

Cytotoxic, Hypoglycemic Activity and Phytochemical Analysis of *Rubus imperialis* (Rosaceae)

Márcia Kanegusuku^c, Jean Carlo Benassi^a, Rozangela Curi Pedrosa^a, Rosendo Augusto Yunes^b, Valdir Cechinel Filho^c, Alcibia Azevedo Maia^c, Márcia Maria de Souza^c, Franco Delle Monache^d and Rivaldo Niero^{c,*}

^a Departamento de Bioquímica and

^b Departamento de Química, Universidade Federal de Santa Catarina (UFSC), 88.040-900, Florianópolis, SC, Brazil

^c Núcleo de Investigações Químico – Farmacêuticas (NIQFAR)/CCS, Universidade do Vale do Itajaí (UNIVALI), 88.302-202, Itajaí, SC, Brazil.

Fax: +47-341-7601. E-mail: niero@ccs.univali.br

^d Centro Chimica Recettori (CNR), Largo F. Vito 1, Rome, Italy

* Author for correspondence and reprint requests

Z. Naturforsch. **57c**, 272–276 (2002); received October 11/December 20, 2001

Rubus imperialis, *Artemia salina*, 3-O-methylelagic-4'-O- α -rhamnose Acid

Screening of different extracts, fractions and compounds from *Rubus imperialis* Chum. Schl. (Rosaceae) has been conducted using the brine shrimp microwell cytotoxicity assay. Three parts of the plant (methanolic extract from leaves, roots and stems), three fractions from roots (hexane, ethyl acetate and butanol) and three isolated compounds (niga-ichigoside F1, 23-hydroxytormentonic acid, ellagic acid derivative) were tested. The most promising material ($LC_{50} < 1000 \mu\text{g/ml}$) were the methanolic extract and ethyl acetate fraction from roots. However, there was little correlation observed in the degree of toxicities observed between the isolated compounds. On the other hand, the cytotoxicity and *in vivo* assays confirmed the hypoglycemic activity of methanolic extract and validated the Brazilian popular use of *R. imperialis* as an antidiabetic agent.

Introduction

The utilization of bioassays for the monitoring of extracts, fractions and compounds obtained from plants, is frequently incorporated in phytochemical research. A rapid and inexpensive test, brine shrimp (*Artemia salina*) (BST), has been used for screening of biological and cytotoxicity activities (De Rosa *et al.*, 1994). The fractions or active compounds in this assay, are further tested in cultured tumoral cells, antimicrobial and anti-parasitic assays, generally with good correlation (Sahpaz *et al.*, 1994; Colman-Saizarbitoria *et al.*, 1995; Siqueira *et al.*, 1998).

The genus *Rubus* (Rosaceae) is employed in some countries to treat different diseases, especially diabetes (Alonso *et al.*, 1980). Previous studies have confirmed that some of these species produce compounds that exert hypoglycemic, antibacterial, anti-allergic and anti-asthmatic activities (Swanson-Flatt *et al.*, 1990; Richards *et al.*, 1994; Nakahara *et al.*, 1996).

R. imperialis grows abundantly in the south of Brazil, being known as “mora-branca” or “mora-

do-mato” and is frequently used in traditional medicine as a remedy to treat diabetes and dolorous process (Cirilo, 1993). We have recently shown that some extracts and a compound denominated Niga-ichigoside F1 exhibit interesting antinociceptive profile (Niero *et al.*, 1999; Niero, 2000). We have now extended our studies and evaluated different extracts from distinct parts from *R. imperialis* which were subjected to cytotoxic studies using brine shrimp (*Artemia salina*) model. In addition, we have tested niga-ichigoside (**1**), 23-hydroxy-tormentonic acid (**2**) and an ellagic acid derivative (**3**) (Fig. 1), that were isolated of the ethyl acetate extract from roots.

Results and Discussion

Considering that a bioassay is the first step necessary for the drug discovery process from ethnomedical systems, some extracts and fractions from *Rubus imperialis*, a reputed Brazilian medicinal plant, were screened for *in vitro* toxicity using the microwell assay. This method allows the use

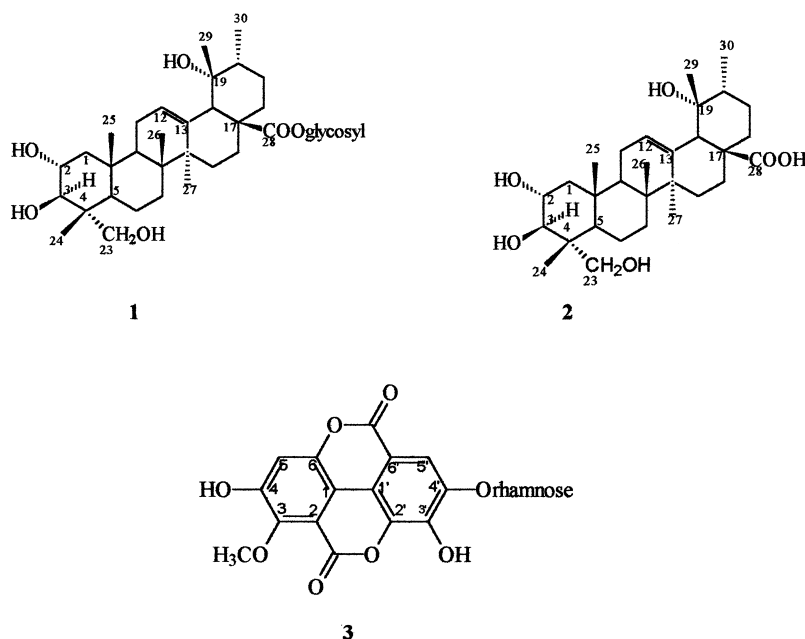


Fig. 1. Molecular structure of the isolated compounds from *R. imperialis* (**1** = nigaichigoside; **2** = 23-hydroxytormentonic acid; **3** = 3-O-methylelagic-4'-O- α -rhamnose acid).

of smaller quantities of extracts, fractions or pure compounds, and permits a larger number of samples and dilutions within a shorter time than using the original test vials.

The LC_{50} (half-inhibition) values obtained after the analysis of some extracts and fractions with different polarities are shown in Table I. Three extracts or fractions demonstrated toxicity to BST ($LC_{50} < 1000 \mu\text{g/ml}$) and extraction with methanol yielded a larger proportion of bioactive extract than hexane or ethyl acetate. These results suggested the presence of bioactive compounds and required further examination using elaborated bioassays for detection of more specific pharmaco-

logical proprieties. It was important, also, to separate and chemically identify the major constituents present in this plant. Surprisingly, the pure compounds (**1**, **2** or **3**) were practically inactive, showing a LC_{50} values higher than $1000 \mu\text{g/ml}$, which suggested that further investigations are necessary to determine if other compounds are responsible for the cytotoxic activity or the existence of a synergic effect.

On the other hand, based on the possible relationship between BST cytotoxicity and plant bioactivity (Mongelli *et al.*, 1996; Alkofahi *et al.*, 1996; Shimada *et al.*, 1997), this work encouraged the research on biological assays *in vivo* of this plant.

Table I. LC_{50} and 95% confidence interval ($\mu\text{g/ml}$) of extracts, fractions and compounds obtained from *R. imperialis* tested at 1000, 100 and $10 \mu\text{g/ml}$ in the *Artemia salina* bioassay. Quinidine sulfate ($200 \mu\text{g/ml}$) was used as a positive control.

Tested material	LC_{50} [$\mu\text{g/ml}$]	95% confidence interval
Methanolic extract (leaves)	387.10	(362.83–412.43)
Methanolic extract (roots)	111.10	(76.32–144.88)
Methanolic extract (stems)	203.40	(154.60–252.40)
Hexane fraction (roots)	>1000	n.d.
Ethyl acetate fraction (roots)	680.00	(592.00–768.00)
Niga-ichigoside F1	>1000	n.d.
23-hydroxytormentonic acid	>1000	n.d.
3-O-methylelagic-4'-O- α -rhamnose acid	>1000	n.d.

n.d., not determined.

A great number of plants have documented "folk Brazilian medicine" reputations as hypoglycemic agents and some of these have been evaluated under controlled conditions, including *Rubus imperialis* (Novaes *et al.*, 2001). These hypoglycemic effects were produced after oral administration of extracts to diabetic rats. In our test, the oral administration of the methanolic extract from aerial parts of *R. imperialis* (300 mg/kg body weight) to normoglycemic rats produced a significant decrease of blood glucose compared with the control group (Fig. 2), whose hypoglycemic effect was produced after a 10-days treatment. According to our results, *R. imperialis* exhibits a hypoglycemic activity that validate the popular use of this plant for the treatment of *diabetes mellitus* symptomatology and confirm the results obtained with BST test.

Studies are currently in progress in our laboratories to determine the active principles of *R. imperialis*.

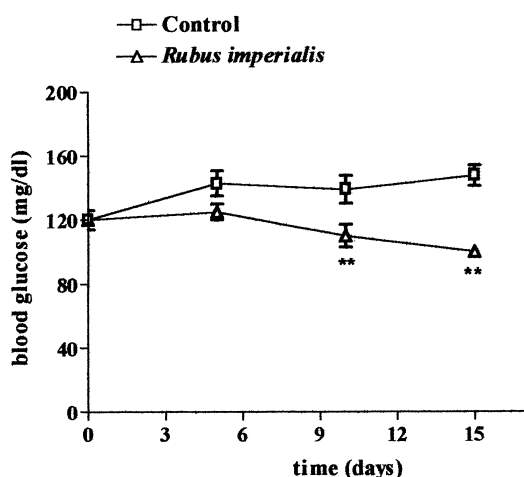


Fig. 2. Effect of the methanolic extract of *Rubus imperialis* (300 mg/kg, via oral) on plasma glucose level in normoglycemic rats treated during 15 days. All values are mean \pm SEM, $n = 6$, (**) significant differences between treated and control group were evaluated by ANOVA and Tukey-Kramer multiple comparison test, $p < 0.01$.

Material and Methods

Plant material

R. imperialis was collected in Florianópolis, Brazil, in June 1997 and identified by Dr. Ademir Reis (Department of Botany, UFSC). A voucher speci-

men was deposited at Barbosa Rodrigues Herbarium (Itajaí) under number V. C. Filho 012.

Extraction and isolation

The dried parts of the plant (leaves, roots and stems, 100 g each), were powdered and macerated with MeOH (100 ml) for seven days at room temperature. After evaporation of the solvent under reduced pressure, the respective methanolic extracts were obtained. Considering that the methanolic extract from roots (8.4 g) showed the better activity, it was successively partitioned with hexane and ethyl acetate affording 0.88 g and 4.3 g (residue dry) of each fraction, respectively.

The EtOAc fraction exhibited activity (Table I), and was selected for phytochemical studies. Thus, it was fractionated on chromatographic column over Silica gel 60, by elution with CHCl_3 -MeOH (80:20), given 150 mg of niga-ichigoside F_1 (1), 250 mg of 23-hydroxy-tormentic acid (2) and 350 mg of 3-O-methylelagic-4'-O- α -rhamnose acid (3), which were identified by spectroscopic data (IR, ^1H and ^{13}C NMR, MS) and compared with literature values (Niero *et al.*, 1999; Niero, 2000; Durham *et al.*, 1996; Seto *et al.*, 1984; Malhotra and Misra, 1981).

Brine shrimp microwell cytotoxicity assay

The test was performed as described by Meyer *et al.*, (1982). Each extract or fraction solutions was tested at a concentration level of 1000, 100 and 10 $\mu\text{g/ml}$ dispensing 100 μl in six replicates into wells of a 24-well microplate. Brine shrimp eggs (*Artemia salina* Leach) were purchased in the locality and hatched in artificial sea water (solution of NaCl 3.8%) at room temperature. After 48 h, the larvae (nauplii) were collected. A suspension of 10 nauplii (100 μl) was added to each well and the covered microplate was incubated for 24 h at room temperature. After this period, the number of dead nauplii in each well was counted using a E. Leitz Wetzlar binocular microscope (10X). Quinidine sulfate (200 $\mu\text{g/ml}$, Sigma, St. Louis, USA) was used as a positive control. Finney's (1971) statistical method of probit analysis was used to calculate the concentration of the extract or fractions that would kill 50% of brine shrimps within the 24 h exposure, i.e. the LC_{50} with the 95% confidence intervals. In cases where

data were insufficient for this technique, the dose-response data were transformed into a straight line by means of a logit transformation; the LC_{50} was derived from the best fit line obtained by linear regression analysis. The extract, fraction or isolated compounds were considered bioactive when LC_{50} was 1000 $\mu\text{g/ml}$ or less.

Normoglycemic rats glucose assay

The experimental animals used in these studies were adult male Wistar rats (*Rattus norvegicus*, 200–250 g) maintained under a 12h/12h light/dark cycle at an ambient temperature of $24^{\circ} \pm 2^{\circ}\text{C}$ with free access to standard commercial food and tap water *ad libidum*. After completing one week of acclimatization, the rats were divided into two groups of 6 animals and used in the experiments. The animals were allowed free access to both food and drinking water throughout the experimental periods. A dose of 300 mg/kg of the methanolic plant extract was administered by oral application

during 15 days. Every five days the rats were anaesthetized with diethylether, and blood samples were collected by ocular puncture and centrifuged at $1000 \times g$, 4°C to 10 min. The fasting glucose level was determined by the glucose oxidase method using commercial kits.

Statistical analysis

Statistical significance of the data was analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Values corresponding to $p < 0.01$ were considered statistically significant.

Acknowledgements

The authors are grateful to Prof. Dr. A. Reis for the botanical identification and to CNPq, CAPES, and UNIVALI/Brazil for financial support. Thanks are expressed to Department of Chemistry and Biochemistry of the Federal University of Santa Catarina for technical support.

- Alkofahi A., Batshoun R., Owais W. and Najib N. (1996), Biological activity of some Jordanian medicinal plant extracts. *Fitoterapia* **67**, 435–442.
- Cirilo V. K., (1993), *Manual de Plantas Mediciniais*, 44^a ed. Assessor, Francisco Beltrão: Paraná, Brasil.
- Colman-Saizarbitoria, T., Gu Z. M., Zhao Z. X., Zeng L., Kozlowski J. F. and McLaughlin J. L. (1995), Venezenin: A new bioactive annonaceous acetogenin from the bark of *Xylopia aromática*. *J. Nat. Prod.* **58**, 532–539.
- De Rosa S., De Giulio A. and Iodice C. (1994), Biological effects of prenylated hydroquinones: structure-activity relationship studies in antimicrobial, brine shrimp, and fish lethality assays. *J. Nat. Prod.* **57**, 1711–1716.
- Durhan D. G., Liu X., Richards E. and Michael R. (1994), A triterpene from *Rubus pinfaensis*. *Phytochemistry* **36**, 1469–1472.
- EP 0 727 218 A2 – EUROPEAN PATENT APPLICATION – Date of publication: 21.08.1996 Bulletin 1996/34. Application number: 96101937.9. Date of filing: 09.02.1996.
- Finney D. (1971), *Probit Analysis*, 3rd ed. Cambridge University Press, Cambridge, UK. pp.76–80.
- Malhotra S. and Misra K. (1981), 3,3'-Di-O-Methyl-lagic acid 4-O-rhamnoside from the roots of *Prosopis juliflora*. *Phytochemistry* **20**, 2043–2044.
- Meyer B. N., Ferrigni N. R., Putnam J. E., Jacobsen L. B., Nichols D. E. and McLeughlin J. L. (1982), Brine shrimp: a convenient general bioassay for active plant constituents, *Planta Med.* **45**, 31–34.
- Mongelli E., Martino V., Coussio J. and Ciccio G. (1996), Screening of argentine medicinal plants using the brine shrimp microwell cytotoxicity assay, *Intern. J. Pharmacog.* **34**, 249–254.
- Niero R., Montanari J. L., Cechinel-Filho V., De Souza M. M., Delle Monache F. and Yunes R. A. (1999), Antinociceptive activity of niga-ichigoside F1 from *Rubus imperialis*. *J. Nat. Prod.* **62**, 1145–1149.
- Niero, R. Obtenção de novas moléculas com atividade analgésica e antiinflamatória a partir de plantas medicinais brasileiras. Tese de doutorado. Universidade Federal de Santa Catarina, 2000.

- Novaes A. P., Rossi C., Poffo C., Pretti Junior E., Oliveira A. E., Schlemper V., Niero R., Cechinel-Filho V. and Bürger C. (2001), Preliminary evaluation of the hypoglycemic effect of some Brazilian medicinal plants. *Thérapie* **56**, 156–159.
- Richards R. M., Durhan, E. D. G. and Liu X. (1994), Antibacterial activity of compounds from *Rubus pinfaensis*. *Planta Med.* **60**, 471–473.
- Sahpaz S., Bories C., Loiseal P. M., Cortes D., Hocquemiller R., Laurens A. and Cavé A. (1994), Cytotoxic and antiparasitic activity from *Annona senegalensis* seeds. *Planta Med.* **60**, 538–540.
- Siqueira M. J., Bomm, D. M., Pereira N. F. G., Garcez W. S. and Boaventura M. A. D. (1998), Estudo fitoquímico de *Unonopsis lindmanii*-Annonaceae, biomonitorado pelo ensaio de toxicidade sobre *Artemia salina* LEACH. *Quim. Nova* **21**, 557–559.
- Seto T., Tanaka T., Tanaka O. and Naruhashi, N. (1984), β -Glucosyl esters of 19 α -hydroxyursolic acid derivatives in leaves of *Rubus* species. *Phytochemistry* **23**, 2829–2834.
- Shimada H., Tyler V. E. and McLaughlin J. L. (1997), Biologically active acylglycerides from the berries of saw palmetto (*Serenoa repens*). *J.Nat. Prod.* **60**, 417–418.
- Suffness M. and Douros J., (1979), In: *Methods in Cancer Research*, vol. **26**, Cancer Drug Development, Part A, De Vita V. T. (Bush H. Ed). Academic Press, New York, pp.73–77.
- Swanson-Flatt K., Day C., Bailey C. J. and Flatt P. R. (1990), Traditional plant treatments for diabetes. Studies in normal and streptozotocin diabetic mice. *Diabetologia* **33**, 462–465.