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Genotoxicity of Goji Berry (Lyciumbarbarum) In Vivo Mammalian Cells

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ABSTRACT: *Lyciumbarbarum (Gojji berry) belongs to family Salonaceae which is found in China and Himalayan. This herb is used to prevent various diseases and in medical treatments as an alternative medicine being widely used for its antioxidant and revitalizing potential effects. In recent years, Gojji has become increasingly popular in Europe and North America as a "superfruit" and dietary supplement. The belief that herbal products do not bring any risk to health, is part of popular culture. However the term "natural" assigned to many products cannot assure no health risk. The aim of this study was to evaluate the possible genotoxic effects of aqueous extract of Lyciumbarbarum (Gojji berry) by micronucleus test and comet assay. Thirty Rattus norvegicus were divided into three equal groups: 1) experimental group, submitted to Gojji berry (200mg/kg orally); 2) positive control group (cyclophosphamide), and; 3) negative control group (distilled water). Micronucleus Tests were done by smear method of bone marrow cells performed after 48h for acute, and 72h for chronic exposure. The comet assay was performed on peripheral blood taken from the tail of each animal 4h, and 24h after intervention. Cytotoxicity was assessed by observing the DNA damage measuring the percentage of DNA in the tail (% DNA- measurement of the proportion of the total DNA present in the tail) and the tail moment (TM-tail length times the percentage of DNA in the tail), calculated by 100 nucleoids per animal and the presence of micronuclei in 2,000 polychromatic erythrocytes per animal. Analysis of variance (ANOVA) followed by Tukey test at 5% significance was used comparing the results. The data showed no significant difference in the frequency of DNA damage and the number of micronuclei between the experimental group and the negative control group. The results also suggest that the aqueous extract of Lyciumbarbarum (Gojji berry) at the dose of 200 mg/kg showed no genotoxic effect, which could, to a certain point, justifies its use.*

Keywords: *Comet assay, Genotoxicity, Lyciumbarbarum, Micronucleu test.*

I. Introduction

Herbal medicine is the use of internal or external *in natura* plants or as medicines for treating diseases. Various parts of the plants is used such as roots, bark, leaves, fruits, seeds, and also by-products, such as essential oils, defined as volatile complex substances, lipophilic, and usually odoriferous and liquid, derived from the secondary metabolism of plants. These can be applied for several purposes, such as antibacterial, antiviral, antifungal, insecticidal, and as anti-inflammatory drug [1]. The belief that herbal products do not bring any risk to health is part of popular culture. However the term "natural" assigned to many products cannot assure no health risk [2].

Lyciumbarbarum (goji berry) is a plant of family *Solanaceae* found in China and Himalayan, for thousands years at the top of a table of the 8000 Chinese herbs believed to be healing foods. This fact is owed to its nutritional content and its ORAC (Oxygen-radical absorbance capacity), being effectively used to prevent various diseases, and also as an alternative medicine for medical treatments. Early studies performed in China praise its broad advantages for health, quoting from its effects on vitality and longevity to the strengthening and restoration of important organs like eyes, liver and kidneys. The polysaccharides presented in it can inhibit the arising and growth of sarcoma S180, and stimulate lymphocyte proliferation. These polysaccharides may also block cell cycle of liver tumors due to its antioxidant properties increasing intracellular calcium in their apoptotic systems, treat male infertility, promote decrease in blood glucose levels and intracellular cholesterol, and apparently its protective effects depend mainly on their antioxidant properties [3-5].

Once there are widespread reports of the use of this herb by many people worldwide, despite the scarce scientific evidence, this study aimed to evaluate the likely genotoxic effects of aqueous extract of *Lyciumbarbarum* (goji berry), by the micronucleus and comet test in Wistar rats.

II. Materials And Methods

2.1 Ethical aspects

The study was approved by the Ethics Committee for Animal Experimentation of the University of Piauí State (CEUA / UESPI) protocol number: 00101/2016.

2.2 Animals

Were used 30 *Rattus norvegicus*, aged 8 weeks and weighing 200g ± 20%, proceding from the vivarium of the College of Medical Sciences, University of PiauíState. During the experiment, each animal were randomly organized in appropriate polypropylene cages under controlled conditions (temperature around 22 °C to 24 °C, 40-60% humidity, and light/dark cycles of 12h each), with food and drink *ad libitum*.

2.3 Aqueous extract

The infusion was performed at a concentration of $1g/L^{-1}$ in hot water (70 °C) decanted into a bottle containing specimens of *Gogi Berry*, previously powdered and weighted for fifteen minutes [6]

2.4 Study groups

The animals were divided into three equal groups of 10 animals each distributed as follows: EG (experimental group); NC (negative control group), and; PC (positive control group). EG was orally by gavage subjected to the aqueous extract of *Lyciumbarbarum* (Goji Berry) at a dose of 200 mg/kg/day. NC group ingested distilled water and PC group ingested intraperitoneal cyclophosphamide at a dose of 50 mg/kg [7]. The experiment was carried out at the Laboratory of Mutagenicity (LABMUT) and at the Laboratory of Molecular Biology and Biological Injuries Study (LABMINBIO) of the Biotechnology and Biodiversity Research Core (NPBIO), University of Piauí State (UESPI).

2.5 Comet Assay

The comet assay was performed using peripheral blood taken from the tail of each animal after 4h after intervention to check the damage, and 24h after intervention to check the repair. At the end of each period 40 uL were collected, transferring them to microtubes containing 120 uL of low melting-point agarose (1.5%) at 37 °C. The mixture was homogenized and transferred to microscope slides pre-covered with Agarose and them covered with coverslips in order to homogeneously spread the content, than stored at 4 °C for 30 minutes [8]. Then the coverslips were removed and immersed in vertical glass cuvettes containing Lysing solution [NaCl (2.5M), EDTA (ethylenediaminetetraacetic acid) (100 mM), and 1.2g of TRIS (hydroxymethylaminomethane) (10mM)]. Upon use, was added 1% of Triton^{TM} X-100 and 10% of DMSO (Dimethyl sulfoxide). The slides were placed in a vessel containing electrophoretic buffer pH> 13.0 (300 mMNaOH and 1mM EDTA), prepared by10N NaOH and 200 mM EDTA, pH 10.0 stock solution), remaining at rest for 20 minutes. The electrophoretic separation was performed with 25 V and 300 mA at a temperature of 4 °C lasting 15 minutes, in the dark. After electrophoresis, the slides were removed from the chamber and immersed in neutralizing solution (0.4 M TRIS, pH 7.5, for 5 minutes). This process was repeated 3 times. Finally, they were rinsed with distilled water and then dried, and stained with GelRed (2.0:10,000 uLdilution for 10 minutes). All slides were analyzed by immunofluorescence microscopy (40x magnification) with excitation filter (420-490 nm) and barrier filter (520 nm). The images were obtained by the Opton^{TM} system (CCD 5.0 mega pixel digital camera for immunofluorescence). Were assessed the DNA damage measuring the tail % DNA (proportion of the total DNA present in the tail) and the tail moment (tail length times the percentage of DNA in the tail) [9]. These parameters were calculated by 100 nucleoids/animal (two slides per animal). For this, were used the software OpenComet^{TM}[10].

2.6 Micronucleus test

Half of the animals were euthanized by cervical dislocation for bone marrow extraction and subsequent micronucleus (MN) count in polychromatic erythrocytes (PCE), 48h after the intervention to verify the acute effect, and the other half 72h after the intervention to check the chronic effect. This procedure was based on the methods outlined by Schmid[11] and Hayashi [12]. Medullary components were removed using a subcutaneous syringe containing fetal bovine serum introduced through the medullary canal, pushing its contents toward the other edge, towards a 15 ml Falcon tube. Then, the sample was centrifuging it for 5 minutes at 1,000 rpm with fetal bovine serum to be homogenized. The supernatant was discarded and two drops of the sample were put at the end of a matte slide, using a Pasteur pipette, smearing it. Three slides were also made for each animal staining with Giemsa. Finally, the slides were assessed by an optical microscope, 100x zoom in immersion oil. The number of micronuclei in 2,000 polychromatic erythrocytes were counted for each sample.

2.7 Statistical analysis

Statistical analyzes were performed using GraphPadPrism™ version 5.0 (GraphPad Software, La Jolla, CA, USA), by analysis of variance (ANOVA) with Tukey post-hoc test, at a 5% significance level.

III. Results And Discussion

The genotoxicity is characterized by types of DNA damage (adduct of DNA basis, alkaline hydrolysis, double-strand breaks) and mutations, ranging from gene to structural or numerical chromosome abnormalities such as aneuploidy and polyploidy [7]. Carcinogenic potential studies using genotoxicity tests are on the rise. The expression of the modified gene, abnormal cell growth, disturbances in the functioning of a normal cell, all of these facts can be related to the genotoxic effects of industrial carcinogens and other potential genotoxic agents [2]. These phenomena can result in genomic instability and can possibly be carcinogenic [13]. To evaluate the risk for cancer, genetic damage can be determined by genotoxicity assays, including comet test, micronucleus test, chromosomal aberration test, gamma-H2AX and bacterial reverse mutation assay [14]. *In vitro* tests are considered as a genotoxicity assay for substances screening such as candidate drugs, herbal extracts, chemicals substances, etc. being also used to evaluate their initial security, while *in vivo* assays provide detailed biological and physiological information [15]. Genotoxicity tests officially approved by the Organization for Economic Cooperation and Development (OECD) include the bacterial reverse mutation assay, chromosomal aberration test, micronucleus test and comet assay [13].

In this study, two methods were used to investigate *in vivo* genotoxicity of the aqueous extract of *Lyciumbarbarum* (Goji Berry) at a dose of 200 mg/kg/day: the comet assay and micronucleus test. The comet assay quantifies injuries to DNA in individual cells and the micronucleus test indicates chromosomal instability [16]. Comet assay results did not indicate an increase in DNA damage at the firts 4h evaluated by the tail % DNA. This finding remained low even after 24h of the intervention. Once there were no sign of genotoxicity the substance cannot be classified as genotoxic. These findings is consistent with tail moment and the tail length (Table 1). On PC group, the tail % DNA tail, the tail length and tail moment were significantly higher compared to NC group which provides evidence that the study was clearly conducted and under suitable conditions [17].

Table 01: Comet test by tail % DNA, tail length and tail moment.

Parameters	PC group N= 05	NC group N=05	4h after intervention N=05	24h after intervention N=05
Tail % DNA	6.8± 1.1	1.1 ± 0.02[a]	0.8± 0.20[b]	0.76 ± 0.08[b]
Tail legth (μM)	17.0± 2.2	7.6 ±2.0[a]	8.1±1.8[b]	7.1± 1.12[b]
Tail Moment	1.9± 0.1	0.8± 0.63[a]	0.56± 0.88[b]	0.68± 0.66[b]

[a]p<0.05 compared to positive group;
[b] p<0.05 compared to positive group.

Tables 02 and 03 show MN average values found in each group for acute (table 02) and chronic (table 03) exposure. The results presented in both tables show that there is no significant difference comparing EG group to NC group but significant differences comparing to PC group (cyclophosphamide). This result corroborates to the findings of the comet assay (table 01), with no genotoxicity induction.

Table 02: MN average in 2000 polychromatic erythrocytes for acute exposure.

Treatments	Number of MNPCE per animal MNPCE PCE/NCE					(Mean ± SD)
	R1	R2	R3	R4	R5	
NC group	7	6	7	5	8	6.6± 1.140[a]
EG group	5	6	4	6	7	5.6± 1.140[a]
PC group	31	32	36	37	36	34.4± 2.702

[a]p<0,001 compared to PC group.

Table 03: MN average in 2000 polychromatic erythrocytes to chronic exposure.

Treatments	Number of MNPCE per animal MNPCE PCE/NCE					(Mean ± SD)
	R1	R2	R3	R4	R5	
NC group	13	14	13	12	9	12.20± 1.920[a]
EG group	12	15	13	15	9	12.80± 2.490[a]
PC group	49	45	50	31	51	45.20± 8.250

[a]p<0,001 compared to positive group.

The micronucleus test was first reported in 1970 by Boller and Schmid and was later used by Heddle in 1977. The micronucleus is an additional nucleus, separate from the main nucleus of a cell, during its division. It is

composed of whole chromosomes or chromosomal fragments that remain from other chromosomes after the completion of mitosis [18]. The micronuclei result from structural changes in the chromosome, spontaneous or experimentally induced, or even by cell fusion errors. However, these micronuclei are excluded from the new nuclei renewed at telophase [19-21].

On PC group, tail % DNA, tail length, tail moment, and micronucleus number of ECP was significantly higher compared to NC group. Thus, those findings suggest that all experimental tests were conducted clearly and under suitable conditions [17].

The data suggest that aqueous extract of *Lyciumbarbarum* (Goji Berry) at a dose of 200 mg/kg/day had no genotoxic effect, which could, to a certain point, justifies its use. However, it is suggested new studies and new methodologies to evaluate the safety in using the medical herb.

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