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

Patrícia Scotini Freitas\(^1\), Sérgio Faloni de Andrade\(^2\) and Edson Luis Maistro\(^3\)

\(^1\)Faculdade de Enfermagem, Universidade José do Rosário Vellano, Poços de Caldas, MG, Brazil.
\(^2\)Núcleo de Investigações Químico-Farmacêuticas, Universidade do Vale do Itajaí, Itajaí, SC, Brazil.
\(^3\)Departamento de Fonoaudiologia, Faculdade de Filosofia e Ciências, Universidade Estadual Paulista, Marília, SP, Brazil.

Abstract

The northern highbush blueberry (\textit{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 \textit{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 \textit{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, \textit{Vaccinium corymbosum}.

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 \textit{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 \textit{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 \textit{Vaccinium} species has revived due to the pharmacological properties of anthocyanins, which includes antioxidant activity (Wang \textit{et al}., 1997; Mazza \textit{et al}., 2002; Lohachoompol \textit{et al}., 2004), anti-inflammatory effects (Youdim \textit{et al}., 2002), cardiovascular protection, anti-diabetic properties, improvements to vision and inhibition of carcinogenesis (Cabrita and Andersen, 1999; Camire, 2000; Katsube \textit{et al}., 2003). The fruits of the economically important northern highbush blueberry (\textit{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 \textit{et al}., 1998; Ehlenfeldt and Prior, 2001).

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

Material and Methods

Plant material and chemicals

Northern highbush blueberry (\textit{Vaccinium corymbosum} L. Ericales, Ericaceae) berries were collected in an experimental field in the town of Videira...
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 were used in the present study. The mice were kept in polyethylene boxes in groups of six mice (three females and three males) in a climate-controlled environment (25 ± 5°C, 55 ± 5% humidity) with a 12 h (0700 to 1900) day-length. All the mice were fed ad libitum and had free access to drinking water. The mice were kept in the animal house of the Jose do Rosario Vellano University (UNIFENAS), Alfenas, Minas Gerais, Brazil. The mice were kept in polyethylene bags at -20 °C until extract preparation. The mice were kept in polyethylene bags at -20 °C until extract preparation. The mice were euthanized by decapitation and the livers were excised and weighed. The liver weight was used as an index of liver size.

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 mice used in the comet assay and blood-smear slides were 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 (F1 to F15) and male (M1 to M15) 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). |
|---|---|---|---|---|---|---|---|---|
| Treatments† | Females Mouse Comet score‡ | Males Mouse Comet score‡ | Females Mouse Comet score‡ | Males Mouse Comet score‡ |
| Negative control, water | F1 16 M1 15 | | F1 5 M1 11 | |
| | F2 5 M2 6 | | F2 2 M2 4 | |
| | F3 10 M3 14 | | F3 5 M3 7 | |
| | (10.33 ± 5.50) | (11.66 ± 4.93) | (4.00 ± 1.73) | (7.33 ± 3.51) |
| Extract, 1 g kg⁻¹ bw | F4 16 M4 23 | F4 9 M4 17 | |
| | F5 8 M5 28 | F5 6 M5 9 | |
| | F6 5 M6 14 | F6 6 M6 6 | |
| | (9.66 ± 5.68) | (21.66 ± 7.09) | (7.00 ± 1.73) | (10.6 ± 5.68) |
| Extract, 1.5 g kg⁻¹ bw | F7 5 M7 11 | F7 10 M7 10 | |
| | F8 3 M8 4 | F8 9 M8 4 | |
| | F9 20 M9 6 | F9 14 M9 7 | |
| | (9.33 ± 9.29) | (7.00 ± 3.60) | (11.0 ± 2.64) | (7.00 ± 3.0) |
| Extract, 2 g kg⁻¹ bw | F10 1 M10 14 | F10 25 M10 21 | |
| | F11 5 M11 5 | F11 5 M11 4 | |
| | F12 10 M12 9 | F12 9 M12 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 | F13 176 M13 162 | F13 122 M13 121 | |
| | F14 171 M14 174 | F14 110 M14 107 | |
| | F15 174 M15 151 | F15 115 M15 99 | |
| | (533 ± 2.5) | (162 ± 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).
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.

### 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).
Mutagenic evaluation of *V. corymbosum*

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 catechin 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.

**Acknowledgments**

We thank the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG – Rede Mineira de Ensaios Toxicológicos e Farmacológicos de Produtos Terapêuticos, EDT - 1879/02) for their financial support for this study. We also thank Lucimara Maria da Silva for her technical assistance.

**References**

Blumenthal M, Goldberg A and Brinckmann J (2000) Herbal Medicine – Expanded Commission E Monographs. American Botanical Council, Newton pp 519.

Cabrita L and Andersen OM (1999) Anthocyanins in blue berries of *Vaccinium padifolium*. Phytochemistry 52:1693-1696.

Camire ME (2000) Bilberries and blueberries as functional foods and nutraceuticals. In: Mazza G and Oomah BD (eds) Functional Foods: Herbs, Botanicals and Teas. Technomic Publishing, Lancaster, pp 289-319.

Ehlenfeldt MK and Prior RL (2001) Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentra-

tions in fruit and leaf tissues of highbush blueberry. J Agric Food Chem 49:2222-2227.

Faria A, Oliveira J, Neves P, Gameiro P, Santos-Buelga C, Freitas V and Mateus N (2005) Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. J Agric Food Chem 53:6896-6902.

Hartmann A and Speit G (1997) The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). Toxicol Lett 90:183-188.

Hartmann A, Elhajouji A, Kiskinis E, Poetter F, Martus HJ, Fjallman A, Frieauff W and Suter W (2001) Use of the alkaline comet assay for industrial genotoxicity screening: Comparative investigation with the micronucleus test. Food Chem Toxicol 39:843-858.

Huberman E, Sachs L, Yang SK and Gelboin V (1976) Identification of mutagenic metabolites of benzo(a)pyrene in mammalian cells. Proc Natl Acad Sci USA 73:607-611.

Hoyos LS, Au WW, Heo MY, Morris DL and Legator MS (1992) Evaluation of the genotoxic effects of a folk medicine, *Petiveria alliacea* (Anamu). Mutat Res 280:29-34.

Katsube N, Iwashita K, Tsushima T, Yamaki K and Kobori M (2003) Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. J Agric Food Chem 51:68-75.

Klaude M, Eriksson S, Nygren J and Ahnström G (1996) The comet assay: Mechanisms and technical considerations. Mutat Res 363:89-96.

Krishna G and Hayashi M (2000) *In vivo* rodent micronucleus assay: Protocol, conduct and data interpretation. Mutat Res 455:155-166.

Lohachoompol V, Srzednicki G and Craske J (2004) The change of total anthocyanins in blueberries and their antioxidiant effect after drying and freezing. J Biomed Biotechnol 5:248-252.

Matkowski A and Wolniak D (2005) Plant phenolic metabolites as the free radical scavengers and mutagenesis inhibitors. BMC Plant Biol 5(Suppl 1):S23.

Mazza G, Kay CD, Cottrell T and Holub BJ (2002) Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J Agric Food Chem 50:7731-7737.

Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H, Sutou S, Suzuki T, Wakata A, Sofuni T et al. (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B). The summary report of the 6th collaborative study of the micronucleus group test. Mammalian mutagenicity study group. Mutat Res 389:3-122.

Prior RL, Cao G, Martin A, Sofic E, McEwen J, O’Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G et al. (1998) Antioxidant capacity is influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J Agric Food Chem 46:2686-2693.

Ragunathan I and Panneerselvam N (2007) Antimutagenic potential of curcumin on chromosomal aberrations in *Allium cepa*. J Zhejiang Univ Sci B 8:470-475.

Rojas E, Lopez MC and Valverde M (1999) Single cell gel electrophoresis assay: Methodology and applications. J Chromatogr Biomed Sci Appl B 722:225-254.

Salamone M, Heddle J, Stuart E and Katz M (1980) Towards an improved micronucleus test: Studies on 3 model agents,
mitomycin C, cyclophosphamide and dimethylbenzanthrace. Mutat Res 74:347-356.

Sekihashi K, Yamamoto A, Matsumura Y, Ueno S, Watanabe-Akanuma M, Kassie F, Knasmuller S, Tsuda S and Sasaki YF (2002) Comparative investigation of multiple organs of mice and rats in the comet assay. Mutat Res 517:53-75.

Singh NP, McCoy MT, Tice RR and Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191.

Singh NP, Danner DB, Tice RR, Brant L and Schneider EL (1990) DNA damage and repair with age in individual human lymphocytes. Mutat Res 237:123-130.

Snyder R and Hedli CC (1996) An overview of benzene metabolism. Environ Health Perspect 104(Suppl. 6):1165-1171.

Sokal RR and Rohlf FJ (1995) Biometry. W.H. Freeman, San Francisco, 887 pp.

Speit G and Hartmann A (1999) The comet assay (single-cell gel test). In: Henderson DS (ed) Methods in Molecular Biology, v. 113, DNA Repair Protocols: Eukaryotic Systems. Humana Press Inc., Totowa, pp 203-212.

Surh YJ and Ferguson LR (2003) Dietary and medicinal anti-mutagens and anticarcinogens: Molecular mechanisms and chemopreventive potential – Highlights of a symposium. Mutat Res 523-524:1-8.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC and Sasaki YF (2000) Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206-221.

Torri E, Lemos M, Caliari V, Kassuya CAL, Bastos JK and Andrade SF (2007) Anti-inflammatory and antinociceptive properties of blueberry extract (Vaccinium corymbosum). J Pharm Pharmacol (in press).

Wang H, Cao G and Prior RL (1997) Oxygen radical absorbing capacity of anthocyanins. J Agric Food Chem 45:304-309.

Youdim KA, McDonald J, Kalt W and Joseph JA (2002) Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. J Nutr Biochem 13:282-288.

Zadernowski R, Naczk M and Nesterowicz J (2005) Phenolic acid profiles in some small berries. J Agric Food Chem 53:2118-2124.

Associate Editor: Catarina S. Takahashi

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.