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Antimutagenic activity of *Carica papaya* L. assayed *in vivo* by micronucleus test

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ABSTRACT

There is little information about *in vivo* toxic effects of the ethanolic extract of leaves of *Carica papaya* L. (ECP). Therefore, in this study ECP was characterized chemically by HPLC-RP and the antimutagenic and cytotoxic activities of an aqueous solution of ECP (CA) were assessed by the micronucleus (MN) bioassay. The extract consisted mainly of polar substances, one of which was rutin. The MN test was performed on groups of Wistar rats, as follows: negative control (NC) - vehicle; positive control (CP40) - cyclophosphamide (40 mg/kg, ip), 24h; extract-treated (CA) - ECP (500 mg/kg, po), 24h; extract + cyclophosphamide-treated (CACP40) - ECP (500 mg/kg, po), 48, 36 and 24 h, plus cyclophosphamide (40 mg/kg, ip), 24h. The MN index was 3.2 ± 1.79 and 1.6 ± 0.5, for NC and CA, and the PCE-to-NCE ratio was 1.38 ± 0.52 and 1.13 ± 0.28, respectively, indicating low cytotoxicity of CA. CP40 showed a high MN index of 20 ± 4.9, but CACP40 only 3.0 ± 1.6, the same as NC, indicating an antimutagenic effect. The study suggests that ECP has low toxicity and possesses an antimutagenic protective effect in which rutin may be involved.

Keywords: Carica papaya. HPLC-RP. Micronucleus bioassay.

The use of medicinal plants to treat many diseases has been recorded in all regions of the world (WHO, 2000). The importance of popular knowledge about medicinal plants cannot be overestimated. However, their pharmacological properties and safety should be tested (WHO, 2000).

The safety of either a synthetic drug or an herbal medicine may be assessed by several toxicological assays. The 50% lethal dose (LD_{50}), chronic toxicity, genotoxic effect and mutagenicity assays are the most frequently used (Paulo et al., 2009). The *in vivo* micronucleus (MN) assay, performed on mouse bone marrow erythrocytes, has been officially used to evaluate damage caused by xenobiotic substances in the body (Schmid, 1976).

The MN bioassay can be used to test the chemopreventive action of drugs, when the drug is given before the administration of the mutagenic agent (Salvadori et al., 2003). Chemopreventive agents are synthetic or natural substances that are used to inhibit, retard or reverse the carcinogenic process (Sporn & Suh, 2000).

Cyclophosphamide is a cytotoxic drug that acts by alkylating DNA. The drug thus blocks DNA duplication completely, resulting in cell death, or incompletely, causing mutation (Vahlsing et al., 1977).

The fruits of *Carica papaya* L., known as papaya or pawpaw, are widely consumed, worldwide and in Brazil (Castro & Anjos, 2008). L-Lycopene and benzylisothiocyanate (Rossetto et al., 2008) were isolated and identified in the fruit of this species and α-tocoferol, carotenoid (Ching & Mohamed, 2001), lycopene (Breemen & Pajkovic, 2008) and flavonoids (Miean & Mohamed, 2001) were identified in the leaves of *C. papaya.* Several studies have evidenced the chemopreventive action of lycopene, flavonoids and benzylisothiocyanate (Etherton-Kris et al., 2002; Ferrari & Torres, 2002; Kuroiwa et al., 2006).

Biological activity has been reported in the stems, fruits and leaves of *C. papaya*, such as weak inhibition of angiotensin-converting enzyme (ACE), immunomodulation and cytotoxicity (Braga et al., 2007; Otsuki et al., 2010). Recently, the immunomodulatory and cytotoxic activities of an aqueous extract of *C. papaya* were detected in tumor cell and human peripheral blood cell lines (Otsuki et al., 2010).

However, data have not been published about either the *in vivo* cytotoxic effect or the antimutagenic effect of *C. papaya*. Therefore, the aims of the present study were to characterize the ethanolic extract of *C. papaya* by chromatographic fingerprint analysis and assess its

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antimutagenic and cytotoxic activities by the micronucleus assay.

The leaves of *Carica papaya* L. (Caricaceae) were cultivated without herbicide or pesticide and collected in the rural district of Venda Nova do Imigrante, ES, Brazil, in May 2010. A voucher specimen was deposited at the Herbarium of the Universidade Vila Velha (UVVES 2035) and identified by Solange Zanotti Schneider, MSc.

The plant material (26g) was macerated (22 mg/mL) with 96 vol% ethanol, under sonication (42 Hz), for 30min. The ethanolic extract was concentrated under reduced pressure in a rotary evaporator, at 50ºC, to a residue, ECP (2.46g). Part of the extract (1.25g) was resuspended in ultrapure water and the whole was carefully transferred to a 20 mL volumetric flask and diluted to the mark (62.5 mg/mL). This aqueous solution of ECP (CA) was administered orally to the experimental rats. Both ECP and CA were analyzed by chromatography.

The chromatographic analyses were performed at room temperature on a Waters XBridge™ C-18 column (150 x 4.6mm i.d., 3.5µm), in combination with the XBridge™C-18 guard column (20 x 4.6mm i.d., 3.5µm, Waters), at a flow rate of 0.80 $mL.min^{-1}$. The UV detector was set at 254nm and 365nm. The column was eluted isocratically with MeOH : H_2O (95:0.5, 1% phosphoric acid, pH 4.0). Solvents used were HPLC grade (Merck, Germany), water was ultrapure (ELGA 18.2Ω) and liquids were degassed by sonication before use. Standards (rutin and lycopene) and samples (ECP and CA) were dissolved in MeOH to final concentrations of 2 and 10mg/mL, respectively. After centrifugation at 8,400 rpm for 5 min, the sample solutions (20µL) were manually injected into the chromatograph (Waters 1515 system, binary pump, UV/Vis detector 2489, Breeze software). The calibration curves were prepared with rutin (1.95-250 mg/mL) and lycopene (2 – 250 mg/mL).

The ultraviolet spectra (190 to 700 nm) of the samples (ECP, CA) and the standards, lycopene and rutin, were recorded against a reagent blank (MeOH). The UV/Vis spectra were collected in a T80+ spectrophotometer (PG Instruments), with a tungsten-deuterium lamp as light source.

The MN assay was performed with female Wistar-Kyoto rats (WKY; n=29), four months old (100–250g). The animals lived under conditions of controlled temperature, maintained around 22º, and a light cycle of 12 h light : 12 h dark. Relative humidity was between 40 and 70%.

The experimental protocol was approved by the ethical committee for animal welfare at UVV (protocol 120/2010). The animals were divided into four groups. The negative control (NC, n =6) received the vehicle (distilled water). The positive control group (CP40, n=6) received cyclophosphamide (40mg/kg i.p.), 24 hours before euthanasia. The treated group (CA, n=6) received a dose of 500 mg/kg of ECP, *per os* (p.o.), 24 hours before euthanasia. The other treated group (CACP40, n=6) received p.o. a dose of 500 mg/kg of ECP (CA), 48, 36 and 24 hours before euthanasia, the last dose being given simultaneously with cyclophosphamide (40mg/kg i.p.). The dose of ECP was calculated from the concentration used *in vitro* (Otsuki et al., 2010). The surgical procedure was performed as previously described (Schmid, 1976). The blood smear was prepared from two drops of a bone marrow suspension (Schmid, 1976). Three slides were prepared per animal. On each slide, a total of 1000 cells were counted. For the first 200 cells, both mature/normochromatic (NCE) and immature/polychromatic (PCE) erythrocytes were counted. After the first 200 cells, only polychromatic cells were counted. The criterion to identify micronuclei was their size, shape and color. The micronucleus shoΩ™κuld have 1/10 to 1/20 the size of the PCE. In all, 2000 PCEs were counted per animal, from at least five animals per treated group (Schmid, 1976).

The rat bone marrow bioassay data were subjected to one-way analysis of variance (ANOVA), followed by the post-hoc Tukey test, with a significance level of $p<0.05$. The group results were expressed as mean ± standard deviation.

The numbers of NCEs, PCEs and the frequency of micronucleated polychromatic erythrocytes (MN-PCE) in 2000 PCEs, as well the PCE-to-NCE ratio, for each treatment assayed in this study, are shown in Table 1. The group treated with cyclophosphamide (CP40) showed a high frequency of micronuclei (20 ± 4.9; $p<0.01$). In the negative control (NC) and in the extract-treated group (CA), the MN rates were 3.2 ± 1.79 and 1.6 ± 0.5, respectively, and the PCE-to-NCE ratios were 1.38 ± 0.52 and 1.13 ± 0.28 (Table 1). The MN rate of the group treated with both ECP and cyclophosphamide (CACP40) was 3.0 ± 1.6 and the PCE-to-NCE ratio was 0.39 ± 0.07.

Table 1: Numbers of polychromatic (PCE) and normochromatic (NCE) erythrocytes in 200 cells and micronucleated polychromatic erythrocytes (MN-PCE) in 2000 PCEs, and the ratio PCE/NCE, in the bone marrow of Wistar-Kyoto rats.

Group	PCE	NCE	MN-PCE	PCE/NCE
NC	113.8 ± 17.8	88 ± 18.32	3.2 ± 1.79	1.38 ± 0.52
CP40	61.6 ± 20.2	139 ± 20.9	20 ± 4.9**	0.47 ± 0.21**
CA	105.6 ± 13.5	95.6 ± 12.7	1.6 ± 0.5##	1.13 ± 0.28##
CACP40	57.0 ± 6.38	143.0 ± 6.38	3.0 ± 1.6##	0.39 ± 0.07**,++

CN: Negative control; CP40: Positive control (cyclophosphamide 40mg/kg); CA: Group treated with ethanol extract of *C. papaya* L.*;* CACP40: group treated with ethanol extract of *C. papaya* L. and cyclophosphamide . The values expressed are the averages of each group ± standard deviation (S.D.). **$p<0.01$ compared to negative control and ##$p<0.01$ compared to positive control and ++$p<0.01$ compared to group treated with extract of *C. papaya* alone. Data analyzed by one-way ANOVA, followed by *post-hoc* Tukey test.

The chemical profiles of the ethanolic extract from *C. papaya* leaves (ECP) and the aqueous solution of ECP (CA), analyzed by HPLC-RP, were simple, dominated by peaks assigned to polar substances, with a retention time of two minutes, whether eluted with MeOH:H_2O (95:05, pH4) or MeOH:H_2O (50:50, pH4). However, the aqueous solution of ECP (CA) had a distinct chemical profile, with other peaks at a retention time of 2.23 minutes. A rutin standard injected under the same chromatographic conditions produced a peak with a retention time identical to the main peak of ECP and CA. The proportion of

flavonoids, calculated as rutin, was 2.26 ± 0.3 % (w/w) in ECP and 1.65 ± 0.07 % (w/w) in CA.

The UV-Vis spectrum of rutin exhibited absorption peaks at 245 nm, 285 nm and 380 nm and that of lycopene at 294 nm, 442nm, 469 nm and 499 nm. The ECP spectrum had absorption maxima at 242 nm, 254 nm, 270 nm, 315 nm, 360 nm, 389 nm, 431 nm and 685 nm and CA had a spectrum similar to that of rutin, with peaks at 268 nm, 293 nm, 320 nm, 343 nm and 359 nm, and the highest peak was at 380 nm (Figure 1).

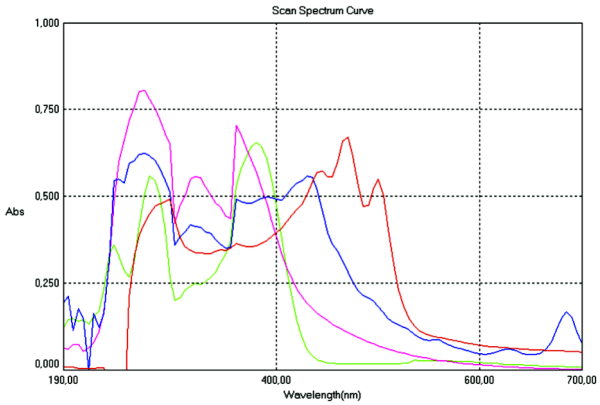

Figure 1. UV-Vis spectra (from 190 to 700 nm) of lycopene (red line), rutin (green line), aqueous solution of ethanolic extract of *Carica papaya* (CA) (pink line) and ethanolic extract of *Carica papaya* (blue line).

The high frequency of micronuclei in the cyclophosphamide group (CP40) indicates the induction of chromosome damage by this reagent (Krishna & Hayashi, 2000), while the PCE-to-NCE ratio for shows its cytotoxicity (0.47 ± 0.21; $p<0.01$) (Krishna & Hayashi, 2000).

The PCE-to-NCE ratio for the *C. papaya* leaf extract (CA) indicated the absence of any cytotoxicity under these experimental conditions, as the results for groups CA and NC were not substantially different. The fraction of MN-PCE did not increase during the treatment with CA, so there was no evidence of mutagenic activity in the *C. papaya* extract (Table 1).

The MN-PCE rate and PCE-to-NCE ratio for the group treated with both CA and cyclophosphamide (CACP40) indicated that the ethanolic extract of leaves from *C. papaya* had an antimutagenic and chemoprotective effect (Krishna & Hayashi, 2000). The number of micronuclei fell from 20 (for CP40) to 3.0 (same value as NC) when the extract was administered before the cyclophosphamide (Table 1). There is some published data on the antimutagenic and chemopreventive action of plant species, such as black beans (*Phaseolus vulgaris* L.) (Azevedo et al., 2003) and guaco (*Mikania glometara* Sprengel) (Costa et al., 2008). A protective mechanism that could explain the potential of various plants to prevent DNA damage involves cell-cycle control. The presence of MNs indicates that mutations have occurred during the cell cycle (Fenech, 1977). However, in the present case, DNA repair could not have happened in the CACP40 group, since ECP did not inhibit the cytotoxic effect of cyclophosphamide (Table 1); in fact, the micronucleated polychromatic erythrocytes were probably eliminated. The rate of cell death in CACP40 was close to the positive control.

Recently, the chemopreventive and immunomodulatory effects of an aqueous extract of *C. papaya* leaves have been reported in cultures of human cancer cells from the cervix, breast, liver, lung and pancreas (Otsuki et al., 2010). Immunomodulatory effects of the aqueous extract of *C. papaya* were also observed on human peripheral blood cell lines, promoting the production of type 1 cytokines and thus increasing the cytotoxicity against tumor cells (Otsuki et al., 2010). The aqueous extract of *C. papaya* leaves showed *in vitro* antiproliferative action on the tumor cells (Otsuki et al., 2010). The antiproliferative (Otsuki et al., 2010) and chemopreventive (Weinert, 1998) effects of *C. papaya* are potentially useful, both reducing the multiplication of carcinogenic cells and blocking the tumor process.

The rutin-like flavonoids detected in the ethanolic extract from *C. papaya* could be the compounds responsible for the antimutagenic and chemopreventive activities of the extract. The chemopreventive effect of natural products has been demonstrated in many *in vitro* (Otsuki et al., 2010, Endringer et al., 2009) and *in vivo* (Ferrari & Torres, 2002) models. Rutin was disclosed as a cancer chemopreventive constituent of *Hancornia speciosa* in the TPA-induced NF-κB inhibition assay (Endringer et al., 2009). Quercetin, the aglycone of rutin, has also been reported as a cancer chemopreventive compound (Dihal et al., 2006; Ichimatsu et al., 2007). Some studies have shown that rutin works as a quercetin deliverer to the large intestine and that its anti-inflammatory action in rats with induced colitis may be through quercetin-mediated inhibition of induced NF-κB activation (Kim et al., 2005; Kwon et al., 2005). The similarity between the spectrum of the aqueous solution of ethanolic extract of *C. papaya* leaves and that of rutin indicate that this could be the majority constituent of CA (Figure 1).

The results of this study indicated that an aqueous solution of the ethanolic extract of leaves of *C. papaya*, under the experimental conditions used, has low toxicity and an antimutagenic effect; flavonoids, such as rutin, may be the substances involved in this action.

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RESUMO

Atividade antimutagênica de Carica papaya L. pelo ensaio do micronúcleo in vivo

Há poucas informações sobre o efeito tóxico *in vivo* do extrato etanólico das folhas de *Carica papaya* (ECP). Portanto, esse estudo caracteriza quimicamente ECP por meio de HPLC-RP e avalia a atividade antimutagênica e citotóxica da fração aquosa de ECP

(CA), empregando ensaio do micronúcleo (MN). O extrato tem predominância de substâncias polares, entre elas a rutina. O ensaio do MN foi realizado em ratos *Wistar*. Grupos: Controle Negativo (CN), veículo; Controle Positivo (CP40), ciclofosfamida (40 mg/Kg i.p.), 24 horas; Grupo tratado (CA), ECP (500 mg/Kg, p.p.) 24h; Grupo Tratado (CACP40), CA (500 mg/Kg, p.o.) 48, 36 e 24 horas , a última dose de ECP foi administrada junto com ciclofosfamida (40 mg/Kg, i.p.). O índice de MN para CN e CA foi de 3,2 ± 1,79 e 1,6 ± 0,5, e razão PCE/NCE foi de 1,38 ± 0,52 e 1,13 ± 0,28, respectivamente, indicando baixa toxicidade. Por outro lado o índice de MN foi de 20 ± 4,9 para CP40 e 3,0 ± 1,6 para o CACP40, indicando efeito antimutagênico de ECP. Este estudo sugere que ECP possui efeito antimutagênico e baixa toxicidade e que a rutina pode estar envolvida nesse efeito.

Palavras-chave: Carica papaya. HPLC-RP, ensaio do micronúcleo.

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