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Research Paper

In vivo evaluation of the genetic toxicity of *Rubus niveus* Thunb. (Rosaceae) extract and initial screening of its potential chemoprevention against doxorubicin-induced DNA damage

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ABSTRACT

Ethnopharmacological relevance: *Rubus niveus* Thunb. plant belongs to Rosaceae family and have been used traditionally to treat wounds, burns, inflammation, dysentery, diarrhea and for curing excessive bleeding during menstrual cycle. The present study was undertaken to investigate the *in vivo* genotoxicity of *Rubus niveus* aerial parts extract and its possible chemoprotection on doxorubicin (DXR)-induced DNA damage. In parallel, the main phytochemicals constituents in the extract were determined.

Materials and methods: The animals were exposed to the extract for 24 and 48 h, and the doses selected were 500, 1000 and 2000 mg/kg b.w. administered by gavage alone or prior to DXR (30 mg/kg b.w.) administered by intraperitoneal injection. The endpoints analyzed were DNA damage in bone marrow and peripheral blood cells assessed by the alkaline alkaline (pH > 13) comet assay and bone marrow micronucleus test.

Results and conclusion: The results of chemical analysis of the extract showed the presence of tormentic acid, stigmasterol, quercitinglucoronide (miquelianin) and niga-ichigoside F1 as main compounds. Both cytogenetic endpoints analyzed showed that there were no statistically significant differences ($p > 0.05$) between the negative control and the treated groups with the two higher doses of *Rubus niveus* extract alone, demonstrating absence of genotoxic and mutagenic effects. Aneugenic/clastogenic effect was observed only at 2000 mg/kg dose. On the other hand, in the both assays and all tested doses were observed a significant reduction of DNA damage and chromosomal aberrations in all groups co-treated with DXR and extract compared to those which received only DXR. These results indicate that *Rubus niveus* aerial parts extract did not revealed any genotoxic effect, but presented some aneugenic/clastogenic effect at higher dose; and suggest that it could be a potential adjuvant against development of second malignant neoplasms caused by the cancer chemotherapic DXR.

1. Introduction

Considering that many plants can present toxic effects, and that genetic toxicity is associated with an increased overall risk of cancer (Hagmar et al., 1998), it is essential to perform studies evaluating the risk of the use of plants with medicinal potential for human health. In parallel, is also essential to identify plants that confer chemical prevention against genotoxic agents to decrease the overall risk of cancer and also to protect against secondary cancers caused by traditional treatments with chemotherapy (Meadows et al., 1985).

The genus *Rubus* is very diverse including over 750 species distributed in 12 subgenera, and is found on all continents except Antarctica (Finn, 2008). Several of the *Rubus* plant species are

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grown as ornamentals and specially for their fruits, are used as medicinal herbs, due to several of them present tannins accumulation. *Rubus niveus* Thunb. (Rosacea family) is native from Indian to southeastern Asia, the Philipines, and Indonesia (Gerrish et al., 1952). Fruits and other parts of the plant from *Rubus* genus have been used traditionally to treat wounds, burns, inflammation (Mann et al., 1994), diabetes (Patel et al., 2004), as antispasmodic (Tanira et al., 1996), and chemical and pharmacological studies have shown that some active principles of them to present antigastropathic, antiinflammatory and antioxidant properties (Choi et al., 2003; Nam et al., 2006). Other activities also have been reported, like antimicrobial, analgesic (Richards et al., 1994; Niero et al., 1999; Thiem and Goslinska, 2004), hypoglycaemic (Novaes et al., 2001; Kanegusuku et al., 2002), cytotoxicity against viruses and antinociceptive effects (Niero et al., 1999). Concerning to traditional use of *Rubus niveus*, the fruits and other parts have been used by local people for the treatment of various ailments. The ripen fruits are eaten by some local people (Singh, 2008; Srivastava et al., 2010). The fresh root tips are used for curing excessive bleeding during menstrual cycle. The root tips are made into a paste with water and small pills are made. one pill per day, preferably with butter made from buffalo milk, is taken empty stomach in the morning for 7 days (Uniyal et al., 2006). Root is also used for the treatment of dysentery and diarrhea (Pfoze et al., 2012). Chemical and pharmacological studies have shown that products from aerial parts and some extracts have nematicidal activity (Sultana et al., 2010); antimicrobial activity of extracts and fractions from aerial parts Melin et al. (2013); and root extract have anti-inflammatory, analgesic, antipyretic (George et al., 2013), antitumor and wound healing properties (George et al., 2014).

Knowing that assessing the genetic toxicity of *Rubus niveus* extract in an *in vivo* system is essential for establishing its safety, we investigated this genotoxicity in different cells of mice by the comet and micronucleus assays. Furthermore, the possible protective effect of *Rubus niveus* extract against doxorubicin (DXR)-induced genotoxicity was also investigated to contribute with new knowledge that could to explore the characteristics of this plant as a promoter of good health.

2. Material and methods

2.1. Chemicals

Doxorubicin (DXR) (IMA S.A.I.C. Laboratories) was used as the positive control substance due to its potential as DNA damaging agent in the comet assay and micronucleus test using Swiss mice. The other main chemicals were obtained from the following suppliers: normal melting point (NMP) agarose (Cat. no. 15510-019—Invitrogen), low melting point (LMP) agarose (Cat. no. 15517-014—Invitrogen), sodium salt *N*-lauroyl sarcosinate (L-5125—Sigma) and ethylenediaminetetraacetic acid (EDTA—Merck).

2.2. Plant material

Rubus niveus Thunb. (Rosaceae) was collected in proximities of Universidade do Vale do Itajaí (SC—Brazil; Latitude 26°54′28″S and longitude 48°39′43″W), in July 2010 and identified by MSc. Renê Arthur Ferreira. A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí—SC) under number V.C. Filho 035. The material was originated from plantation private local, who does not require specific permissions and does not involve endangered or protected species.

2.3. Phytochemical analysis

Air-dried material from *Rubus niveus* aerial parts (700 g) was cut into small pieces and macerated with methanol at room temperature for seven days. The material was filtrated and the solvent totally removed by rotary evaporator under reduced pressure, yielding the respective methanolic extract (ME, 35 g).

Rubus niveus crude extract was solubilized in a methanol:water (1:1) added with 0.1% formic acid solution for positive mode analysis. The same procedure was used to the negative mode analysis, using methanol:water (1:1) added with 0.1% ammonium hydroxide solution.

The solutions were directly infused into the mass spectrometer electrospray ionization source (ESI). The ESI-MS and ESI-MS/MS were acquired using the positive mode for the acidic solution and negative mode for the alkaline solution.

The chromatographic separation was performed using a Hybrid BEH (EthyleneBridgedHybrid) Acquity UPLC C18 column, with 1.7 μm particle size. The mobile phase consisted of methanol: formic acid 0.1% (65:35 v/v), and was delivered at a 0.7 mL/min flow rate at room temperature. The injection volume was 5 μL.

Equipament: The UPLC used consisted of a Waters Acquity UPLC. A Waters triple–triple TDQ MS/MS mass spectrometer with an electrospray ionization source (ESI) was used as a detector. The sequential mass analysis used Argonium as collision gas. Analyst software Masslynx was used for the control of equipment, acquisition and data analysis. The analyses were monitored in the full-scan mode and the mass lines intended to be analyzed were chosen for dissociation induced by collision.

2.4. Animals and treatments

The experiments were carried out using 12-week-old male Swiss albino mice (*Mus musculus*), weighing 25–30 g. The animals were acquired from the Universidade Estadual Paulista (UNESP), Botucatu, São Paulo State, Brazil, and housed in polyethylene boxes in a climate-controlled environment (25 ± 4 °C, 55 ± 5% humidity) with a 12-h light/dark cycle (7:00 am to 7:00 pm). Food (Nuvilab CR1, Nuvital) and water were available *ad libitum*. The mice were divided into 8 experimental groups of 11 animals each. *Rubus niveus* extract was dissolved in 1% Tween 80 aqueous solution and administered in a single dose of 0.3 mL by gavage at concentrations of 500, 1000 and 2000 mg/kg body weight. These doses were selected based on our preliminary acute toxic studies in mice, which was higher than 2000 mg/kg, and following the limit dose recommended by OECD (2001, 420). At these doses, the animals showed no signs of toxicity such as locomotor alterations, diarrhea or piloerection. The negative control group received 1% Tween 80 aqueous solution by gavage, and the positive control group received an intraperitoneal injection of doxorubicin (DXR) at 30 mg/kg body weight. Simultaneous treatments for antigenotoxic evaluation were carried out by administering the extract and then DXR 1 h later. The animals used in this study were sacrificed by cervical dislocation without anesthesia, to avoid possible alterations in the DNA damage analysis. The Animal Bioethics Committee of the Faculdade de Medicina de Marília (CEP/FAMEMA, Marília, São Paulo state, Brazil) approved the present study on 28 March 2011 (protocol number 105/11), in accordance with federal government legislations on animal care.

2.5. Comet assay

The comet assay (or single cell gel electrophoresis (SCGE)) was carried out by the method described by Speit and Hartmann (1999) and reviewed by Burlinson et al. (2007). Peripheral blood samples from the tail vein were obtained from six Swiss mice of each group, at