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Research Article

# Clastogenicity of *Piper cubeba* (Piperaceae) seed extract in an *in vivo* mammalian cell system

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## Abstract

The plant *Piper cubeba* is widely distributed in tropical and subtropical regions and is used medically for various purposes but has not yet been evaluated for genotoxicity. We used male and female Swiss mice and Wistar rats and the comet assay and micronucleus test to investigate the mutagenic potential of a crude extract of *P. cubeba* seeds. The rodents were administered 0.5 g $kg^{-1}$, 1.0 g $kg^{-1}$ and 1.5 g $kg^{-1}$ of the extract by gavage. For the Swiss mice, peripheral blood was collected 24 h after treatment for the comet assay, and at 48 and 72 h for the micronucleus test. For the Wistar rats, peripheral blood and hepatic cells were collected for the comet assay and bone marrow cells were collected for the micronucleus test 24 h after treatment. At 1.5 g $kg^{-1}$, the highest dose tested, the extract induced a statistically significant increase in both the mean number of micronucleated polychromatic erythrocytes and the level of DNA damage in the rodent cell types analyzed. Under our experimental conditions, the *P. cubeba* seed extract was genotoxic *in vivo* when administered orally to mice and rats.

*Key words:* micronucleus test, *Piper cubeba,* single cell gel electrophoresis (SCGE).

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## Introduction

A number of natural products are used in the traditional medicine of many countries and alternative medicines for the treatment of various diseases are increasing in popularity. Many medicinal plants provide relief of symptoms comparable to that obtained by allopathic medicines. Therefore, an assessment of their genotoxic potential is necessary to ensure a relatively safe use of medicinal plants (Surh and Ferguson, 2003).

The genus *Piper* belongs to the family Piperaceae and has over 1000 species distributed in both hemispheres, where they grow in the form of erect or scadent (climbing) herbs, shrubs, or, less frequently, trees. Throughout the tropics, members of the genus *Piper* are used for many purposes, such as foods and spices, fish bait, fish poison, hallucinogens, insecticides, oils, ornaments, perfumes and for many medicines (Barrett, 1994; Joly 1981). The phytochemical profile of *Piper* species is characterized by the production of typical classes of compounds such as amides, benzoic acids, and chromenes, as well as terpenes, phenylpropanoids, lignans, other phenolics and a series of alkaloids (Jensen *et al.*, 1993; Parmar *et al.*, 1997; Wu *et al.*, 1997).

The species *Piper cubeba* L., known in Brazil as "pimenta de Java" ("Java pepper") and in English as Cubeb pepper, is a popular medicinal plant which has been extensively used in Europe since the Middle Ages, as well as in many other countries, including Arabia, India, Indonesia and Morocco. The fruits are used as a spice and have also been used for the treatment of abdominal pain, asthma, diarrhea, dysentery, gonorrhea, enteritis and syphilis (Eisai, 1995; Sastroamidjojo, 1997) and has also been reported to have an inhibitory effect on hepatitis C virus protease (Januario *et al.*, 2002). Choi and Hwang (2003) demonstrated significant anti-inflammatory and analgesic activities of the methanolic extract from fruits of *P. cubeba*.

In view of the potential therapeutic use of *P. cubeba* extracts and the absence of any data on its genetic toxicity in eukaryotes, the study described in this paper was under-

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taken to evaluate the potential *in vivo* mutagenic effects of *P. cubeba* seed extract in terms of DNA damage in hepatic and peripheral blood leukocytes and the induction of micronuclei in bone marrow polychromatic erythrocytes of rodents.

## Material and Methods

### Plant material

Seeds of *Piper cubeba* L. were imported from India country, air-dried to constant weight at 55 °C and powdered. A simple extract was made by macerating 150 g of powdered seeds in three litres of a 70% (w/v) ethanol in water mixture for 48 h, after which the macerate was filtered and a further 150 g of seed powder was added to the filtrate and macerated for a further 48 h to produce a crude ethanolic aqueous extract which was concentrated under reduced pressure to produce 54 g of crude extract (yield 18%).

### Animals and assay procedures

We used 25-30g twelve-week old Swiss mice (*Mus musculus*) and 100 g six-week old Wistar rats (*Rattus norvegicus*) obtained from the of Jose do Rosario Vellano University, Alfenas town, Brazil, animal house. The rodents were housed in polyethylene cages ($n$ = 6) in a climate-controlled environment (25 ± 4 °C, 55 ± 5% humidity) with a 12 h (07:00 to 19:00) day length and had *ad libitum* access to food (Labina, Purina) and water.

The rats and mice were divided into negative control, extract and positive control experimental groups, each containing three females and three males housed as described above. The *P. cubeba* seed extract was administered by gavage at concentrations of 0.5 g $kg^{-1}$, 1 g $kg^{-1}$ and 1.5 g $kg^{-1}$ in single 0.5 mL doses, these concentrations being chosen based on a *P. cubeba* seed extract 50% lethal dose ($LD_{50}$) value of 2 g $kg^{-1}$ for Swiss mice (Perazzo F.F., data not shown). The negative control groups (both mice and rats) received distilled water by the same route. The positive control group for mice received 0.05 g $kg^{-1}$ of N-nitroso-N-ethylurea (ENU, Chemical Abstracts Service (CAS) No. 759-73-9, Sigma) dissolved in pH 6 phosphate-buffer while the positive control group for rats received 0.05 g of cyclophosphamide (CP, CAS: C 0768, Sigma) $kg^{-1}$ dissolved in the saline solution (NaCl 0.9%), intraperitoneal injections being administered in both cases.

The comet assay was carried out by the method described by Speit and Hartmann (1999), which is based on the original work of Singh *et al.* (1988) and includes modifications introduced by Klaude *et al.* (1996) as well as additional modifications. Mouse peripheral blood leukocytes and rat peripheral blood leukocytes and liver cells were sampled 24 h after treatment. Liver samples were washed in saline solution in an ice bath and a fragment transferred to a Petri dish containing 1 mL of Hank's solution (pH 7.5) and then gently homogenized with small forceps. A 10 µL aliquot of cells from each animal was mixed with 120 µL of 0.5% (w/v) low melting point agarose (Invitrogen, Cat. No. 15517-014) at 37 °C and the mixture rapidly spread onto microscope slides pre-coated with 1.5% normal melting point agarose (Invitrogen,. Cat. No. 15510-019). Coverslips were added and the slides were allowed to gel at 4 °C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA, Merk), 10 mM Tris, pH set to 10 with ~8 g NaOH, 890 mL of distilled water and 1% (w/v) sodium N-lauroylsarcosine (Sigma, L-5125)), plus 1 mL of Triton X-100 (Merck) and 10 mL of DMSO. The slides, which were protected from light, were left to stand at 4 °C for 1 h and then placed in the gel box, positioned at the anode end, and left in a high pH (> 13) electrophoresis buffer (300 mM NaOH per 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10 EDTA) at 4 °C for 20 min prior to electrophoresis, to allow the DNA to unwind. The electrophoresis run was carried out at 4 °C in an ice bath for 20 min at 1.25 V $cm^{-1}$ and 300 mA. The slides were then submerged in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min, dried at room temperature, fixed in 100% ethyl alcohol for 10 min, dried and stored overnight or longer. For staining, the slides were briefly rinsed in distilled water, covered with 30 µL of 1x ethidium bromide staining solution prepared from a 10x stock (200 µg $mL^{-1}$) and covered with a coverslip. The stained cells were evaluated immediately at 400x magnification using a Nikon fluorescence microscope with a 515 nm to 560 nm excitation filter and a 590 nm barrier filter. The animals used in this study were sacrificed by cervical dislocation. The Animal Bioethical Committee of the UNIFENAS, Brazil, approved the present study on October 25, 2005 (protocol number 15A/2005), in accordance with the Federal Government legislation on animal care.

For the micronucleus assay we collected peripheral blood from the orbital vein of each mouse 48 h and 72 h after treatment and prepared blood-smear slides, while the rats were sacrificed 24 h after treatment and the bone marrow cells from one femur prepared as recommended by Schmid (1976). All the slides were coded, fixed with methanol and stained with Giemsa solution. Two thousand polychromatic erythrocytes from each rat, and four thousand polychromatic erythrocytes from each mouse (2000 cells from the 48 h blood sample and 2000 cells from the 72 h blood sample), were scored. In rats, one thousand cells were analyzed per animal to determine the polychromatic/normochromatic erythrocyte ratio (PCE:NCE).

### Scoring procedures and statistical analysis

The extent and distribution of DNA damage indicated by the comet assay was evaluated by examining at least 100

randomly selected and non-overlapping cells on the slides per animal. These cells were visually scored into comet classes according to tail size: class 0 = no tail; class 1 = tail shorter than the diameter of the head (nucleus); class 2 = tail length 1 to 2x the diameter of the head; and class 3 = tail longer than 2x the diameter of the head. Comets with no heads and those with nearly all the DNA in the tail, or with a very wide tail, were excluded from the evaluation because they probably represented dead cells (Hartmann and Speit, 1997). The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 300 (all maximally damaged).

The micronucleus test and comet assay data were submitted to one-way analysis of variance test (ANOVA) and the Tukey-Kramer multiple comparison test (Sokal and Rohlf, 1995), using the GraphPad Instat® program version 3.01. The results were considered statistically significant at $p < 0.05$.

## Results and Discussion

The comet assay is a sensitive, reliable and rapid method for the detection of double- and single-strand DNA breaks, alkali-labile sites and delayed repair sites in individual eukaryotic cells, and is an important tool for evaluating the *in vitro* and in *vivo* genotoxic potential of compounds (Rojas *et al.*, 1999; Tice *et al.*, 2000; Sekihashi *et al.*, 2002). Our comet assay results are shown in Tables 1, 2 and 3, where the female and male results for the different concentrations of extract and the N-nitroso-N-ethylurea (ENU) and cyclophosphamide (CP) positive controls are compared with the negative control (water). As expected, when the positive controls were compared to the negative controls we found that both ENU and CP induced a statistically significant increase ($p < 0.05$ or greater) in comet assay DNA migration for mouse and rat leukocytes and rat liver cells (Tables 1, 2, 3). Regarding the *Piper cubeba* extracts, we found a statistically significant increase in DNA migration at an extract concentration of 1 g $kg^{-1}$ (50% of the $LD_{50}$value) for leukocytes from male ($p < 0.05$) rats (Table 2) and at 1.5 g $kg^{-1}$ (75% of the $LD_{50}$) for leukocytes from the female mice ($p < 0.05$, Table 1) and female ($p < 0.001$) and male ($p < 0.05$) rats (Table 2). Rat liver cells also showed a statistically significant increase ($p < 0.001$ for females, $p < 0.05$ for males) in DNA damage at an extract concentration of 1.5 g $kg^{-1}$compared to the negative control (Table 3). At extract concentrations that induced a significant increase in DNA damage, the majority of the damaged cells showed minor damage (class 1) and with very few showed a large amount of damage (class 2 and 3).

The mouse micronucleus assay has been used to evaluate aneuploidy and clastogenic chromosome aberrations (Morita *et al.*, 1997), but experiments using erythrocytes from mammalian species other than mice (*e.g.* humans, laboratory rats and wild rodents) have met with less success due to the ability of the spleen to remove micronucleated (MN) erythrocytes from the blood (Simula and Priestly, 1992; Holden *et al.*, 1997).

The results for our mouse micronucleus assay evaluation of the clastogenic potential of *P. cubeba* seed extract are given in Table 4, which shows that, as compared to the negative control (water), there was a statistically significant increase ($p < 0.001$) in the mean number of micronucleated polychromatic erythrocytes (MNPCEs) in blood sampled at 48 and 72 h for the 1.0 g $kg^{-1}$ and 1.5 g $kg^{-1}$ doses of extract and, as expected, 50 mg $kg^{-1}$ ENU positive control.

The rat bone marrow cell micronucleus assay results are summarized in Table 5, which shows that, as compared to the negative control, there was a statistically significant increase in the average number of MNPCEs in rats treated with 1.0 g $kg^{-1}$ ($p < 0.05$) and 1.5 g $kg^{-1}$($p < 0.01$) of extract and, as expected, the positive CP control ($p < 0.01$).

However, the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE:NEC, Table 5) from CP and extract treated groups was not significantly different from the negative control group ($p > 0.05$), indicating that the *P. cubeba* seeds extract did not present cytotoxic properties in rat bone marrow cells at any of the doses tested.

Phytochemical screening of *P. cubeba* extracts has detected alkaloids/amides, lignans, neolignans and terpenes, including aschantin, dihydrocubebin, piperine alkaloid, piperol A, B and C, piperol A-triacetate, the terpenes α-copaene, ρ-cymene, germacrene D and limonene (among others), sesamin and (+)-Zeylinol (Parmar *et al.*, 1997). In a more recent review, Usia *et al.* (2005) reported sixteen known compounds (α-asarone, (-)-clusin, (-)-dihydroclusin, ethoxyclusin, (-)-cubebin, (-)-cubebinin, (-)-cubebininolide, (-)-dihydrocubebin, α-methylcubebin, (-)-hinokinin, magnosalin, medioresinol, 2,4,5-trimetoxiphenylacetone, 1-(2,4,5-trimetoxyphenyl)-1,2-propanedione, (-)-thujaplicatin trimethyl ether, (-)-yatein), two new lignans ((8R,8'R)-4-hydroxycubebinone and (8R, 8'R,9'S)-5-methoxyclusin) and two new sesquiterpenes ((5α,8α-2-oxo-1(10),3,7(11)-guaiatrien-12,8-olide and (1α,2β,5α,8α,10α)-1,10-epoxy-2-hydroxy-3,7(11)-guaiadien-12,8-olide).

Insecticidal properties have been reported for the *Piper* species *P, guineense*, *P. brachystachyum* and *P. nigrum* (Jacobson and Crosby, 1971; Parmar *et al.*, 1997; Jensen *et al.*, 2006), and some compounds present in *P. cubeba* seed extract (aschantin, α-copaene, ρ-cymene, dihydrocubebin, germacrene D, lignans, limonene and sesamin) are also present in *P. guineense* seeds (Parmar *et al.*, 1997). Since several insecticides are known to have mutagenic effects (Jha *et al.*, 2002; Beard, 2006), the clastogenic effects of the *P. cubeba* seed extract observed in the present work could be attributed to some compound with insecticide potential. We intend to investigate some isolated com-

**Table 1** - Comet assay for the *in vivo* assessment of genotoxicity of a *Piper cubeba* seed extract on peripheral blood leukocytes from female ($F_1$ to $F_{15}$) and male ($M_1$ to $M_{15}$) Swiss mice exposed to different concentrations of extract 24h before the assay. Each treatment used three mice (n = 3). The class total for each mouse is the sum of the numbers in classes 1, 2 and 3, whereas the comet score was calculated as the comet class (1, 2 or 3) multiplied by the number in the class (*i.e.* 1 in class 1 = 1, 1 in class 2 = 2 and 1 in class 3 = 3). Negative control = water, positive control = N-nitroso-N-ethylurea (ENU).

| Treat-ments | Females | | | | | | | Males | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mice | Comet class and score | | | | Class total | Total comet score (Mean ± SD in bold)† | Mice | Comet class and score | | | | Class total | Total comet score (Mean ± SD in bold)† |
| | | 0 | 1 | 2 | 3 | | | | 0 | 1 | 2 | 3 | | |
| Water | | | | | | | | | | | | | | |
| | $F_1$ | 97 | 2 | 1 | 0 | 3 | 4 | $M_1$ | 92 | 6 | 2 | 0 | 8 | 10 |
| | $F_2$ | 93 | 5 | 1 | 1 | 7 | 10 | $M_2$ | 91 | 7 | 2 | 0 | 9 | 11 |
| | $F_3$ | 98 | 2 | 0 | 0 | 2 | 2 | $M_3$ | 88 | 9 | 2 | 1 | 12 | 16 |
| | | | | | | | **5.33 ± 4.16** | | | | | | | **12.3 ± 3.21** |
| Extract 0.5 g kg$^{-1}$ extract | | | | | | | | | | | | | | |
| | $F_4$ | 94 | 5 | 1 | 0 | 6 | 7 | $M_4$ | 95 | 4 | 1 | 0 | 5 | 6 |
| | $F_5$ | 93 | 6 | 1 | 0 | 7 | 8 | $M_5$ | 97 | 3 | 0 | 0 | 3 | 3 |
| | $F_6$ | 97 | 3 | 0 | 0 | 3 | 3 | $M_6$ | 80 | 16 | 4 | 0 | 20 | 24 |
| | | | | | | | **6.0 ± 2.64** | | | | | | | **11.0 ± 11.3** |
| Extract 1.0 g kg$^{-1}$ extract | | | | | | | | | | | | | | |
| | $F_7$ | 87 | 12 | 1 | 0 | 13 | 14 | $M_7$ | 83 | 17 | 0 | 0 | 17 | 17 |
| | $F_8$ | 82 | 16 | 2 | 0 | 18 | 20 | $M_8$ | 72 | 25 | 3 | 0 | 28 | 31 |
| | $F_9$ | 63 | 34 | 3 | 0 | 37 | 40 | $M_9$ | 90 | 9 | 1 | 0 | 10 | 11 |
| | | | | | | | **24.6 ± 13.6** | | | | | | | **19.6 ± 10.2** |
| Extract 1.5 g kg$^{-1}$ extract | | | | | | | | | | | | | | |
| | $F_{10}$ | 78 | 20 | 2 | 0 | 22 | 24 | $M_{10}$ | 53 | 47 | 0 | 0 | 47 | 47 |
| | $F_{11}$ | 42 | 55 | 2 | 1 | 58 | 62 | $M_{11}$ | 68 | 30 | 2 | 0 | 32 | 34 |
| | $F_{12}$ | 57 | 41 | 2 | 0 | 43 | 45 | $M_{12}$ | 67 | 32 | 1 | 0 | 33 | 34 |
| | | | | | | | **43.6 ± 19.03*** | | | | | | | **38.3 ± 7.5** |
| ENU 0.05 g kg$^{-1}$ ENU | | | | | | | | | | | | | | |
| | $F_{13}$ | 32 | 63 | 3 | 2 | 68 | 75 | $M_{13}$ | 57 | 40 | 1 | 2 | 43 | 48 |
| | $F_{14}$ | 40 | 57 | 3 | 0 | 60 | 63 | $M_{14}$ | 45 | 52 | 2 | 1 | 55 | 59 |
| | $F_{15}$ | 46 | 60 | 4 | 0 | 64 | 68 | $M_{15}$ | 18 | 76 | 6 | 0 | 82 | 88 |
| | | | | | | | **68.6 ± 6.02***** | | | | | | | **65.0 ± 20.6**** |

†Asterisk indicate significant differences from the negative control by the ANOVA and Tukey test at $p < 0.05$*, $p < 0.01$** or $p < 0.001$***.

**Table 2** - Comet assay for the *in vivo* assessment of genotoxicity of a *Piper cubeba* seed extract on peripheral blood leukocytes from female ($F_1$ to $F_{15}$) and male ($M_1$ to $M_{15}$) Wistar rats exposed to different concentrations of extract 24h before the assay. Each treatment used three rats (n = 3). The class total for each rat is the sum of the numbers in classes 1, 2 and 3, whereas the comet score was calculated as the comet class (0, 1, 2 or 3) multiplied by the number in the class (*i.e.* 1 in class 1 = 1, 1 in class 2 = 2 and 1 in class 3 = 3). Negative control = water, positive control = N-nitroso-N-ethylurea (ENU).

| Treatments | Females | | | | | | Males | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rats | Comet class and score | | | | Class total | Total comet score (Mean ± SD in bold)[†] | Rats | Comet class and score | | | | Class total | Total comet score (Mean ± SD in bold)[†] |
| | | 0 | 1 | 2 | 3 | | | | 0 | 1 | 2 | 3 | | |
| Water | | | | | | | | | | | | | | |
| | $F_1$ | 94 | 6 | 0 | 0 | 6 | 6 | $M_1$ | 98 | 2 | 0 | 0 | 2 | 2 |
| | $F_2$ | 94 | 6 | 0 | 0 | 6 | 6 | $M_2$ | 97 | 3 | 0 | 0 | 3 | 3 |
| | $F_3$ | 96 | 3 | 1 | 0 | 4 | 5 | $M_3$ | 99 | 1 | 0 | 0 | 1 | 1 |
| | | | | | | | **5.66 ± 0.57** | | | | | | | **2.0 ± 1.0** |
| Extract 0.5 g kg$^{-1}$ | | | | | | | | | | | | | | |
| | $F_4$ | 68 | 27 | 5 | 0 | 32 | 37 | $M_4$ | 99 | 1 | 0 | 0 | 1 | 1 |
| | $F_5$ | 98 | 2 | 0 | 0 | 2 | 2 | $M_5$ | 98 | 2 | 0 | 0 | 2 | 2 |
| | $F_6$ | 96 | 4 | 0 | 0 | 4 | 4 | $M_6$ | 99 | 1 | 0 | 0 | 1 | 1 |
| | | | | | | | **14.3 ± 19.6** | | | | | | | **1.33 ± 0.57** |
| Extract 1.0 g kg$^{-1}$ | | | | | | | | | | | | | | |
| | $F_7$ | 73 | 27 | 0 | 0 | 27 | 27 | $M_7$ | 58 | 42 | 0 | 0 | 42 | 42 |
| | $F_8$ | 80 | 20 | 0 | 0 | 20 | 20 | $M_8$ | 51 | 59 | 0 | 0 | 59 | 59 |
| | $F_9$ | 70 | 29 | 1 | 0 | 30 | 31 | $M_9$ | 51 | 48 | 1 | 0 | 49 | 50 |
| | | | | | | | **26.0 ± 5.5** | | | | | | | **50.3 ± 8.5*** |
| Extract 1.5 g kg$^{-1}$ | | | | | | | | | | | | | | |
| | $F_{10}$ | 38 | 50 | 9 | 3 | 62 | 77 | $M_{10}$ | 44 | 53 | 0 | 0 | 53 | 53 |
| | $F_{11}$ | 27 | 63 | 9 | 1 | 73 | 84 | $M_{11}$ | 71 | 24 | 2 | 3 | 29 | 31 |
| | $F_{12}$ | 41 | 59 | 0 | 0 | 59 | 59 | $M_{12}$ | 74 | 23 | 3 | 0 | 26 | 28 |
| | | | | | | | **73.3 ± 12.8*** ** | | | | | | | **37.3 ± 13.6*** |
| ENU 0.05 g kg$^{-1}$ | | | | | | | | | | | | | | |
| | $F_{13}$ | 70 | 25 | 4 | 0 | 29 | 33 | $M_{13}$ | 68 | 30 | 1 | 1 | 32 | 35 |
| | $F_{14}$ | 42 | 53 | 5 | 0 | 58 | 63 | $M_{14}$ | 31 | 61 | 8 | 0 | 69 | 77 |
| | $F_{15}$ | 45 | 49 | 5 | 1 | 55 | 62 | $M_{15}$ | 83 | 16 | 1 | 0 | 17 | 18 |
| | | | | | | | **52.6 ± 17.0**** | | | | | | | **43.3 ± 30.3*** |

[†]Asterisks indicate a significant difference from the negative control by the ANOVA and Tukey test at $p < 0.05$*, $p < 0.01$** or $p < 0.001$***.

**Table 3** - Comet assay for the *in vivo* assessment of genotoxicity of a *Piper cubeba* seed extract on liver cells from female ($F_1$ to $F_{15}$) and male ($M_1$ to $M_{15}$) Wistar rats exposed to different concentrations of extract 24h before the assay. Each treatment used three rats (n = 3). The class total for each rat is the sum of the numbers in classes 1, 2 and 3, whereas the comet score was calculated as the comet class (0, 1, 2 or 3) multiplied by the number in the class (*i.e.* 1 in class 1 = 1, 1 in class 2 = 2 and 1 in class 3 = 3). Negative control = water, positive control = N-nitroso-N-ethylurea (ENU).

| Treatments | Females | | | | | | Males | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rats | Comet class and score | | | | Class total | Total comet score (Mean ± SD in bold)[†] | Rats | Comet class and score | | | | Class total | Total comet score (Mean ± SD in bold)[†] |
| | | 0 | 1 | 2 | 3 | | | | 0 | 1 | 2 | 3 | | |
| Water | | | | | | | | | | | | | | |
| | $F_1$ | 95 | 4 | 1 | 0 | 5 | 6 | $M_1$ | 89 | 8 | 3 | 0 | 11 | 14 |
| | $F_2$ | 95 | 5 | 0 | 0 | 5 | 5 | $M_2$ | 96 | 4 | 0 | 0 | 4 | 4 |
| | $F_3$ | 98 | 2 | 0 | 0 | 2 | 2 | $M_3$ | 98 | 2 | 0 | 0 | 2 | 2 |
| | | | | | | | **4.33 ± 2.08** | | | | | | | **6.66 ± 6.42** |
| Extract 0.5 g kg$^{-1}$ | | | | | | | | | | | | | | |
| | $F_4$ | 86 | 14 | 0 | 0 | 14 | 14 | $M_4$ | 89 | 11 | 0 | 0 | 11 | 11 |
| | $F_5$ | 94 | 6 | 0 | 0 | 6 | 6 | $M_5$ | 98 | 2 | 0 | 0 | 2 | 2 |
| | $F_6$ | 94 | 6 | 0 | 0 | 6 | 6 | $M_6$ | 96 | 4 | 0 | 0 | 4 | 4 |
| | | | | | | | **8.66 ± 4.61** | | | | | | | **5.66 ± 4.72** |
| Extract 1.0 g kg$^{-1}$ | | | | | | | | | | | | | | |
| | $F_7$ | 93 | 7 | 0 | 0 | 7 | 7 | $M_7$ | 21 | 78 | 19 | 3 | 0 | 25 |
| | $F_8$ | 84 | 14 | 2 | 0 | 16 | 18 | $M_8$ | 32 | 68 | 29 | 2 | 1 | 36 |
| | $F_9$ | 92 | 7 | 1 | 0 | 8 | 9 | $M_9$ | 50 | 50 | 45 | 4 | 1 | 56 |
| | | | | | | | **11.33 ± 5.85** | | | | | | | **39.0 ± 15.71** |
| Extract 1.5 g kg$^{-1}$ | | | | | | | | | | | | | 65 | 78 |
| | $F_{10}$ | 43 | 40 | 12 | 5 | 57 | 79 | $M_{10}$ | 35 | 55 | 7 | 3 | 51 | 52 |
| | $F_{11}$ | 42 | 51 | 4 | 3 | 58 | 68 | $M_{11}$ | 49 | 50 | 1 | 0 | 26 | 29 |
| | $F_{12}$ | 39 | 52 | 6 | 3 | 61 | 73 | $M_{12}$ | 74 | 23 | 3 | 0 | | **53.0 ± 24.5*** |
| | | | | | | | **73.3 ± 5.5***** | | | | | | | |
| ENU 0.05 g kg$^{-1}$ | | | | | | | | | | | | | 32 | 35 |
| | $F_{13}$ | 70 | 26 | 4 | 0 | 30 | 34 | $M_{13}$ | 68 | 30 | 1 | 1 | 61 | 69 |
| | $F_{14}$ | 31 | 64 | 4 | 1 | 69 | 75 | $M_{14}$ | 39 | 53 | 8 | 0 | 37 | 42 |
| | $F_{15}$ | 40 | 52 | 7 | 1 | 60 | 69 | $M_{15}$ | 63 | 32 | 5 | 0 | | **48.66 ± 17.95*** |
| | | | | | | | **59.33 ± 22.1***** | | | | | | | |

[†]Asterisks indicate a significant difference from the negative control by the ANOVA and Tukey test at $p < 0.05$* or $p < 0.001$***.

**Table 4** - Micronucleus (MN) test results showing the number of micronucleated polychromatic erythrocytes (MNPCEs) in peripheral blood samples from female (n = 3; $F_1$, $F_2$ and $F_3$) and male (n = 3; $M_1$, $M_2$ and $M_3$) Swiss mice treated with a *Piper cubeba* seed extract. For each blood collection time 2000 cells were scored per mouse. SD = standard deviation. Negative control = water, positive control = N-nitroso-N-ethylurea (ENU).

| Treatments | Blood collection time (h) | Number of MNPCEs per mouse | | | | | | Mean number of MNPCEs (± SD) |
|---|---|---|---|---|---|---|---|---|
| | | $F_1$ | $F_2$ | $F_3$ | $M_1$ | $M_2$ | $M_3$ | |
| Water | 48 | 1 | 2 | 3 | 1 | 2 | 2 | 1.83 ± 0.75 |
| | 72 | 1 | 3 | 2 | 2 | 2 | 1 | 1.83 ± 0.75 |
| Extract 0.5 g $kg^{-1}$ | 48 | 4 | 6 | 5 | 6 | 0 | 4 | 4.16 ± 2.22 |
| | 72 | 4 | 5 | 5 | 4 | 6 | 2 | 4.33 ± 1.36 |
| Extract 1.0 g $kg^{-1}$ | 48 | 9 | 5 | 7 | 7 | 8 | 4 | 6.66 ± 1.86* |
| | 72 | 8 | 11 | 6 | 4 | 8 | 7 | 7.33 ± 2.33* |
| Extract 1.5 g $kg^{-1}$ | 48 | 5 | 8 | 6 | 6 | 7 | 7 | 6.50 ± 1.04* |
| | 72 | 7 | 6 | 6 | 7 | 7 | 7 | 6.66 ± 0.51* |
| ENU 0.05 g $kg^{-1}$ | 48 | 10 | 9 | 8 | 8 | 7 | 7 | 8.16 ± 1.16* |
| | 72 | 9 | 8 | 9 | 8 | 9 | 7 | 8.33 ± 0.81* |

*Significantly different from negative control by the ANOVA and Tukey test at $p < 0.05$.

**Table 5** - Micronucleus (MN) test results showing the number of micronucleated polychromatic erythrocytes (MNPCE) bone-marrow samples from female (n = 3; $F_1$, $F_2$ and $F_3$) and male (n = 3; $M_1$, $M_2$ and $M_3$) Wistar rats treated with *Piper cubeba* seed extract, 2000 cells being scored per rat. The ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE:NCE) was also calculated, 1000 cells being scored per rat. SD = standard deviation. Negative control = water, positive control = cyclophosphamide (CP).

| Treatments | Number of MNPCE per rat | | | | | | MNPCE (Mean ± SD)† | PCE:NCE (Mean ± SD) |
|---|---|---|---|---|---|---|---|---|
| | $F_1$ | $F_2$ | $F_3$ | $M_1$ | $M_2$ | $M_3$ | | |
| Water | 2 | 1 | 3 | 4 | 0 | 1 | 1.83 ± 1.47 | 0.84 ± 0.07 |
| Extract 0.5 g $kg^{-1}$ | 3 | 1 | 2 | 1 | 1 | 3 | 1.83 ± 0.98 | 0.78 ± 0.10 |
| Extract 1.0 g $kg^{-1}$ | 6 | 4 | 7 | 5 | 5 | 6 | 5.5 ± 1.04* | 0.84 ± 0.07 |
| Extract 1.5 g $kg^{-1}$ | 8 | 3 | 7 | 5 | 5 | 9 | 6.16 ± 2.22** | 0.78 ± 0.05 |
| CP 0.05 g $kg^{-1}$ | 8 | 5 | 1 | 9 | 8 | 7 | 6.33 ± 2.94** | 0.75 ± 0.05 |

†Asterisks indicate significant differences from the negative control by the ANOVA and Tukey test at $p < 0.05$* or $p < 0.01$**.

pounds from *P. cubeba* seed extract to verify this hypothesis.

To our knowledge, this is the first report of the mutagenic effect of *P. cubeba* extract on Swiss mice and Wistar rat cells. Our results indicate that when given at high doses of 1.0 g $kg^{-1}$ and 1.5 g $kg^{-1}$ *P. cubeba* extract induced a significant increase in the mean number of cells with DNA damage and micronuclei, indicating that the extract, or its metabolites, show moderate genetic toxicity in rodent cells and that caution is required regarding the indiscriminate use of high dose *P. cubeba* extracts by the public.

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