ginseng extract, which is different from what was found in studies with tumor cells. Besides, the treatments in our study were performed *in vivo* and not *in vitro*.

Regarding the cytotoxic activity of *G. biloba*, Hecker et al. (2002) corroborate these results and found no cytotoxic effects for EGb761 in human keratinocytes (HaCaT) and epithelial cells from monkey kidney (LLC-MK_2). The authors also identified substances with

high cytotoxic potential that were derived from a ginkgolic acid (alkylphenols). However, these substances appear in concentrations up to 5 ppm in commercial extracts, which, according to the authors, would be insufficient to promote cytotoxicity.

In the mutagenicity test, we evaluated the potential of the Brazilian ginseng and *G. biloba* solutions to induce chromosomal aberrations. Additionally, in the antimutagenicity test, we evaluated the antimutagenic and protective or reductive activity of the cyclophosphamide-induced clastogenicity in the bone marrow cells of Wistar rats. The major chromosomal abnormalities found in the analysis of metaphase were chromatic (cb) or isochromatic (ib) breaks, chromatic (cg) or isochromatic (ig) gaps, acentric fragments (af), minutes (mn), and double minutes (dm).

Despite the lack of cytotoxicity in this study, the chemotherapeutic drug cyclophosphamide, which is highly clastogenic and pro-oxidant (Colvin, 1999), was effective in inducing chromosomal damage in the mutagenicity and antimutagenicity tests (Figure 1) when compared to control (CP: χ^2 = 499.60 and 1336.36, respectively). The aberrations observed in this treatment were 6 cg + 46 cb + 27 af for mutagenicity, and 29 cg + 4 ig + 16 cb + 14 ib + 20 af + 30 mn + 15 dm for antimutagenicity tests.

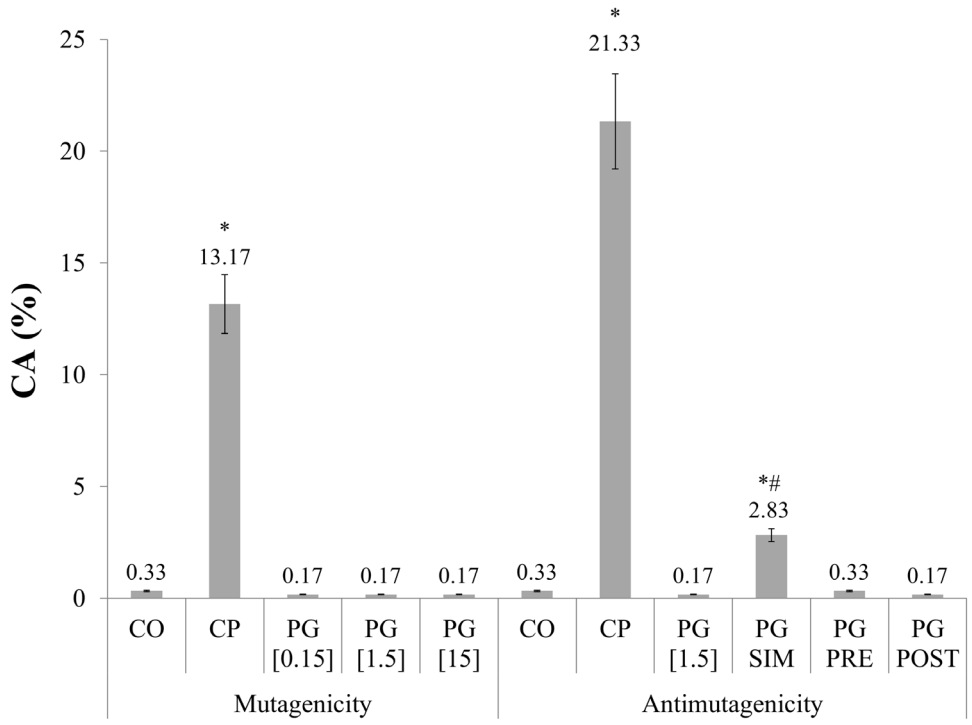

Figure 1. Chromosomal aberration (CA) (mean percentage and standard deviation) for the mutagenicity and antimutagenicity tests with *Pfaffia glomerata* (PG) aqueous solutions in bone marrow cells of Wistar rats. Mutagenicity test - CO: control group (1 mL water/100 g bw); CP: cyclophosphamide 1.5 mg/mL; PG: 0.15, 1.5, or 15 mg/mL per 100 g bw. Antimutagenicity test - CO: control group (1 mL water/100 g bw); CP: cyclophosphamide 1.5 mg/mL; PG: 1.5 mg/mL per 100 g bw, in simultaneous treatment (SIM), pretreatment (PRE), and post-treatment (POST) cyclophosphamide. *Results were statistically significant compared to CO. #Results were statistically significant compared to CP.

Figure 1 shows the percentage of chromosomal aberrations found in animals treated with the three concentrations of Brazilian ginseng, which showed no mutagenic potential and was statistically similar to the control (PG: [0.15] $\chi^2 = 0.08$; [1.5] $\chi^2 = 0.08$; [15] $\chi^2 = 0.08$; damage was represented, respectively, by 1 cg, 1 cb, and 1 cg). In the antimutagenicity test, the results show that the plant extract significantly reduced the percentage of damage induced by cyclophosphamide (PG: SIM $\chi^2 = 16.04$; PRE $\chi^2 = 20.67$; POST $\chi^2 = 20.99$). Cyclophosphamide-induced damage was reduced by 87% for simultaneous treatment, 98% for pretreatment, and 99% for post-treatment with PG. However, only the simultaneous treatment was not statistically equivalent to the control (PG: SIM $\chi^2 = 18.94$; PRE $\chi^2 = 0.09$; POST $\chi^2 = 0.08$). The chromosomal aberrations that were found using PG treatment were 12 cb + 5 cg (SIM), 2 cb (PRE), and 1 cb (POST).

The present study demonstrates, for the first time, that Brazilian ginseng aqueous solution lacks mutagenic effects and exhibits antimutagenic potential. The combined effect of its components may have been responsible for the protective action of the medicinal plant against cyclophosphamide-induced chromosomal damage in the bone marrow cells of Wistar rats *in vivo*. Studies performed with substances isolated from this plant strengthen the protective effect reported here. Nakai et al. (1984) isolated pfaffic acids and pfaffosides and verified their inhibitory activity on the proliferation of melanoma cell lines *in vitro*. Villaseñor et al. (2002) did not observe mutagenic activity and found an antimutagenic effect of sitosterol, observing a significant reduction (61.7%) in the number of micronuclei induced by tetracycline in mouse polychromatic erythrocytes. Furthermore, Wangari-Talbot et al. (2012) studied the effects of ecdysone, an important component of the Brazilian ginseng root, and elucidated the mechanism of RNA interference that inhibits membrane receptors related to the proliferation of human melanoma cells *in vivo* and *in vitro*, showing great therapeutic potential for this disease.

Figure 2 shows the percentage of chromosomal abnormalities found for the treatment with the aqueous solution of *G. biloba*, which, at the three concentrations tested, also showed no mutagenic potential and was statistically similar to the control (GB: [1] $\chi^2 = 0.07$; [2] $\chi^2 = 0.07$; [3] $\chi^2 = 0.08$; GB [3] damage was represented by 1 cg and GB [1] and [2] showed no alterations. Therefore, a single damage event was attributed to statistical calculations). In the antimutagenicity test, the results showed that the plant significantly reduced the percentage of cyclophosphamide-induced damage (GB: SIM $\chi^2 = 12.00$; PRE $\chi^2 = 13.01$; POST $\chi^2 = 12.00$). The decrease in the alterations caused by cyclophosphamide for *G. biloba* was 75% for simultaneous and post-treatment and 78% for the pretreatment. However, this reduction was not statistically equivalent compared to control (GB: SIM $\chi^2 = 75.76$; PRE $\chi^2 = 57.08$; POST $\chi^2 = 75.76$). The alterations found in GB treatment were: 6 cg + 3 ig + 10 cb + 5 ib + 1 af + 5 mn + 2 dm (SIM), 4 cg + 3 ig + 10 cb + 4 ib + 1 af + 5 mn + 1 dm (PRE), and 7 cg + 3 ig + 7 cb + 1 ib + 6 af + 8 mn (POST).

The time of exposure to a particular agent can be crucial for the occurrence of adverse effects and have even more serious consequences for the health of the individual. In contrast to the results presented here with acute *G. biloba* treatment, the National Toxicology Program (NTP) presented an extensive study performed with mice and rats chronically treated with commercial *G. biloba* extracts for 3 months or 2 years. It was found that a significant number of animals developed several types of cancer involving organs such as the thyroid, liver, bone marrow, and respiratory system, and severe nephropathology (NTP - National Toxicology Program, 2013). Lin et al. (2014) also found evidence of carcinogenesis in rodents

induced by commercial *G. biloba* extracts. However, the authors suggest that further studies should be developed, primarily to ensure the quality of these products that are extensively commercialized around the world.

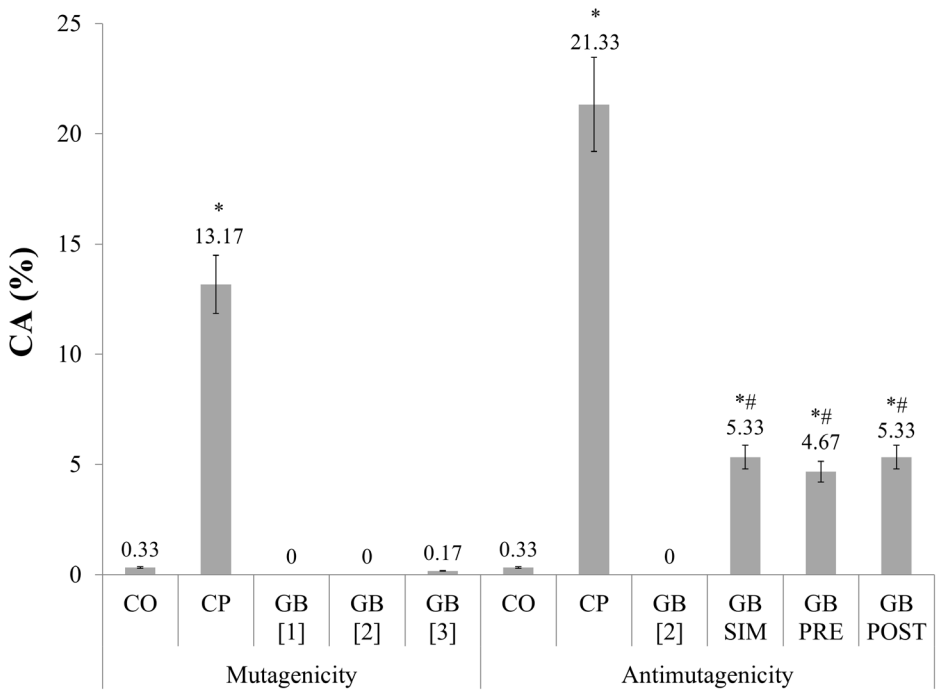

Figure 2. Chromosomal aberration (CA), reported as a mean percentage and standard deviation, for the mutagenicity and antimutagenicity tests with *Ginkgo biloba* (GB) aqueous solutions in Wistar rats' bone marrow cells. Mutagenicity test: CO: control group (1 mL water/100 g bw); CP: cyclophosphamide 1.5 mg/mL; GB: 1, 2, or 3 mg/mL per 100 g bw. Antimutagenicity test: CO: control group (1 mL water/100 g bw); CP: cyclophosphamide 1.5 mg/mL; GB: 2 mg/mL per 100 g bw), in simultaneous treatment (SIM), pretreatment (PRE), and post-treatment (POST) to cyclophosphamide. *Results were statistically significant compared to CO. #Results were statistically significant compared to CP.

Despite the findings of adverse effects, other studies show beneficial effects from the use of *G. biloba* extracts, which corroborate the antimutagenic effects presented in this paper. The anticlastogenic effect of the EGb761 extract was verified by the micronucleus test in patients with hyperthyroidism that underwent radiation therapy with iodine-131. It was found that supplementation reduces the genotoxic damage induced by radiation, without compromising the clinical recovery of the patients (Dardano et al., 2007). Sener et al. (2006) analyzed the same radioprotective effect in which the *G. biloba* extract reduced the oxidative damage induced by ionizing radiation and the level of DNA fragmentation in the ileum of rats supplemented for 15 days before exposure to radiation.

The antimutagenic activity of the EGb761 extract was also evaluated in the photosynthetic flagellate *Euglena gracilis* (Krizková et al., 2008). In this test, the extract was capable of efficiently adsorbing the acridine orange molecule, which is responsible for inducing DNA damage in chloroplasts. This adsorption capacity occurred primarily through the carbohydrates

present in the extract, which can also be present in the aqueous solution of the plant and have aided in reducing the cyclophosphamide-induced mutagenicity observed in this study.

Based on the mechanism of action for the chemotherapeutic drug, it is possible that the protective effects observed in the antimutagenicity test were caused by the antioxidant activity of the components of the analyzed medicinal plants, mainly pfaffic acids and ginkgoflavonoids, which have the ability to capture the reactive oxygen species produced by cyclophosphamide. Moreover, these results corroborate those of Zhang et al. (2008), who used cyclophosphamide as the damaging agent and identified antigenotoxic and antiapoptotic effects of saponins isolated from *Panax ginseng* (also present in *P. glomerata*) in mouse bone marrow cells and peripheral blood lymphocytes using the comet assay. According to the authors, they also observed the increased activity of antioxidant enzymes that are normally inhibited in the presence of cyclophosphamide.

In the present study, the antimutagenic effects of aqueous solutions from both medicinal plant extracts and their phytocomplexes were evident. However, the protective actions on the DNA exerted by the Brazilian ginseng were higher than those observed for *G. biloba*, particularly when administered before (PRE) or following (POST) the application of cyclophosphamide, presenting results similar to the control. According to the mechanism of action, plant components can protect the sites in DNA that would be affected by the agent, inhibit the metabolic activation of pro-mutagens or scavenge reactive molecules in simultaneous and pretreatment. During post-treatment, the antimutagenic substance acts upon the process that induces the formation of mutations or on the mechanisms that repair DNA damage (Kada et al., 1978; Kuroda et al., 1992). The analysis of these results showed that both mechanisms of action might have contributed to the effective antimutagenic activity of Brazilian ginseng and *G. biloba*.

CONCLUSIONS

The results presented here at the chromosomal and cytological levels in the mammalian *in vivo* model, *Rattus norvegicus*, which has a metabolism similar to that of humans, indicate that the aqueous solutions of the dried extracts of medicinal plants, *Pfaffia glomerata* and *Ginkgo biloba*, do not present cytotoxic or mutagenic activity. These extracts are popular worldwide for increasing cognitive, stimulant, and antioxidant capacity in humans. Most importantly, the results showed that these two plants have an effective antimutagenic/protective action, significantly reducing the DNA damage induced by the chemotherapeutic drug cyclophosphamide. This beneficial property of the medicinal plants presented here can efficiently contribute to improving the quality of life and the recovery of people undergoing chemotherapeutic treatment, or assist those looking for health and preventive habits.

Conflicts of interest

The authors declare no conflict of interest.

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