**In vivo genotoxic potential of the seed oleaginous extract of Carapa guianensis Aublet using the comet assay**

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**Abstract**

**Objective:** The seed oil extract of *Carapa guianensis* has various biomedical applications. Recently this extract was evaluated for expressing great potential as antioxidant on *in vivo* assays. Besides its safety has been evaluated in several genotoxicity assays, not being toxic in different models of DNA damage. The aim of this research was to evaluate the genotoxic potential of seed oil extract of *C. guianensis* on peripheral blood leukocytes of Sprague-Dawley rats using the comet assay.

**Methods:** Five experimental groups were formed consisting of five male and five female Sprague-Dawley rats in each group. A placebo group (2% Tween 65), three dose levels of the extract (400, 1000 and 2000 mg/kg) administered orally for 14 days, and a positive control group treated with cyclophosphamide (C\textsubscript{P}) at a dose of 50 mg/kg intraperitoneally 48 and 24 h before euthanasia were established. After the experimental period the animals were anesthetized and a blood drop was extracted for performing the comet assay of peripheral blood leukocytes; then the rats were euthanized under ether atmosphere.

**Results:** The results presented no differences between controls and extract-treated animals of both sexes in the percentage of nucleoids at different levels, arbitrary units and length of DNA migration. However, validating our result, the CP-treated animals showed significant DNA damage.

**Conclusion:** The seeds oil extract of *C. guianensis* did not exert genotoxic effect as measured through the comet assay on peripheral blood leukocytes of male as well as female Sprague-Dawley rats.

**INTRODUCTION**

*Carapa guianensis* from Meliaceae family, is a very popular medicinal plant in several countries of the world; in Cuba it is known as Cedro Macho. The characterization of the *C. guianensis* seed oil has revealed the presence of different fatty acids and some tetraterpenoids. Phytochemical studies of the extracts show a majority presence of polyunsaturated fatty acids and phenolic compounds such as tannins and limonoids [1-5].

It has been observed that tetraterpenoids obtained from *Carapa guianensis* seed has a significant antiallergic activity, given by nuclear factor (NF)-κB inhibition, interleukin (IL)-5 and chemokine ligand (CCL)-11 (eotaxin) suppression [4, 5]. Recently, the antioxidant potential, the capacity for protecting the skin from the sun and the insecticide effect of the oleaginous extract of *C. guianensis* have been described [6-10].

The antioxidant effect of the oleaginous extract of *C. guianensis* has been demonstrated previously by means of the oral administration of the extract during three weeks in Sprague-Dawley rats from both sexes co-administered with an oxidizing substance [10]. However, it is already necessary to carry out the evaluation in other levels of security, such as in classic toxicity assay of first and second barrier [11].
It is known that the extract does not induce micronucleus formation in bone marrow cells in mice [12] neither interferes in mitosis nor increases spontaneous frequency of chromosome aberrations in hematopoietic cells using Balb/c mice from both sexes as biomodel at 2000 mg/kg oral administration for 14 days [13]. However, when evaluated in the sperm, head morphology assay represented cytotoxicity in the spermatic cells, but it was not genotoxic at 2000 mg/kg dose, administered by oral route for 35 days in Balb/c male mice [14].

The present research aimed to evaluate the genotoxic potential of the *C. guianensis* oleaginous seed extract to DNA primary structure of peripheral blood leukocytes in Sprague-Dawley rats from both sexes detected by comet assay. Sprague-Dawley rats were used since they are the best biomodel for this assay according to different studies comparing Balb/c mice to other lines of rats. This rat line showed the lowest results in DNA damage by alkaline comet assay [15].

**MATERIALS AND METHODS**

**Animals and environmental conditions**

During the entire experimental process the established ethical principles for the research with laboratory animals were respected. Young adult Sprague-Dawley rats from both sexes were used (6-8 weeks old) of which corporal weight ranged from 180-200 g at the end of quarantine. They were kept under controlled conditions: temperature 23 ± 2°C, relative humidity 60 ± 5% and cycles of light-darkness of 12 h. Access to water and food (CENPALAB, Havana, Cuba) was *ad libitum*. These characteristics were common for all the experimental groups evaluated in this assay.

**Obtainment of the oleaginous extract**

N-Hexane was added to a ratio of 2 kg of seed of *C. guianensis* previously dried and crushed. It was allowed to stand for 60 min and then it was vacuum filtered. The solvent was added until the botanical sample of the oil was saturated and then, it was removed by reducing pressure. The yield of the extract was 29% (v/w). The apparent density of the oil was calculated (0.81 g/ml) and this result was used to define the exact volume that each animal received. The oil was stored at 20°C until its use.

**Administration and dosage**

The oleaginous extract was suspended in Tween 65 (2%) 2 h before the administration and the concentrations were adjusted weekly in function of the corporal weight increase. It was decided to use the oral route because it matches with the therapeutic proposal and also that will be used in the rest of the preclinical toxicology evaluations.

Animals were distributed randomly (5 rats/group/sex) into 5 experimental groups: one group was the vehicle control (Tween 65, 2%), three groups were treated with *C. guianensis* oleaginous extract (400, 1000 and 2000 mg/kg), and a positive control group was treated with cyclophosphamide (CP).

Emulsions performed with the extract and vehicle were administered by gastric intubations (2 ml/kg) for 14 days, schedule from 10:00 to 11:00 a.m. CP was administered in two doses (50 mg/kg) intraperitoneally (i.p.) 48 and 24 h before euthanasia, in the schedule from 10:00 to 11:00 a.m [16, 17]. The low dose of the extract has been used in non clinical pharmacology studies [4, 8, 18, 19], which has demonstrated to be effective in the model against *Leishmania*, uterus cancer and possess antioxidant effects; the additional two superior levels (1000 and 2000 mg/kg) were multiples of the pharmacological dose [18, 19].

**Clinical observations**

Two daily clinical observations were performed, in the schedule of 8:30-10:30 a.m and in the afternoon at 3:00-4:30 p.m, which allowed the observation of the general clinical state of the animals including palpation to detect lesions, possible affectations of the nervous, cardiovascular and gastrointestinal systems, skin state, hair, coloration of the mucous and the eyes.

**Assessments of the comet assay of peripheral blood leukocytes**

The method of euthanasia selected was ether atmosphere to verify the total loss of the reflections. After anesthetizing, 15-20 µl of tail total blood were extracted from the animals. Alkaline electrophoresis of individual cells was performed according to standardized protocols and adjusted as described earlier [20]. The total of 15-20 µl of samples were suspended in 140 µl of low melting point agarose at 0.5%; then agarose was added to previously prepared sheets. They were submerged in lysis solution at pH 10 for 1.5 h at 4°C and subjected to 20 min of denaturalization in electrophoresis regulatory solution pH >13. Electrophoresis was carried out at 30 mA and 1 V per cm from 18 to 20 min [21, 22]. The sheets were washed with neutralization regulatory solution using Tris buffer 0.4 M at pH 7.5 and clarified with distilled water and later they were tinted with silver nitrate at 0.05% [20, 21].

The nucleoids were evaluated using a light transmission microscope (Olympus BH-2) by three independent analysts, to establish an average between readings. The visual analysis includes the quantification of 100 comets per animal from the center of each gel [21]. Each comet was classified into DNA damage from 0 to 4. The magnitude of the DNA damage was expressed in arbitrary units (AU) [20, 21].
The comparisons investigating E differed statistically, with values among 74.42-105.71±2xTCG for both sexes. This value varied from 79.14 to 80.1±5.02 in animals treated with C at the lowest dose (400 mg/kg). Higher doses (1000-2000 mg/kg) resulted between 76.44-80.46% for both sexes.

The percentage grade 4 damaged nucleoids in the control groups resulted between 0.98-1.02. The animals treated with the minimum dose of the extract (400 mg/kg) exhibited 1.22-1.31%, and those treated with the maximum dose (2000 mg/kg) presented 1.44-161% of grade 4 nucleoids; these values are close to the control levels and do not differ statistically (P > 0.05).

In animals treated with CP, grade 0 damage was 30.25-31.33% and grade 4 damage 3.55-3.9%; all values were significantly higher than those of the extract-treated animals (P < 0.05). The highest values of DNA damage achieved with the administration of CP were recorded as damage grade 1 and 2 (see Table 1), as it is described for this mutagen.

The AU value in the animals of control groups was among 30.51-35.03 and in the animals treated with the maximum dose of the extract among 35.61-37.27; these values did not differ statistically (P > 0.05). However, the AU levels of the animals treated with CP were much higher than controls and extract-treated animals, varying among 105.71-106.93 in both sexes (P < 0.05).

The migration longitudinal of DNA was also similar between control animals and those treated with the extract values from 50.41-57.10 µm for both sexes (P > 0.05). However, animals treated with CP experienced a considerable increase in the longitudinal of damage, inferring bigger number of ruptures in single DNA chains, with values among 74.42-79.69 µm (P < 0.05).

In summary, none of analyzed variables showed significant statistical differences between sexes, neither at the minimum nor at the highest dose of the extract. Fig.1 shows some representative comet images taken during the study; briefly, a total degradation of DNA was observed in Fig.10 obtained from an animal treated with two doses of CP.

### Table 1. Comet assay results in peripheral blood leukocytes of Sprague-Dawley rats treated with Carapa guianensis extract or cyclophosphamide (positive control)

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Sex</th>
<th>Migration longitude of DNA (µm)</th>
<th>Arbitrary units (AU)</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>F</td>
<td>50.41±3.99</td>
<td>30.51±14.1</td>
<td>71.98±4.35</td>
<td>10.47±3.18</td>
<td>5.17±2.9</td>
<td>2.26±1.43</td>
<td>0.98±0.44</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>52.88±4.28</td>
<td>35.03±8.9</td>
<td>78.21±9.1</td>
<td>13.19±4.77</td>
<td>4.98±2.33</td>
<td>2.6±1.26</td>
<td>1.02±0.83</td>
</tr>
<tr>
<td>CP</td>
<td>F</td>
<td>79.69±8.11*</td>
<td>106.93±15.66*</td>
<td>31.33±3.64*</td>
<td>45.09±2.33*</td>
<td>12.80±3.32*</td>
<td>6.88±1.34*</td>
<td>3.9±1.23*</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>74.42±10.08*</td>
<td>105.71±13.11*</td>
<td>30.25±2.41*</td>
<td>46.99±3.26*</td>
<td>13.11±2.97*</td>
<td>6.1±1.06*</td>
<td>3.55±2.5*</td>
</tr>
<tr>
<td>OECG (400 mg/kg)</td>
<td>F</td>
<td>54.41±4.21</td>
<td>34.80±10.24</td>
<td>79.14±4.32</td>
<td>11.86±4.02</td>
<td>5.37±3.1</td>
<td>0.98±0.44</td>
<td>1.31±1</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>57.1±1.28</td>
<td>33.46±7.51</td>
<td>80.1±5.22</td>
<td>11.23±5.56</td>
<td>5.02±0.89</td>
<td>2.45±1.2</td>
<td>1.22±0.98</td>
</tr>
<tr>
<td>OECG (1000 mg/kg)</td>
<td>F</td>
<td>55.72±2.12</td>
<td>33.19±10.31</td>
<td>80.46±6.59</td>
<td>10.78±5.11</td>
<td>5.17±3.15</td>
<td>2.29±1.51</td>
<td>1.33±0.99</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>52.33±5.01</td>
<td>33.58±8.11</td>
<td>79.2±3.42</td>
<td>12.09±4.16</td>
<td>4.85±3.28</td>
<td>2.51±1.1</td>
<td>1.35±0.68</td>
</tr>
<tr>
<td>OECG (2000 mg/kg)</td>
<td>F</td>
<td>55.91±3.44</td>
<td>35.61±7.33</td>
<td>77.46±5.1</td>
<td>15.12±2.96</td>
<td>3.21±3.01</td>
<td>2.72±1.33</td>
<td>1.44±1.12</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>57.01±2.21</td>
<td>37.27±5.82</td>
<td>76.44±6</td>
<td>15.67±2.31</td>
<td>3.88±2.99</td>
<td>2.5±1.03</td>
<td>1.61±0.53</td>
</tr>
</tbody>
</table>

All values were presented as average ± SD. F, female; M, male; VC, vehicle control (Tween 65, 2%); CP, cyclophosphamide; OECG, oil extract of Carapa guianensis. *P < 0.05 for comparisons with the negative control group in the same sex (Mann Whitney U test).
DISCUSSION

The present and earlier results demonstrate that the evaluated extract does not cause damages in the primary structure of the DNA [15]. Several researchers have reported several biomedical uses of oleaginous extract of \textit{Carapa guianensis}, and its systemic toxicity in single and repeated doses [18, 19] has also been evaluated by various authors; however, the present report of the damage it causes to the primary structure of the DNA, determined by the alkaline comet assay in leukocytes of peripheral blood, is novel. Since results are not different between treatments, the non-existence of dose-dependence is also demonstrated.

The genotoxicity results obtained so far demonstrated that the maximum dose of 2000 mg/kg, did not modify micronucleus frequency, chromosomal aberrations, cells with polyploidy and mitotic index in bone marrow cells of Balb/c mice [12, 13]. The results in all group treated with the extract were similar to basal DNA damage levels of Sprague-Dawley biomodels [15].

The results of damage induced with CP, also match the literature when this mutagen is used with positive control in genotoxicity assays using the same route and rat line [15]. CP duplicated the results of both the controls and the animals treated with the extract in almost all analyzed variables, but this increase was mainly observed for grade 1 and 2 nucleoides [15, 22]. The significant increase in leukocytes of the animals treated with CP in the longitude of DNA revealed bigger DNA ruptures; it has also been demonstrated that these variables are directly proportional [22]. On the other hand, it is known that oleaginous extracts are rich in unsaturated fatty acids and terpenoids as major components have not caused damage to the DNA as measured in this assay. It has been demonstrated that some terpenoids are usually genotoxic, though it does not mean that the extracts of this compound also have this characteristic.

\textit{In vivo} studies using Sprague-Dawley rats have shown that the extract was able to protect DNA from the damage for oxidative stress generated by CP [10]. The extract was able to reverse the damage for oxidative stress induced by CP in all cases; results already obtained with natural products with majority components very similar to those of the evaluated extract [23].

The D-004 lipid extract from the Cuban royal palm (\textit{Roystonea regia}) is rich in polyunsaturated fatty acid and this extract was not genotoxic when evaluated on \textit{in vivo} alkaline comet assay in NMRI mice, administered in repeated oral doses for 14 days [23]. The Vimang® extract obtained from \textit{Mangifera indica} bark is rich in terpenoids was also not genotoxic when evaluated using the complete battery of genotoxicity at different levels of DNA damage expression, that includes cellular and non-cellular comet assays, moreover, it was also evaluated in SOS assay and it did not cause damage to prokaryotes cells [24, 25].
The results obtained in this assay in the animals treated with the extract match also those of the D-003 extract which is rich in aliphatic alcohols. The antioxidant properties have been demonstrated, where the capacity of inducing damage on DNA of Sprague-Dawley rats in the alkaline comet assay was evaluated [26].

We have not determined yet the mechanisms of the oxidizing effect of the extract, nevertheless experimental results show a decrease in the levels of DNA damage when oxidative stress has been induced with CP [10]. It is known that the metabolism at hepatic level of the fatty acids generates ROS as consequence of the beta-oxidation that can damage DNA [27].

Obtained results could assure that the treatment with the oleaginous extract from C. guianensis under experimental conditions did not cause increase of ROS or any other compound up to high concentrations that could increase the strand breaks and alkali-labile sites formation on DNA. Changes in the activity of the catalase enzyme, the most important enzyme in detoxification of peroxide of hydrogen and main ROS generated during beta oxidation [28], were observed in studies conducted to demonstrate the antioxidant effects of this extract.

It has been shown that some flavonoids and highly antioxidant substances as terpenoids, such as dioximnium and quercetin, correct oxidizing effect of the extract, nevertheless we have not determined yet the mechanisms of the detoxification of peroxide of hydrogen and main ROS. The antioxidant substances as terpenoids, such as diosminum and quercetin, correct the oxidizing effect of this extract. [10].

In conclusion, the seed oil extract from Carapa guianensis did not have DNA genotoxic potential measured by the alkaline comet assay in peripheral blood leukocytes of male and female Sprague-Dawley rats.

REFERENCES

COMPETING INTERESTS
Authors declare that this work was carried out on the bases of good practices of preclinical laboratory present in the national regulation of protocols approval of research in the Cuban republic. It is also declared on the part of the authors that the consent of protocol approval was obtained when this research began. The authors not declare any conflicts of interest.

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