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Research Article

Evaluation of the *in vivo* mutagenic potential of hydroalcoholic extracts of the northern highbush blueberry (*Vaccinium corymbosum* L. Ericales, Ericaceae) on peripheral blood cells of Swiss mice (*Mus musculus* Rodentia, Muridae)

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Abstract

The northern highbush blueberry (*Vaccinium corymbosum* L. Ericales, Ericaceae) is very rich in anthocyanins, natural pigments which have strong antioxidant properties and potential health benefits, resulting in the worldwide use the blueberry as a medicinal plant. We investigated the mutagenic potential of simple hydroalcoholic extracts of *V. corymbosum* acutely administrated by gavage to Swiss mice at doses of 1 g kg^{-1}, 1.5 g kg^{-1} and 2 g kg^{-1}. Peripheral blood cells were collected 4 h and 24 h post-gavage and assessed by the alkaline comet assay, with further blood samples being collected at 48 h and 72 h for assessment using the micronucleus (MN) assay. Our results show that the *V. corymbosum* extracts did not induce any statistically significant increase in the average amount of DNA damage in peripheral blood leukocytes. However, we did record a significant increase in the frequency of micronucleated polychromatic erythrocytes at the three doses tested.

Key words: comet assay, micronucleus, mutagenicity, *Vaccinium corymbosum*.

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Introduction

The medicinal use of plants is of great antiquity, with current records showing that more than 150,000 plant species have been studied for their medicinal properties. Many plants contain therapeutic substances (Hoyos *et al.*, 1992; Surh and Ferguson, 2003) which can be extracted and used in the preparation of medicines, or the plant itself can be used directly as a medication, a practice that is particularly popular in developing countries. However, many plants contain compounds which are known to cause ill-health, or even death, in animals and humans, there is considerable interest in determining the health risks associated with plants used for medicinal purposes.

The genus *Vaccinium* (Ericales, Ericaceae) comprises about 200 species, some of which produce edible fruits of economic importance. In recent decades interest in the anthocyanin content of some *Vaccinium* species has revived due to the pharmacological properties of anthocyanins, which includes antioxidant activity (Wang *et al.*, 1997; Mazza *et al.*, 2002; Lohachoompol *et al.*, 2004), anti-inflammatory effects (Youdim *et al.*, 2002), cardiovascular protection, antidiabetic properties, improvements to vision and inhibition of carcinogenesis (Cabrita and Andersen, 1999; Camire, 2000; Katsube *et al.*, 2003). The fruits of the economically important northern highbush blueberry (*Vaccinium corymbosum* L.) are consumed in many countries throughout the world because they contain large amounts of antioxidants thought to be beneficial to health (Prior *et al.*, 1998; Ehlenfeldt and Prior, 2001).

We assessed the *in vivo* mutagenic potential of three different concentrations of simple hydroalcoholic extracts of *V. corymbosum* using Swiss mice peripheral blood cells subjected to the alkaline comet assay and the micronucleus (MN) assay.

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Material and Methods

Plant material and chemicals

Northern highbush blueberry (*Vaccinium corymbosum* L. Ericales, Ericaceae) berries were collected in an experimental field in the town of Videira

(27°00'04.76" S, 51°07'29.77" W), the field belonging to the Agricultural Research and Extension Education Company (Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A., EPAGRI) in the southern Brazilian State of Santa Catarina. The berries were picked in October 2005 at the commercially ripe stage and all damaged, diseased or pest-infested fruits, stems or leaves were removed. The berries were kept in polyethylene bags at -20 °C until extract preparation. Prior to extraction, the frozen berries were crushed using a food processor and 1 kg of berries macerated with 70% (v/v) aqueous ethanol at room temperature (22 °C) for seven days. The simple hydroethanolic extract was obtained by filtration and concentration of the filtrate under reduced pressure using a rotary evaporator. The yield was 138 g, or 13.8% (w/w) with reference to the original weight of the berries.

The principal chemicals used were obtained from the following suppliers: dimethyl sulphoxide (DMSO, Merck, CAS No. 67-68-5); ethidium bromide (Sigma, CAS No. 1239-45-8); ethylenediaminetetraacetic acid (EDTA, Merck); low melting point (LMP) agarose (Invitrogen 15517-014:); N-nitroso-N-ethylurea (ENU, Sigma, CAS n. 759-73-9); normal melting point (NMP) agarose (Invitrogen 15510-019); sodium *N*-lauroylsarcosine (Sigma L-5125:) and tris(hydroxymethyl)aminomethane (TRIS, Merck, CAS No. 77-86-1) and Triton X-100 (Merck).

Animals and assay procedures

We used 12-week old male and female Swiss mice (*Mus musculus*) with a body weight (bw) of 25 g to 30 g which had been acquired from the animal house of the Jose do Rosario Vellano University (UNIFENAS), Alfenas, Minas Gerais, Brazil. The mice were kept in polyethylene boxes in groups of six mice (three females and three males) in a climate-controlled environment (25 ± 4 °C 55 ± 5% humidity) with a 12 h (0700 to 1900) day-length. All the mice had *ad libitum* access to water and a commercial laboratory rat food (Labina: Purina Co., Brazil), and were euthanized 72 h after the respective treatments. The UNIFENAS Animal Bioethical Committee approved this study under protocol number 03A/2006 in accordance with the Federal Government legislation on animal care.

The *V. corymbosum* extract was administered to each mouse as a single 0.5 mL gavage dose adjusted to contain extract equivalent to 1 g kg^{-1} bw, 1.5 g bw kg^{-1} or 2 g kg^{-1} bw, this range being based on a previous Swiss mice acute toxicity study involving doses of extract in excess of the equivalent of 2 g kg^{-1} bw (data not shown). The negative control group received distilled water by gavage. The positive control group received 50 mg of N-nitroso-N-ethylurea/kg (ENU) dissolved in pH 6 phosphate buffer and administered as a single intraperitoneal injection.

The alkaline comet assay, was carried out using the method described by Speit and Hartmann (1999) based on the original work of Singh *et al.* (1988) and includes modifications introduced by Klaude *et al.* (1996) as well as some additional modifications. Briefly, for each mouse peripheral blood was collected from the orbital vein at 4 h and 24 h post-gavage and a 10 µL aliquot mixed with 120 µL of 0.5% (w/v) LMP agarose at 37 °C and rapidly spread on a microscope slide pre-coated with 1.5% (w/v) normal melting point agarose. Coverslips were added and the slides were allowed to gel at 4 °C, for 20 min before gently removing the coverslips and immersing the slides in cold freshly-prepared lysing solution consisting of 89 mL of a stock solution (890 mL of distilled water containing 2.5 M NaCl, 100 mM EDTA, 10 mM TRIS and 1% (w/v) sodium lauryl sarcosine with the pH set to 10.0 by the addition of about 8 g of solid NaOH) plus 1 mL of Triton X-100 and 10 mL of DMSO. After treatment the slides to stand at 4 °C for 1 h protected from light and then placed at the anode of a gel electrophoreses box containing high pH (> 13) electrophoresis buffer (300 mM NaOH per 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10) at 4 °C for 20 min before electrophoresis to allow the DNA to unwind. Electrophoresis was performed at 25 V and 300 mA for 20 min at 4 °C in an ice bath, after which the slides were submerged in neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethyl alcohol for 10 min. The slides were dried and stored at least overnight before staining by covering the preparation with 30 µL of 1x ethidium bromide staining solution (prepared from a 10x stock solution containing 200 µg mL^{-1} ethidium bromide) and then covered with a coverslip. The material was 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. For each mouse, 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. The cells were visually scored, according to tail size, into four classes: (1) class 0, undamaged and with no tail; (2) class 1, tail shorter than the diameter of the head (nucleus); (3) class 2, tail length 1 to 2 times the head diameter; and (4) class 3, tail longer than twice the head diameter. Comets with no heads and images with nearly all DNA in the tail, or with a very wide tail, were excluded from evaluation because they probably represent 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 (MN) assay followed the general protocol recommended by Krishna and Hayashi (2000) with slight modifications. At 48 h and 72 h post-gavage peripheral blood was collected from the orbital vein of the same mice used in the comet assay and blood-smear slides prepared, all slides being coded, fixed with methanol and stained with Giemsa solution. For each mouse we scored 4000 polychromatic erythrocytes (2000 from the 48 h

blood sample and 2000 from the 72 h blood sample) for micronuclei and 1000 cells per mouse were also scored to determine the polychromatic to normochromatic erythrocytes frequency.

Statistical analysis

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

Results and Discussion

Investigations into the nature of DNA damage and repair have provided valuable insights into aging, human genetics and cancer (Singh *et al.*, 1990). The alkaline comet assay is increasingly used in industrial *in vitro* genotoxicity testing (Rojas *et al.*, 1999; Hartmann *et al.*, 2001) and has also been used an important tool to evaluate the genotoxic potential of compounds *in vivo* (Rojas *et al.*, 1999; Sekihashi *et al.*, 2002). Our comet assay results for peripheral blood leukocytes from blood taken from 12-day old female and male Swiss mice 4 h and 24 h after treatment are summarized in Table 1. As expected, the ENU positive control results showed some fragmentation and migration of the fragments and the mean comet score was significantly higher ($p < 0.001$) than for the negative control group and the extract groups (Table 1). However, no significant effects on DNA migration were found for the three extract concentrations tested and there was no significant difference between the mean comet scores for the negative control group and the groups treated with extract (Table 1). Furthermore, there was no significant difference in DNA migration among the three extract concentrations tested. Comet class data is not shown in Table 1 (but this is available upon request), but when exposed to the test extract

Table 1 - Comet assay for the *in vivo* assessment of genotoxicity of a *Vaccinium corymbosum* hydroethanolic extract on peripheral blood leukocytes from 12-week old female (F_1 to F_{15}) and male (M_1 to M_{15}) Swiss mice (body weight (bw) 25 g to 30 g) exposed to different concentrations of extract 4 h and 24 h before the assay. Each treatment used three mice (n = 3). For each mouse total 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). The negative control was water and the positive control was N-nitroso-N-ethylurea (ENU).

	4 h post-gavage				24 h post-gavage			
	Females		Males		Females		Males	
Treatments[†]	Mouse	Comet score[‡]	Mouse	Comet score[‡]	Mouse	Comet score[‡]	Mouse	Comet score[‡]
Negative control, water	F_1	16	M_1	15	F_1	5	M_1	11
	F_2	5	M_2	6	F_2	2	M_2	4
	F_3	10	M_3	14	F_3	5	M_3	7
		(10.33 ± 5.50)		(11.66 ± 4.93)		(4.00 ± 1.73)		(7.33 ± 3.51)
Extract, 1 g kg^{-1} bw	F_4	16	M_4	23	F_4	9	M_4	17
	F_5	8	M_5	28	F_5	6	M_5	9
	F_6	5	M_6	14	F_6	6	M_6	6
		(9.66 ± 5.68)		(21.66 ± 7.09)		(7.00 ± 1.73)		(10.6 ± 5.68)
Extract, 1.5 g kg^{-1} bw	F_7	5	M_7	11	F_7	10	M_7	10
	F_8	3	M_8	4	F_8	9	M_8	4
	F_9	20	M_9	6	F_9	14	M_9	7
		(9.33 ± 9.29)		(7.00 ± 3.60)		(11.0 ± 2.64)		(7.00 ± 3.0)
Extract, 2 g kg^{-1} bw	F_{10}	1	M_{10}	14	F_{10}	25	M_{10}	21
	F_{11}	5	M_{11}	5	F_{11}	5	M_{11}	4
	F_{12}	10	M_{12}	9	F_{12}	9	M_{12}	4
		(5.33 ± 4.50)		(9.33 ± 4.50)		(13.0 ± 10.5)		(9.66 ± 9.81)
Positive control, ENU 0.05 g kg^{-1} bw	F_{13}	176	M_{13}	162	F_{13}	122	M_{13}	121
	F_{14}	171	M_{14}	174	F_{14}	110	M_{14}	107
	F_{15}	174	M_{15}	151	F_{15}	115	M_{15}	99
Mean comet score ± SD		(173.6 ± 2.5)*		(162.3 ± 11.5)*		(115.6 ± 6.02)*		(109.0 ± 11.1)*

[†]bw = body weight. Doses shown are 'equivalent to', the actual amounts administrated being smaller since each mouse weighed 25 g to 30 g.
[‡]Mean comet score ± SD in parentheses.
*Significantly different to the negative control (ANOVA and Tukey test, $p < 0.001$).

Table 2 - Peripheral blood micronucleus assay results for 12-week old Swiss mice treated with *Vaccinium corymbosum* hydroethanolic extract. Blood samples were taken 48 and 72 h after administration of different concentrations of extract. The negative control was water and the positive control was N-nitroso-N-ethylurea (ENU). For each mouse 12000 polychromatic erythrocytes (PCE) were scored for the number of micronucleated polychromatic erythrocytes (MNPCE) and the percentage calculated. The percentage of PCE per 1000 blood cells is given in the last column. Values shown are Means ± standard deviations for six mice per dose.

Treatments and sample time (h)†	MNPCEs		%PCE per 1000 cells
	Number	%MNPCE	
Negative control, water			
48 h	21	0.17 ± 0.09	43.3 ± 6.75
72 h	16	0.13 ± 0.02	40.3 ± 3.79
Positive control, ENU 0.05 g kg^{-1} bw			
48 h	54	0.45 ± 0.07***	37.3 ± 6.45
72 h	47	0.39 ± 0.05***	37.8 ± 3.24
Extract, 1 g kg^{-1} bw			
48 h	40	0.33 ± 0.12*	40.5 ± 3.26
72 h	37	0.30 ± 0.08*	39.4 ± 3.56
Extract 1.5 mg kg^{-1} bw			
48 h	46	0.38 ± 0.06**	36.5 ± 2.23
72 h	41	0.34 ± 0.09**	37.5 ± 5.22
Extract 2 g kg^{-1} bw			
48 h	29	0.24 ± 0.08	34.7 ± 4.07
72 h	43	0.35 ± 0.05***	36.2 ± 3.50

†bw = body weight. Doses shown are `equivalent to', the actual amounts administrated being smaller since each mouse weighed 25 g to 30 g.
*Significantly different to the negative control (Tukey-test, $p < 0.05$).
**Significantly different to the negative control (Tukey-test, $p < 0.01$).
**Significantly different to the negative control (Tukey-test, $p < 0.001$).

most cells examined were undamaged comet class 0 cells, a few were comet class 1 cells showing minor damage, and a very few were comet class 2 and 3 showing a large amount of damage.

The MN assay using small rodents is considered the best-documented *in vivo* assay for chromosome aberrations (clastogenic effects) in relation to the number of tested chemicals (Morita *et al.*, 1997). Our MN assay results are given in Table 2, which shows the frequency of micronucleated polychromatic erythrocytes (MNPCE) and the percentage of polychromatic erythrocytes (%PCE) in relation to normochromatic erythrocytes (NCE) for the control and treated mice. In our laboratory over the last five years the historical negative control for MN frequency ranges from 0.03% to 0.25% with a mean of 0.17%, Table 2 showing that the negative control fell within this range. As expected, the ENU positive control showed a highly significant increase in the MNPCE frequency ($p < 0.001$). The MN assay of *V. corymbosum* extract revealed that for all the doses tested the MNPCE frequency was increased and, in most cases, doubled (Table 2). No dose response was observed for the extract, this could be occurred by a possible saturation of *V. corymbosum* metabolism for the three high dosages tested. This hypothesis can be tested by assaying a range of less concentrated extracts. The %PCE showed a slight decrease but this was not significantly at any of the doses tested, indicating that the extract did not present cytotoxic properties regarding erythropoiesis (Table 2).

In this study, the comet and MN assay data showed contrasting results. The alkaline comet assay (pH > 13) is capable of detecting double and single-strand DNA breaks, alkali-labile sites, DNA-DNA/DNA-protein cross-linking and single-strand breaks associated with incomplete excision repair sites, the general advantage of this assay being its sensitivity for detecting low levels of DNA damage (Tice *et al.*, 2000). The micronuclei observed in the MN assay usually arises from loss of chromosomal fragments during the division of the nucleated precursor cells (Salamone *et al.*, 1980). This type of damage is also detectable by the comet assay but, however, micronuclei may also be formed if whole chromosomes are lost and this type of mutation is not detected by the comet assay. Our MN data suggest that *V. corymbosum* hydroethanolic extract could produce some aneugenic effects on erythrocyte precursors cells.

Phytochemical studies of *Vaccinium* berries have demonstrated the presence mainly of phenolic acid compounds (*e.g.*, caffeic, *m*-coumaric *o*-coumaric, *p*-coumaric, 3,4-dimethoxycinnamic, ferulic, gentisic, gallic, hydroxycaffeic *p*-hydroxyphenil-acetic, *o*-pyrocatechuic, protoca-

techuic, salicylic, sinapic, syringic, vanillic and veratric acids) along with many anthocyanin compounds (*e.g.*, cyanidin-3-, delphinidin-3-, malvidin-3-, peonidin-3- and petunidin-3-glucose and their arabinose and galactose analogs) as discussed by various authors (Blumenthal *et al.*, 2000; Faria *et al.*, 2005; Zadernowski *et al.*, 2005).

In related work we have attempted to quantify the total phenolics and anthocyanins in *V. corymbosum* simple hydroethanolic extract and found that the total concentration of phenolic compounds, expressed as catequin equivalents) 5.66 ± 0.01 mg g^{-1}, while the anthocyanin content was 0.099 ± 0.05 mg g^{-1} for cyanidin, 0.063 ± 0.06 mg g^{-1} for delphinidin and 0.131 ± 0.06 mg g^{-1} for malvidin (Torri *et al.*, 2007, in press). Despite the proven antioxidant effect of these anthocyanins (Lohachoompol *et al.*, 2004) and the fact that several phenolic compounds have also shown antimutagenic properties (Matkowski and Wolniak, 2005; Ragunathan and Panneerselvam, 2007) the mutagenic potential of some phenolic compounds is well known (Huberman *et al.*, 1976; Snyder and Hedli, 1996). Although further investigations will be necessary, is possible that some phenolic compounds present in *V. corymbosum* extract could be responsible for the clastogenic or aneugenic effect observed by us with the MN assay.

In summary, under our test conditions the comet assay indicated that *V. corymbosum* simple hydroethanolic extract did not induce *in vivo* DNA damage in peripheral blood cells of 12-day old female or male Swiss mice but the micronucleus assay indicated that the extract presented clastogenic or aneugenic effects, thus warranting further investigation of such extracts.

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