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Antigenotoxicity protection of *Carapa guianensis* oil against mitomycin C and cyclophosphamide in mouse bone marrow

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ABSTRACT

The aim of this study was to evaluate the possible protective of *C. guianensis* oil against MMC and CP, which are direct- and indirect-acting chemical mutagens, using the micronucleus test. Three experiments were performed. First the *C. guianensis* oil was co-administered to mice at doses of 250, 500 and 1000 mg/kg bw with 4 mg/kg bw MMC or 50 mg/kg bw CP. Second, the mutagenic drug (CP) was administered ip 50 mg/kg bw and after 6 and 12 hours 250 and 500 mg/kg bw of *C. guianensis* oil were administered. In the last, *C. guianensis* oil was administrated (250 and 500 mg/kg bw) during five days and after it was administered ip 50 mg/kg bw CP. The results obtained showed that the *C. guianensis* oil is not cytotoxic neither genotoxic to mouse bone marrow. Regarding the antimutagenic effect, all doses of *C. guianensis* oil were significantly ($p < 0.05$) effective in reducing the frequency of micronucleated polychromatic erythrocytes, when compared with MMC or CP alone. Based on these results, our results suggest that the *C. guianensis* oil shows medicinal potential as an antimutagenic agent, modulating the mutagenicity caused by both direct- and indirect-acting chemical mutagens, in a mammalian model.

Key words: antigenotoxic, *Carapa guianensis*, micronucleus, oil.

INTRODUCTION

Medicinal plants have been used in folk medicine for a long time and they have played a promising role in the treatment and prevention of various human diseases (Al-Asmari et al. 2014). Besides of the folk use, the medicinal plants also are abundant sources of biologically active compounds, many of which have been used as material to develop new pharmaceuticals products (Palombo 2011). Among these compounds, there are phytochemicals with potential application as natural antigenotoxic or antimutagens compounds. The antigenotoxic

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substances might prevent cancer because they could destroy mutagens in or out of body cells or block mutagens which damage DNA and cause mutation in cells (Hong et al. 2011). Actually, a growing number of studies have identified different plants extracts with antigenotoxic potential, such as: *Mentha longifolia* (Al-Ali et al. 2014), *Dioscorea pentaphylla* (Prakash et al. 2014), *Lilium candidum* (Jovtchev et al. 2014), *Eucalyptus Gunnii* (Bugarin et al. 2014), *Camellia sinensis* (Bhattacharya et al. 2014), *Curcuma longa* (Liju et al. 2014), *Celtis iguanaea* (Borges et al. 2013), *Solanum paniculatum* (Vieira et al. 2013), *Synadenium umbellatum* (Melo-Reis et al. 2011), *Ginkgo biloba* (Vilar et al. 2009), and others. Thus, there has been growing interest in finding and using natural plant products to reduce genotoxic and/or carcinogenic effects.

Among the various plants used in folk medicine all over the world there is *Carapa guianensis*. In Brazil, it is known as Andiroba, Carapa or Carapinha (Corrêa 1984). The specie has several uses and the quality of its wood and the oil extracted from its seeds are well-known (Hammer and Johns 1993). Especially the oil extract from seeds of *C. guianensis* has been empirically used in the treatment of arthritis, rheumatism, infections, wounds, bruises, skin diseases and as an insect repellent (Brito et al. 2013, Hammer and Johns 1993). Scientific studies also identify antiplasmodial (Bickii et al. 2000, Miranda et al. 2012), analgesic (Penido et al. 2005), anti-inflamatory (Penido et al. 2006), anti-allergic (Ferraris et al. 2011) activities from the *C. guianensis* oil. Besides the widespread use of *C. guianensis* by the population, there is little information about the mutational potential.

One way to evaluate the genotoxic or antigenotoxic potential of natural compound extracted of medicinal plant is the micronucleus assay (MN). The MN assay is based on the frequency of MN, structures that originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division (Fenech et al. 1999). Thus, MN may arise from either DNA breakage leading to acentric chromosome fragments or from chromosome/chromatin lagging in anaphase (Fenech et al. 2011). The formation of MN is considered to be an effective biomarker of diseases and processes associated with the induction of DNA damage (Garaj-Vrhovac et al. 2008, Yadav and Jaggi 2015).

The aim of this study was to evaluate the antigenotoxic potential of different concentrations of of *C. guianensis* oil on mouse bone marrow cells, to provide some clues about the preferential way of action that could occur when co-administered with mitomycin C (MMC), a direct-acting agent that does not require any molecular activation, or cyclophosphamide (CP), an indirect-acting agent, which needs to be converted enzymatically to its active metabolites. In addition, we evaluate the antigenotoxic effect of pos and pre-administration of *C. guianensis* oil in animals exposed to CP.

MATERIALS AND METHODS

CHEMICAL AND DRUGS

The chemical and drugs used in the experiment were the following: mitomycin C (MMC, 5 mg, Bristol-Myers Squibb®, New York, NY, USA, Lot No. 2.JO1299), cyclophosphamide (CP, $C_7H_{15}Cl_2N_2O_2P$, Baxter®), Leishmann solution dye, fetal bovine serum (Laborclin Products Laboratories, Paraná, Brazil)

ANIMALS

This study was approved by the Ethics Committee on the use of animals at the Pontifícia Universidade Católica de Goiás (Protocol nº 0003-1/2014). Seventy five healthy male outbred mice of the species *Mus musculus* belonging to the Swiss Webster strain were used. The mice had body weight varying from 30 to 40 g, and they were 45 to 60 days

old on the day of the experiment. The animals were placed in standard individual polypropylene cages with solid floors that were covered with sterilized wood chips according to international standards. The animals were housed in an environment with an average ± SD temperature of 24 ± 2°C and a relative humidity of 55 ± 5%. The light-dark cycle was 12 h:12 h, and water and food were available *ad libitum*.

EXPERIMENTAL PROCEDURE (MICRONUCLEUS ASSAY)

The seventy five animals were divided in 15 groups with five animals each. Three experiments were performed to evaluate the mutagenicity and anti mutagenicity potential *C. guianensis* oil: co-treatment, post treatment and pre-treatment.

To evaluate the antimutagenic potential of andiroba oil, three groups of animals were intraperitoneally (ip) co-treated with 250, 500 and 1000 mg/kg bw *C. guianensis* oil and 4 mg/kg bw of MMC. In addition, three other groups were ip co-treated with 250, 500 and 1000 mg/kg bw of *C. guianensis* oil and 50 mg/kg bw of CP. Two groups of positive controls were included in the experiment: one was administered 4 mg/kg bw of MMC and the other 50 mg/kg bw of CP. In addition, two groups of negative controls were included: one receiving soy oil (0.1 ml/10g bw) and the other *C. guianensis*.

The procedure selected was based on the study by Arrebola et al. (2012), who used the micronucleus assay to assess *C. guianensis* oil in rats (orally administered for 14 days) at doses of 400, 1000 and 2000 mg/kg. Of the three doses administered in the presente study, one was lower (250 mg/kg) and the other higher (500 mg/kg) than the minimum dose (400 mg/kg) applied by Arrebola et al. (2012).

In the post-treatment experiment, two groups of five animals were initially ip administered 50 mg kg b.w. of CP and after 6 and 12 hours, received 250 and 500 mg/kg bw of *C. guianensis* oil, respectively. A positive control (50 mg/kg bw of CP) and a negative control (0.1 ml/10g bw of soy oil) were included. In the pre-treatment experiment 250 and 500 mg/kg bw of *C. guianensis* oil were administered for five days to two groups of five animals. Two hours after the last oil treatment, 50 mg/kg bw of CP was ip administered. The negative control was treated with 0.1 ml/10g bw of soy oil for five days and the positive control with 50 mg/kg bw of CP. For all experiments, after the treatment period (24 hours), mice femurs were dissected and the bone marrow gently flushed out with fetal calf serum, and centrifuged (300 g, 5 minutes). The bone marrow cells were smeared on glass slides, coded for blind analysis, air-dried, and fixed with absolute methanol for 5 minutes. To detect MNPCE frequency, we fixed the smears with Leishmann solution (Ribeiro et al. 2003), prepared two slides for each mouse, and scored 1000 polychromatic erythrocytes (PCE) per slide. The results were the average of two slides. To determine cytotoxic activity, we simultaneously computed 1000 normochromatic erythrocyte and polychromatic erythrocyte frequency.

STATISTICAL ANALYSIS

In order to analyze the mutagenic and antigenotoxicity activity of *C. guianensis* oil, was using one way analysis of variance (ANOVA), followed by a multiple comparison procedure (Tukey test). To evaluate the cytotoxicity of *C. guianensis* oil, the polychromatic erythrocytes/normochromatic erythrocytes ratio (PCE/NCE) of all treated groups was compared to the result obtained in the mutagenic effect evaluation for the negative control group, and the results found in the antimutagenic effect evaluation for the positive control, using qui-square test ($\chi 2$).

For both tests the P values lower than 0.05 ($p < 0.05$) were considered indicative of statistical significance.

RESULTS

The results of the co-treatment with *C. guianensis* oil and MMC or CP are shown in Table I. Negative control group (soya oil) showed low MNPCE values, as expected, and positive control group showed a significant increase in MNPCE compared with the negative control group ($p < 0.05$) and no significant difference in the frequency of MNPCE and EPC/ENC was observed between mice treated with positive controls (MMC and CP alone), these data confirm the sensitivity of the test.

No significant difference in the frequency of MNPCE and EPC/ENC ($p > 0.05$) was observed between mice treated with *C. guianensis* oil (1.000 mg/kg bw) and the negative control (soya oil). The PCE/NCE ratio is an indicator of cytotoxicity. Then, our results suggest that *C. guianensis* oil is not cytotoxicity to mouse bone marrow.

The *C. guianensis* genotoxic activity assessment showed that none of the doses tested (250, 500 and 1.000 mg/kg bw) caused increases in the MNPCE frequency when compared with that CP and MMC groups ($p > 0.05$), indicating no genotoxic effect under these experimental conditions. In addition, groups which were co-treated with 250, 500 and 1.000 mg/kg bw of *C. guianensis* oil and mutagenic drugs (CP and MMC) showed a significant reduction in the frequency of MNPCE and EPC/ENC ratio when compared to CP and MMC ($p < 0.05$). These results showed that *C. guianensis* oil modulated genotoxic activity of CP and MMC at all tested concentrations, demonstrating its antigenotoxic effect.

The results of post-treatment with *C. guianensis* oil and CP are shown in Table II. Negative control group (soya oil) and positive control (CP) showed a significant difference in the frequency of MNPCE and EPC/ENC ratio which confirm the sensitivity of the test. In addition, the MNPCE and EPC/ENC ratio in the *C. guianensis* oil group (250 and 500 mg/kg bw) were significant smaller that the CP group. This result suggests that *C. guianensis* showed antigenotoxic activity when compared with the positive controls.

The results of pre-treatment with *C. guianensis* oil and CP are shown in Table III. The control test, *C. guianensis* oil (500 mg/kg bw), showed no significant difference in the frequency of MNPCE and EPC/ENC ($p > 0.05$) when compared with negative control. Also, it was found that doses of 250 and 500 mg/kg of *C. guianensis* oil and CP (50 mg/kg) showed significant difference in the frequency of MNPCE when compared with the positive control. The greater reduction in the number of micronuclei was found at 500 mg/kg dose (average 7.6) and the highest EPC/ENC ratio (0.75).

DISCUSSION

Identifying substances that provide protection against mutations is a topic of great interest, once antigenotoxic substances might prevent cancer because they could destroy mutagens in or out of body cells or block mutagens which damage DNA and cause mutations in cells (Hong et al. 2011).

As result, both drugs are genotoxic agents because they and their metabolites can bind DNA, causing damage that may result in chromosome breaks, micronucleus formation and cell death (Ahmadi et al. 2008).

The results obtained in the present study showed that the *C. guianensis* oil is not cytotoxic to mouse bone marrow. The PCE/NCE ratio of the group pre-treated with *C. guianensis* oil (500 mg/kg bw) and co-treated with *C. guianensis* oil (1.000 mg/kg bw) showed no significant difference from the negative controls. Some essential oils showing different levels of cytotoxicity exhibited

TABLE I
Frequency of micronucleated polychromatic erythrocytes (MMPCE) and polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio in mice bone marrow cells treated with different doses of the *C. guianensis* oil and co-treated with cyclophosphamide(CP) or mitomycin C (MMC) and their respective controls.

Groups	Treatment	MNPCE		
		Individual data (MNPCE/1.000PCE)	Means ± SD (MN/1000 PCE)	Ratio PCE/ NCE
1	soy oil (0.1 ml/10g b.w)	3-2-3-5-4	3.4 ± 1.01 [a c]	0.9 [a c]
2	cyclophosphamide (CP, 50 mg/kg bw)	18-20-17-18-19	18.4 ± 1.02	0.5
3	*C. guianensis* oil (250 mg/kg bw) + CP (50 mg/kg)	15-17-17-16-13	15.4 ± 1.49 [a]	0.6 [a]
4	*C. guianensis* oil (500 mg/kg bw) + CP (50 mg/kg)	15-12-16-11-15	13.8 ± 1.93 [a]	0.61 [a]
5	*C. guianensis* oil (1.000 mg/kg bw) + CP (50 mg/kg)	12-11-17-13-15	13.6 ± 2.15 [a]	0.62 [a]
6	Mitomycin C (MMC, 4 mg/kg bw)	19-20-23-21-19	20.4 ± 1.5	0.5
7	*C. guianensis* oil (250 mg/kg bw) + MMC (4 mg/kg)	11-15-13-14-12	16.4 ± 1.41 [c]	0.6 [c]
8	*C. guianensis* oil (500 mg/kg bw) + MMC (4 mg/kg)	14-13-12-14-15	14.0 ± 1.01 [c]	0.62 [c]
9	*C. guianensis* oil (1.000 mg/kg bw) + MMC (4 mg/kg)	13-11-11-12-14	12.2 ± 1.16 [c]	0.7 [c]
10	*C. guianensis* oil (1.000 mg/kg bw)	4-2-3-4-6	3.8 ± 1.32 [f]	0.9 [f]

Negative control = soya oil (0.1 ml/10g bw), Positive controls = cyclophosphamide (CP, 50 mg/kg bw) and Mitomycin C (MMC, 4 mg/kg bw), and Test control = *C. guianensis* oil. All results were compared to the respective control group. [a] Statistical difference compared to CP control group ($p < 0.05$), [b] No statistical difference compared to CP control group control ($p > 0.05$), [c] Statistical difference compared to MMC control group, [d] No statistical difference compared to MMC control group, [e] Significant difference compared to negative control ($p < 0.05$), [f] No significant difference compared to negative control ($p > 0.05$).

TABLE II
MMPCE frequency compared to the PCE/NCE ratio in mice bone marrow cells exposed to CP and post-treated with *C. guianensis* oil in two concentrations.

Groups	Treatments	MNPCE		
		Individual data (MNPCE/1.000PCE)	Means ± SD (MN/1000 PCE)	Ratio PCE/ NCE
1	soy oil (0.1 ml/10g bw)	3-2-3-5-4	3.4 ± 1.01 [a]	0.93 [a]
2	cyclophosphamide (CP, 50 mg/kg.bw)	18-20-17-18-19	18.4 ± 1.02	0.51
3	*C. guianensis* oil (250 mg/kg bw) + CP (50 mg/kg)	15-14-12-16-15	14.4 ± 1.35 [a]	0.61 [a]
4	*C. guianensis* oil (500 mg/kg bw) + CP (50 mg/kg)	12-12-15-14-13	13.2 ± 1.16 [a]	0.63 [a]

Negative control = soya oil (0.1 ml/10g bw), Positive controls = cyclophosphamide (CP, 50 mg/kg bw).
[a] Significant difference compared to positive control ($p < 0.05$), [b] No significant difference compared to positive control ($p > 0.05$).

TABLE III
MMPCE frequency compared to the PCE/NCE ratio in bone marrow cells of mice pre-treated with *C. guianensis* oil by five days and after exposed to CP.

Groups	Treatments	MNEPC		
		Individual data MN /1000EPC	Means ± SD MN/1000 EPC	Ratio EPC/ENC
1	soy oil (0.1 ml/10g bw)	4-3-4-2-3	3.2 ± 0.48 [a]	0.93 [a]
2	cyclophosphamide (CP, 50 mg/kg bw)	18-20-17-18-19	18.4 ± 1.02 [c]	0.51 [c]
3	*C. guianensis* oil (500 mg/kg bw)	3-3-3-2-2	2.6 ± 0.48 [d]	0.92 [d]
4	*C. guianensis* oil (250 mg/kg p.c.) + CP (50 mg/kg)	9-10-12-12-11	10.8 ± 1.16 [a]	0.68 [a]
5	*C. guianensis* oil (500 mg/kg bw) + CP (50 mg/kg)	8-8-7-6-9	7.6 ± 1.02 [a]	0.75 [a]

Negative control = soya oil (0.1 ml/10g bw), Positive controls = cyclophosphamide (CP, 50 mg/kg bw), and Test control = *C. guianensis* oil (500 mg/kg bw).
[a] Significant difference compared to positive control ($p < 0.05$), [b] No significant difference compared to positive control ($p > 0.05$), [c] Significant difference compared to negative control ($p < 0.05$), [d] No significant difference compared to negative control ($p > 0.05$).

different antioxidative capacities depending on the composition of the oil and especially on their phenolic content (Bakkali et al. 2006). The absent of cytotoxicity of *C. guianensis* is in agreement with Pereira et al. (2014) who demonstrated that this oil is not toxic to human fibroblast cells. The cytotoxic property is of great importance in the applications of essential oils not only against certain human or animal pathogens or parasites but also for the preservation of agricultural or marine products.

Several studies with various essential oils or their main components have demonstrated that, generally, most of them did not induce nuclear mutations (Bakkali et al. 2008). However, some exceptions should be identified, such as *Artemisia dracunculus* oil (Zani et al. 1991), *Mentha spicata* oil (Karpouhtsis et al. 1998), *Anethum graveolens* oil and *Pinus sylvestris* oil (Lazutka et al. 2001). In the present study, the MNPCE frequency of *C. guianensis* oil treated groups was very similar to negative control, suggesting that *C. guianensis* oil show no genotoxic activity for all doses tested (250, 500, and 1.000 mg/kg bw). Our result is in agreement with an earlier study that showed the absence of genotoxicity from *C. guianensis* oil in mice (Arrebola et al. 2012).

In addition, the antigenotoxic analysis showed in the co-treatment experiment that *C. guianensis* oil was able to significantly protect ($p < 0.05$) DNA from the mutagens when compared with each positive control (CP and MMC), at all doses tested. Also, the *C. guianensis* oil reduced the genotoxic effects induced by cyclophospamide (CP) in the pre and post-treatment groups. The antigenotoxic activity of the oil might be related to the presence of one or more components identified in *C. guianensis* oil. It is known that essential oils are complex mixtures of numerous molecules, and one might wonder if their antigenotoxic effects are the result of a synergism of all molecules or reflect only those of the main molecules present at the highest levels. Phytochemical analyses showed that *C. guianensis* oil contains natural myristic, palmitic, linoleic, oleic and stearic acids, as well as arachidonic fatty

acids, some tetranortriterpenoids among other components (Pereira et al. 1999, Qi et al. 2004).

The biochemistry of antimutagenic interference with promutagen metabolism to prevent mutagenesis is known and relatively well documented, as well as, the role and reactions of ROS scavengers (De Flora et al. 1999). *In vitro* physicochemical assays characterize most of essential oil as antioxidants (Collins 2005). According Evangelista et al. (2004), olive and canola oils have been showed the antigenotoxic effect. The anticlastogenic effect of olive and canola oils is associated with the antioxidant potential of linolic acids presents in both oils and which inhibit the free radicals. Also, there are reports that oleic acid causes a reduction in the levels of lipid peroxidation and have an antioxidant effect (Trueba et al. 2004) and conjugated linoleic acids have been efficient in inhibition of mammary tumourigenesis (Kritchevsky 2000).

Although this work did not provide the exact mechanism of action against the genotoxic effects of CP and MMC, it may be possible explained, at least partially, by the reduction of alkylation and/or the antioxidant actions exerted by *C. guianensis* oil, since CP and MMC activity is associated with its ability to alkylate the DNA and produce reactive free radicals (Shokrzadeh et al. 2014, Higgins et al. 2014).

According Surai (2015) the antioxidant defenses could include several options, such as: 1) decrease activity of pro-oxidant enzymes and improve efficiency of electron chain in the mitochondria and decreasing electron leakage leading to superoxide production, 2) prevention of first-chain initiation by scavenging initial radicals by inducing various transcription factors and synthesis of direct antioxidant enzymes, 3) activation and synthesis and increased expression of protective molecules, 4) binding metal ions (metal-binding proteins) and metal chelating, 5) chain breaking by scavenging intermediate radicals such as peroxyl and alkoxyl radicals, and others.

Other important result of our work was that *C. guinensis* antigenotoxic potential was dose dependent showed in all experiments. Among the doses tested, the most effective for antigenotoxic activity was 500 mg/kg bw used in pre-treatment, since it had the lowest number of MNs and higher EPC/ENC ratio. In the co-treatment, the dose of 1000 mg/kg bw was more efficient in reduce genotoxic potential of CP and MMC. Ours results is in agreement with different studies that have shown the dose dependent effect of essential oils in medicine (Zanandrea et al. 2004, Mendonça and Onofre 2009).

CONCLUSIONS

In conclusion, our results suggest that the *C. guianensis* oil shows medicinal potential, acting as an antimutagenic agent, modulating the mutagenicity caused by both direct- and indirect-acting chemical mutagens, in a mammalian model.

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