Phytochemical Analysis, in vitro Antioxidant Capacity and Toxicity Assessment of Copaifera paupera Oleoresin

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Introduction

The use of herbal medicinal plant products and phytonutrients had been increased in the last decade; the public interest and acceptance of their use as alternative and complementary medicine is not only in developing countries but also in developed ones. One of the major reasons of the use of herbal medicine on the developed countries is the belief that it will promote a healthier living, a source of natural product medication. That’s the reason why more than half of the natural product–derived compounds that are undergoing clinical trials for ingredients of development drugs are derived from plants, being the plants the source of most of the active ingredients of traditional medications.

The World Health Organization (WHO) reports that almost 80% of the world’s population uses medicinal plants for their primary health care needs, that represents up to four billion people of developing countries. According to Organization Panamericana de la Salud, a medical plant is any wild or cultivated plant used to prevent, relieve, cure or modify a normal or pathological physiological process or as a source of drugs or their precursors. This kind of medicine is based in the inherited knowledge that has been passed down from generation to generation that are culturally linked, providing a cheaper and easier access than the modern drugs and also, the trust on their communities traditions. Therefore, is necessary scientific information of effectiveness and safety of medicinal plants to avoid serious side effects. (opinion of the author is not clear)

ABSTRACT

Introduction: The present study was taken to determine the phytochemical analysis, the antioxidant activity in vitro (FRAP and TBARS methodologies), the oral acute toxicity and antigenotoxicity of the oleoresin Copaifera paupera. Methods: The oleoresin was tested for in vivo cytoprotective capacity using the Micronucleus Test and the Comet Assay in mice bone marrow cells and mice erythrocytes cells, respectively. Results: The oleoresin had low levels of total flavonoids and phenol content and also of antioxidant capacity. Orally, the LD50 appeared to be > 5000 mg/kg (no toxic or low toxic). The results showed all the doses evaluated (180 360, 720 and 1440 mg/kg) antigenotoxic effect by reduce the DNA damage induced by cyclophosphamide, being a 100 % DNA damage reduction at the highest dose evaluated. Conclusion: According to the Micronucleus test, the oleoresin Copaifera paupera had the ability of increase the cell proliferation despite the exposition of cyclophosphamide.

Keywords: Copaifera paupera, Oleoresin, Phytochemical analysis, Antigenotoxicity, Micronucleus test, Comet assay.
double-strand breaks, incomplete excision repair and alkali-labile site in frequencies less than 1 in 10^7 bases. However, the Micronucleus test can detect other types of damage corresponding to the chromosome breaks and aneuploidy and is visualized as a small chromatin mass in the cytoplasm beside the nucleus. Being chromosome breaks and aneuploidy considered of relevance in carcinogenesis events.

The aim of this study was to determine the phytochemical profile, antioxidant capacity, acute oral dose toxicity and also, their protective potential against cyclophosphamide using Micronucleus test and Comet assay of ethanolic extract from _Copaifera paupera._

**MATERIAL AND METHODS**

**Plant material**

The plant material (oleoresin) was collected by the Instituto Nacional de Investigaciones Agrarias (INIA–Pucallpa) in June 2016 from Estación Experimental Forestal “Alexander von Humboldt” (8°23’00’’S 74°33’00’’O/–8.383333, -74.55) at Coronel Portillo – Ucayali. The plant was taxonomically authenticated in situ by Dr. Eloy Cuellar (Instituto Nacional de Investigaciones Agrarias).

**Extract preparation**

The extraction for oleoresin was performed according Herrera-Calderon et al. with modifications. Four grams of oleoresin was extracted with 80 mL ethanol (80%) at 37°C for 3 h with the use of a shaking water bath. The extract was centrifuged at 5000 RPM for 5 min and the supernatant was evaporated with a rotavap and then dried at 39°C for 2 h. Finally, 0.1 mg of solid residue was diluted in a mix of 2 mL of water and 1 mL of DMSO, this mix was used for phytochemical analysis and antioxidant capacity.

**Phytochemical analysis and antioxidant capacity**

The total content of flavonoids and phenolic compounds were analyzed according to the method described by Herrera-Calderon et al. respectively. The antioxidant capacity assessment was determined using different methodologies: FRAP and TBARS.

**Animals**

For Acute Oral toxicity experiment, male Holtzman rats weighing 180–190 g (6 – 8 weeks) were obtained from the School of Philosophy and Science from Universidad Peruana Cayetano Heredia. During the experiment, the bioterium was at 19.7°C, with relative humidity of 67% and a dark natural cycle. For Micronucleus Test and Comet Assay, healthy, young, mice (_Mus musculus_) between 6 -10 weeks old, weighing 25-35 g were supplied by the animal house of the National Institute of Health (Lima, Peru). The animals were brought to the animal facilities of the university seven days before the experiments and kept in boxes in air-conditioned room, at 23°C (±2°C) and relative humidity of 50 % (±10%), with a 12-h light/dark natural cycle, were fed with a pelleted diet (Universidad Agraria La Molina, Perú) and water ad libitum.

The experiments followed national and international standards of management and experimentation with animals.

**General Experiment Procedures**

For Acute Oral toxicity, according to the OECD guideline for testing chemicals 423, the animals were treated and observed for 14 days. The treated group (3 rats) was administrated with 2000 mg/Kg/day by using an intragastric cannula; the control group was fed with 1 mL distilled water. The animals were weighed at the beginning of the experiment, day 7 and at the end; then they (animals) were sacrificed by cervical dislocation for the macroscopic organ analysis. During all the experiment (14 days), abnormal behavior was recorded.

For Micronucleus Test and Comet Assay, 5 mice (3 females, 2 males) were used for each treatment group and control and the experiment lasted 12 days. The extract preparation was administrated to the animals by gavage daily for 11 days. The animals received different doses of plant extract (180, 360, 720 and 1440 mg/kg). One hour before the 11th plant extract dose, cyclophosphamide (50 mg/kg) was administrated by intraperitoneal injection to each animal of each treatment group, also the positive control group. The negative control group was administrated orally with the diluent DMSO 10% and no bioactive substances.

**Micronucleus test**

The Micronucleus test was conducted following the protocol proposed by Schmid with minor modifications. After the sacrifice on 11th day, bone marrow was extracted from the femur with physiological serum enriched with (long form followed by short form) BSA 10% and centrifuged at 1500 rpm for 5 min. The supernatant was eliminated and the pellet resuspended and smeared on slides to let dry and fixed with cold methanol for 10 min. The slides were stained with Giemsa 2% for microscopic analysis at 1000X using a Nikon Eclipse 50i microscope. The criterion for scoring MN was established by the OECD guideline 474. Two-thousand polychromatic erythrocytes (1000 cells per each replicate) were scored for each sample. In addition, 500 cells (polychromatic and normochromic erythrocytes) were scored to determine the Cytotoxic Index (CTI) (number of polychromatic erythrocyte/number of normochromic erythrocyte).

**Alkaline comet assay**

The comet assay was developed as previously described by Singh et al. with minor modifications. 40 uL of blood were carefully resuspended in 110 ul of 0.5% Low Melting point Agarose (LMA), layered onto microscope slides pre-coated with 100 ul of 0.5% normal melting point agarose (NMA) (dried at 65°C) and covered with a coverslip and kept at 4°C until solidification. The coverslips were removed and cells were lysed for 2 h at 4°C in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding and exposure of alkali-labile sites, slides were placed for 20 min in a horizontal gel electrophoresis tank filled with a freshly cold electrophoresis solution. Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. After electrophoresis, slides were neutralized with two washes each 5 min with 0.4 M Tris-HCl (pH 7.5), fixed with cold absolute ethanol for 3 min and stored in the dark at room temperature until scoring. Just before the microscope analysis, slides were stained with 50 uL of Hoescht 33258 (50 ug/mL) a 400X magnification at a Nikon Eclipse 50i fluorescence microscope. One hundred randomly cells (50 cells from each of the two replicate slides) were analyzed per sample. These cells were visually analyzed according to classes, ranging from undamaged (0) to highly damage (4), the arbitrary unit ranged from 0 (completely undamaged; 100 cells x 0) to 400 (completely damaged, 100 cells x 4).

**Statistical analysis**

The difference between the negative control or the positive control and the different treatments were evaluated by the analysis of Medias by using the one-way ANOVA followed by Tukey’s post-hoc test. The statistical significance was 05%, p<0.05. The percentage reduction reflected the reduction of damage of the positive control (cyclophosphamide, CP) induced by the treatment of copaiba oil and it was calculated as following:
RESULTS AND DISCUSSION

Phytochemical analysis and antioxidant capacity

The results of phytochemical analysis and antioxidant capacity are shown in Table 1. It is evident that the total phenols and flavonoid contents are low compared with fruits,24 the bark25 and leaf.26 In fact, Tincusi et al.27 reported twelve known diterpenes and three known sesquiterpenes, along with a new C15–C20 terpenoid, with a structure based on an unprecedented skeleton in which a labadaneterpene is linked to a monocyclic sesquiterpene by an ester bridge, which were isolated from the oleoresin Copaifera paupera from Perú. Baldisera et al.28 told that β-caryophyllene is the major component presents in the essential oil from the oleoresin, although, Bardajie et al.29 argue that the main component is the β-Bisabolene. Leandro et al.30 supported that this oleoresin is a solution of diterpenoids, especially, mono and di-acids, solubilized by sesquiterpene hydrocarbons. The sesquiterpenes and diterpenes (labdane, clerodane and kaurane skeletons) are different for each Copaifera species and have been linked to several reported biological activities, ranging from antitumor to embriotrophic effects. So all the biological activities of the oleoresin come from these compounds and do not relate with phenolic acids and flavonoids.

Acute Oral toxicity

Oral administration of copaiba oil did not produce any mortality or abnormal behavioral changes on rats during the 14 days of experiment. Neither body weight or pathology change compared to the control group was observed. Orally, the LD50 appeared to be > 5000 mg/kg. Based on LD50 value and according to the scale of Ahmed31 and the Global Harmonized system of classification and labelling of chemicals, the copaiba oleoresin would be classified as a Class 5 drug and, low or non-toxic category. This support is based on the knowledge of the millenial use of the oleoresin without report of human toxicity. This was the first report of toxicity of oil resin of C. paupera had done, nonetheless, Gomes32 showed that after 5 days of treatment of copaiba oils (500 mg/kg), no behavioral alterations or lesions or stomach bleeding were observed; with calculations of LD50 values of 3.9 and 4.3 g/kg to C. reticulate and C. multijuga, respectively; supporting the idea of the non-toxicity of copaibas oleoresin. Another kind of toxicity test was done by Sachetti et al.33 they reported that there’s no evidence of teratogenicity at the highest dose (1250 mg/kg of oleoresin C. reticulate).

Antigenotoxic potential (Micronucleus test and alkaline comet assay)

The Micronucleus test and Comet assay were developed to assess the genotoxic effect of chemical products or another substance but now, are used to determine the antigenotoxic potential of natural products too, especially medicinal plants. In one of the protocols to evaluate the antigenotoxic potential10 of a substance, first the biological system had been exposed to the extract plant for several days and in the last day, a exposure to a well know cytotoxin genotoxic had been. To evaluate whether the extract plant has antigenotoxic or cytoprotective potential, the damage reduction analysis is performed. Therefore, identifying possible antigenotoxic agents is important for the risk/benefit assessment of their potential use in humans.

Cyclophosphamide (CP) has been used as a positive control chemical in in vivo genetic toxicity test34 by inducing dominant lethal mutations, micronuclei, DNA damage and generation of free radicals. So, in the present investigation, oleoresin extract of Copaifera paupera was used to study its effect as preventive in the genotoxic damage induced by cyclophosphamide by measuring the capacity of damage reduction on Micronucleus test and Comet assay.

Previous research about the genotoxicity of different species of Copaiba reported the lack of genotoxicity of the genus Copaifera on the Micronucleus test. Maistro et al.35 and dos Santos et al.36 had shown that oleoresin of C. dukes and C. martii, respectively, were no genotoxic at the highest doses evaluated in mice. A recent study of oleoresins and leaves extract of 6 Copaifera species (that include C. paupera) showed no genotoxic effect in vivo and in vitro Micronucleus test. An assessment of a commercial Copaiba oil-resin37 reported no increase of MN frequency in bone marrow and peripheral blood mice, even at the maximum able dose for be evaluated (2000 mg/kg). Alves et al.38 evaluated the genotoxicity of C. langsdorffii hydroalcoholic leaf extract and shown also that the leaf extract was unable to increase MN frequency. All the data available supported the idea of absence of significant risk of Copaibaoleoresin, so it was time to investigate its possible antigenotoxic capacity. The results of the Micronucleus test on mice bone marrow were shown on Table 2, the pre-treatment of mice with oleoresin extract of C. paupera caused a reduction on the frequency of MNPE induced by cyclophosphamide in all the doses evaluated. Treatments of 180, 360, 720 and 1440 mg/kg body weight oleoresin extract of C. paupera caused a significant decrease in the MNPE frequency by 55% (10.80 ± 1.483), 72% (7.40 ± 1.516), 89.0 (10.80 ± 1.483), 72% (7.40 ± 1.516), 120% (0.60 ± 0.22) and 90% (1.20 ± 0.447) respectively; supporting the idea of absence of significant risk of Copaibaoleoresin, so it was time to investigate its possible antigenotoxic capacity. The results of the Micronucleus test on mice bone marrow were shown on Table 2, the pre-treatment of mice with oleoresin extract of C. paupera caused a reduction on the frequency of MNPE induced by cyclophosphamide in all the doses evaluated. Treatments of 180, 360, 720 and 1440 mg/kg body weight oleoresin extract of C. paupera caused a significant decrease in the MNPE frequency by 55% (10.80 ± 1.483), 72% (7.40 ± 1.516), 89.0 (10.80 ± 1.483), 72% (7.40 ± 1.516), 120% (0.60 ± 0.22) and 90% (1.20 ± 0.447) respectively; supporting the idea of absence of significant risk of Copaibaoleoresin, so it was time to investigate its possible antigenotoxic capacity. The results of the Micronucleus test on mice bone marrow were shown on Table 2, the pre-treatment of mice with oleoresin extract of C. paupera caused a reduction on the frequency of MNPE induced by cyclophosphamide in all the doses evaluated. Treatments of 180, 360, 720 and 1440 mg/kg body weight oleoresin extract of C. paupera caused a significant decrease in the MNPE frequency by 55% (10.80 ± 1.483), 72% (7.40 ± 1.516), 89.0 (10.80 ± 1.483), 72% (7.40 ± 1.516), 120% (0.60 ± 0.22) and 90% (1.20 ± 0.447) respectively; supporting the idea of absence of significant risk of Copaibaoleoresin, so it was time to investigate its possible antigenotoxic capacity.
Table 3: Arbitrary Units (AU) of DNA damaged in peripheral blood erythrocytes mice detected by the Comet assay.

<table>
<thead>
<tr>
<th></th>
<th>AU (mean ± SD)</th>
<th>AU reduction (%)</th>
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<tbody>
<tr>
<td>Negative control (DMSO 10%)</td>
<td>39.6 ± 10.87</td>
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</tr>
<tr>
<td>Positive control (CP 50mg/kg)</td>
<td>221.4 ± 20.42</td>
<td>--</td>
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<tr>
<td>Extract (180 mg/kg) + CP</td>
<td>93.8 ± 25.54</td>
<td>70.2</td>
</tr>
<tr>
<td>Extract (360 mg/kg) + CP</td>
<td>97.8 ± 20.57</td>
<td>67.9</td>
</tr>
<tr>
<td>Extract (720 mg/kg) + CP</td>
<td>39.4 ± 11.69</td>
<td>100</td>
</tr>
<tr>
<td>Extract (1440 mg/kg) + CP</td>
<td>20.0 ± 6.04</td>
<td>110.8</td>
</tr>
</tbody>
</table>

The comet assay involved the same groups of animals treated for the MN test. All results are means ± standard deviation (SD) from five mice. A total of 100 nuclei per animal were analyzed.

aSignificant difference from the negative control (p < 0.05).

bSignificant difference from the positive control (p < 0.05).

89% (4.00 ± 2.000) and 103% (1.20 ± 0.447), respectively compared to CP exposure (21.80 ± 5.403), showing the protective capacity of the oleo-resin extract. This antigenotoxic effect was also reported previously by Alves et al.37 with a significant decrease of the number of micronucleus induced by doxorubicin at 80 mg/kg of leaf extract.

Data from comet analysis of peripheral blood erythrocytes in mice appear in Table 3. Like the results of the Micronucleus test, all the doses of oleoresin extract reduced DNA damage induced by CP, being capable of reduce all the damage at 720 mg/kg of pre-treatment. There's no previous assessment of antigenotoxicity evaluated by Comet assay but one had evaluated the genotoxicity of commercial Copaiba-olein38 and did not increase DNA damage in liver cells of mice, even at concentration of 2000 mg/kg. Cavalcanti et al.39 had evaluated the kaurenic acid, a bioactive terpenoid present in Copaifera genus and according to their results, concentrations of 30 μg/mL and upper were genotoxic in V79 cells.

Another parameter that evaluated the Micronucleus test is the PCE/NCE ratio, known as Cytotoxic Index (CTI). This parameter is an indicator of the progression of cells from erythroblast through the PCE stage to NCE and determinates if the substance is able to accelerate or inhibit the erythropoiesis. The results of the present study showed that oleoresin C. paupera can mitigate the cytotoxicity of the CP and promotes the erythropoiesis in mice.

CONCLUSION

Our research is the first report of the antigenotoxicity of the Copaiba oleoresin. Both antigenotoxicity assessments showed the DNA damage repair ability of the oil-resin extract of Copaifera paupera. The kind of damage detected in the comet assay is primary DNA damage, which can be repaired fast and the one detected in the Micronucleus test is fixed DNA damage. So, the results demonstrated the cytoprotective potential of C. paupera oleoresin against two different types of DNA damage. Because it’s the first study carried out, is necessary to continue investigate its potential to confirm the results obtained and also, determine which of the bioactive compounds is responsible of these effects.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

CTI: Cytotoxic index; PCE/NCE: Number of polychromatic erythrocyte/number of normochromic erythrocyte; CP: Cyclophosphamide; MNPC: Polychromatic erythrocytes with micronuclei; FRAP: Ferric reducing antioxidant power; TBARS: Thiobarbituric acid reactive substances; LD50: Lethal dose 50.

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