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Original Article

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

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**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 $LD_{50}$ 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.

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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.[1] One of the major reason 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.[2] 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,[3] being the plants the source of most of the active ingredients of traditional medicaments.[4]

The World Health Organization (WHO) reports that almost 80% of the world's population trust on medicinal plant for their primary health careneeds,[4] that represents up to four billion people of developing countries.[5] According to Organizacion Panamericana de la Salud,[6] 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.[7] Therefore, is necessary scientific information of effectiveness and safety of medicinal plants to avoid serious side effects. (opinion of the author is not clear)

Peru is considered as a country with one of the highest biodiversities in the world, which has 10% of the global plant diversity that makes it a country with millennia history of plant use for treatment of diseases,[8-9] with many of them being used nowadays. According to the Instituto de Investigaciones de la Amazonia Peruana (IIAP), the Peruvian Amazonian jungle holds the greatest biological diversity worldwide, known by the medicinal components in the leafs of its plants but for some authors, is the Andean region where is the highest number of multiple use plant species.[10]

The *Copaifera* genus is distributed in the tropical regions of Latin America (40 species), West Africa (4 species) and Asia (1 species) and the oleoresin (copaiba oil) is produced by exudation of the trunk of the trees.[11] The Peruvian species that predominates in the Amazon region of the country is *Copaifera paupera*, it has been applied in folk medicine for many years by the natives. Recently, studies of *Copaifera* genus have shown anti-inflammatory, antitumor, antitetanus, antiseptic effects and useful in the treatments of various disease like asthma, ulcer, bronchitis, psoriasis and wound healing.[12-13] The single-cell gel electrophoresis (SCGE) assay or comet assay is a very simple, rapid, economic, versatile and sensitive technique for measuring primary DNA damage.[14] This technique is able to detect DNA damage like single and

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double-strand breaks, incomplete excision repair and alkali-labile site in frequencies less than 1 in $10^7$ bases.[15] However, the Micronucleus test can detect other types of damage corresponding to the chromosome breaks and aneuploidyand 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.*[16] 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 methoddescribed by Herrera-Calderon *et al.*[17] respectively.

The antioxidant capacity assessment was determined using different methodologies: FRAPand TBARS.[18]

### 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 12-h light/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.[19]

### General Experiment Procedures

For Acute Oral toxicity, according to the OECD guideline for testing chemicals 423,[20] 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 distillated 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 $11^{th}$ 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[21] with minor modifications. After the sacrifice on $11^{th}$ day ($11^{th}$), 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.[21] 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.*[22] 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);[23] 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:

$$\text{Reduction (\%)} = (A - B / A - C) \times 100$$

A: corresponds the media of damage in positive control; B: media of damage in the treatment (Copaiba oleoresin+ CP); and C: media of damage in the negative control.

## RESULTS AND DISCUSION

### Phytochemical analysis and antioxidant capacity

The results of phytochemical analysis and antioxidant capacity are shown in the Table 1. It is evident that the total phenols and flavonoid contents are low compared with fruits,[24] the bark[25] and leaf.[26] In fact, Tincusi *et al.*[27] reported twelve known diterpenes and three known sesquiterpenes, along with a new $C_{20}$-$C_{15}$ terpenoid, with a structure based on an unprecedented skeleton in which a labdanediterpene 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-caryophylene is the major component presents in the essential oil from the oleoresin, although, Bardaji*et al.*[29] argue that the main component is the β-Bisabolene. Leandro *et al.*[14] suported 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 antitumoral to embriotoxic effects. So all the biological activities of the oleoresin come from these compounds and do not relative with phenolic acids and flavonoids.

### Acute Oral toxicity

Oral administration of copaiba oil did not produce any mortality or abnormal behavioral response on rats during the 14 days of experiment. Neither body weight or pathology change compared to the control group was observed. Orally, the $LD_{50}$ appeared to be > 5000 mg/kg. Based on $LD_{50}$ value and according to the scale of Ahmed[30] 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 classification is supported by the knowledge of the millennial 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, Gomes[31] 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 $LD_{50}$ values of 3.9 and 4.3 g/kg to *C. reticulate* and *C. multiijuga*, respectively; supporting the idea of the non-toxicity of copaiba's oleoresin. Another kind of toxicity test was done by Sachetti *et al.*[32] 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 potential[33] 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 test[34] 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. dukei* 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-resin[36] 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.*[37] evaluated the genotoxicity of *C. langsdorffii* hydroalcaholic 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 MNPCE 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 MNPCE frequency by 55% (10.80 ± 1.483), 72% (7.40 ± 1.516),

**Table 1: Results of phytochemical analysis and antioxidant capacity.**

| | Total phenols (mg GAE/g) | Flavonoids (mg QE/g) | FRAP (umols Fe2+/g) | TBARS (mg MDA/g) |
|---|---|---|---|---|
| Plant extract | 0.49 ± 0.00 | 0.65 ± 0.00 | 2.428 ± 0.084 | 14.17 ± 0.37 |

**Table 2: Incidence of frequency of MNPCE, MNPCE reduction and CTI in mouse bone marrow.**

| | MNPCE/1000PCE (mean ± SD) | MNPCE reduction (%) | CTI (PCE/NCE mean ± SD) |
|---|---|---|---|
| Negative control (DMSO 10%) | 1.80 ± 1.095 | -- | 6.030 ± 1.019 |
| Positive control (CP 50mg/kg) | 21.80 ± 5.403[a] | -- | 0.0607 ± 0.022[a] |
| Extract (180 mg/kg) + CP | 10.80 ± 1.483[a,b] | 55.0 | 1.134 ± 0.407[a,b] |
| Extract (360 mg/kg) + CP | 7.40 ± 1.516[a,b] | 72.0 | 1.721 ± 1.654[a,b] |
| Extract (720 mg/kg) + CP | 4.00 ± 2.000[b] | 89.0 | 2.580 ± 0.269[a,b] |
| Extract (1440 mg/kg) + CP | 1.20 ± 0.447[b] | 103.0 | 3.297 ± 0.152[a,b] |

CP: cyclophosphamide; MNPCE: micronucleus in polychromatic erythrocytes; PCE: polychromatic erythrocytes; NCE: Normochromic erythrocyte; CTI: cytotoxic index. All values are means ± standard deviation (SD) from five mice.

[a]Significant difference from the negative control ($p < 0.05$).

[b]Significant difference from the positive control (($p < 0.05$).

**Table 3: Arbitrary Units (AU) of DNA damaged in peripheral blood erythrocytes mice detected by the Comet assay.**

| | AU (mean ± SD) | AU reduction (%) |
|---|---|---|
| Negative control (DMSO 10%) | 39.6 ± 10.87 | -- |
| Positive control (CP 50mg/kg) | 221.4 ± 20.42[a] | -- |
| Extract (180 mg/kg) + CP | 93.8 ± 25.54[a,b] | 70.2 |
| Extract (360 mg/kg) + CP | 97.8 ± 20.57[a,b] | 67.9 |
| Extract (720 mg/kg) + CP | 39.4 ± 11.69[b] | 100 |
| Extract (1440 mg/kg) + CP | 20.0 ± 6.04[b] | 110.8 |

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.

[a]Significant difference from the negative control ($p < 0.05$).

[b]Significant 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 oleoresin 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 Copaibaoil-resin[38] 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 kaurenoic acid, a bioactive terpenoid present in *Copaifera* genus and according to their results, concentrations of 30 ug/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[40] 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, determinate 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; **MNPCE:** Polychromatic erythrocytes with micronuclei; **FRAP:** Ferric reducing antioxidant power; **TBARS:** Thiobarbituric acid reactive substances; **$LD_{50}$:** Lethal doses 50.

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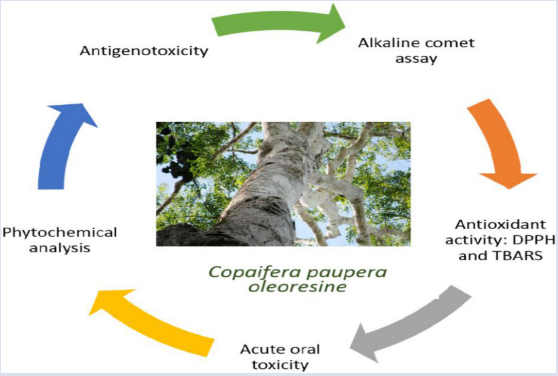
## GRAPHICAL ABSTRACT




## SUMMARY

- Copaiba oleoresin showed antigenotoxic effect *in vitro*.
- The results demonstrated the cytoprotective potential of *C. paupera* oleoresin against two different types of DNA damage.
- Treatments of 180, 360, 720 and 1440 mg/kg body weight oleoresin extract of *Copaifera paupera* caused a significant decrease in the MNPCE frequency by 55% (10.80 ± 1.483), 72% (7.40 ± 1.516), 89 % (4.00 ± 2.000) and 103% (1.20 ± 0.447), respectively compared to CP exposure (21.80 ± 5.403).

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