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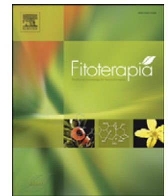

Effect of *Citrus bergamia* juice on human neuroblastoma cells *in vitro* and in metastatic xenograft models

M. Navarra [a], M.R. Ursino [a], N. Ferlazzo [a], M. Russo [a], U. Schumacher [b], U. Valentiner [b,*]

[a] *Department of Drug Sciences and Health Products, University of Messina, Messina, Italy*
[b] *Institute of Anatomy and Experimental Morphology, University Medical Center, Hamburg-Eppendorf, Germany*

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ABSTRACT

Neuroblastoma is the most common extracranial pediatric solid tumor with poor prognosis in children with disseminated stage of disease. A number of studies show that molecules largely distributed in commonly consumed fruits and vegetables may have anti-tumor activity. In this study we evaluate the effect of *Citrus bergamia* (bergamot) juice (BJ) *in vitro* and in a spontaneous metastatic neuroblastoma SCID mouse model. Qualitative and quantitative characterizations of BJ flavonoid fractions were performed by RP-HPLC/PDA/MS. We show that BJ significantly affects SK-N-SH and LAN-1 cell proliferation *in vitro*, but fails to reduce primary tumor weight *in vivo*. Moreover, BJ reduced cell adhesiveness and invasion of LAN-1 and SK-N-SH cells *in vitro* and the number of pulmonary metastases under consideration of the number of tumor cells in the blood in mice inoculated with LAN-1 cells *in vivo*. These effects without any apparent sign of systemic toxicity confirm the potential clinical interest of BJ and lay the basis for further investigation in cancer.

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1. Introduction

Neuroblastoma (NB) is the most common tumor in children less than one year of age worldwide. Each year, approximately 1500 cases occur in Europe and 700 in the USA and Canada, accounting for about 28% of all cancers diagnosed in European and USA infants [1]. NB is a malignant embryonic tumor of the neural crest cells and therefore can develop anywhere along the strands of the sympathetic nervous system [2]. Hematogenous metastasis is present in about half of NB patients and is responsible for many NB deaths. Although aggressive and intensive multimodality therapies (surgery, cytotoxic chemotherapy, radio-metabolic treatment) have produced some improvements in the overall cure rate of NB patients, the prognosis of patients with metastatic NB remains poor. Thus, novel therapeutic strategies to ameliorate the prognosis of NB patients are required.

Plant kingdom has always been an attractive source of novel anticancer drugs resulting in the fact that about 50% of anticancer drugs are natural or semisynthetic products [3]. Epidemiologic studies provided evidence that *Citrus* consumption is associated with a reduced all-cancer incidence, although significant results were obtained only for prostate and pancreatic cancers [4]. In contrast, some studies did not find an association between *Citrus* fruit intake and cancer prevention [5,6]. However, several *in vivo* studies suggest that *Citrus* juices have a potential in cancer prevention. Thus, it has been documented that mandarin juice may reduce both chemically-induced rat colon carcinogenesis [7] and lung cancer formation [8]. Moreover, orange juice may decrease both chemically-induced mammary tumor burden and azoxymethane-induced colon cancer in rats [9,10]. Further, grapefruit juice shows a suppressive effect on *in vivo* induced colon carcinogenesis [11].

Citrus bergamia Risso et Poiteau (bergamot) is a small tree belonging to the family Rutaceae. 90% of bergamot worldwide

* Corresponding author at: Center for Experimental Medicine, Institute of Anatomy and Experimental Morphology, University Hospital Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Tel.: +49 40 7410 53587; fax: +49 40 7410 55427.
E-mail address: valentiner@uke.de (U. Valentiner).

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production occurs in the southern coasts of Calabria region (Italy), where the microclimatic characteristics are particularly suitable for its cultivation. Bergamot fruit is used mostly for the extraction of its essential oil from the peel, a basic component of fragrances. Bergamot juice (BJ), which is obtained from the endocarp after essential oil extraction, is considered just as a secondary and discarded product with disposal costs. Over the past few years BJ attracted large attention as a result of its remarkable content of flavonoids, known for their beneficial effects.

Flavonoids are polyphenolic compounds present in vegetables, especially in the genus *Citrus*, characterized by a common benzo-γ-pyrone structure. More than 8000 compounds of flavonoid structure have been described. The large number of molecules arises from various combinations of multiple hydroxyl and methoxyl groups substituting the basic flavonoid skeleton. Numerous pre-clinical and epidemiological studies point to a possible protective effect of flavonoids against cardiovascular and malignant diseases, linked to their abilities to inhibit enzymes involved in cell activation [12].

Recently, we have shown that BJ reduces growth rates of different cancer cell lines, by mechanisms that in SH-SY5Y neuroblastoma cells are linked to an early impairment in cell adhesive and migratory processes [13]. However, until now the anticancer properties of BJ *in vivo* have not been investigated. On these bases, the present study was designed to evaluate the effect of BJ both *in vitro* and in an experimental model of spontaneous metastasis formation of human NB cells xenografted into immunodeficient mice.

2. Materials and methods

2.1. Plant material

Bergamot fruits were collected from *C. bergamia* cultivation located in Bovalino (Reggio Calabria, Italy). The fruits were hand-squeezed and small aliquots of the juice (BJ) were stored at −20 °C. BJ was defrosted and filtered (0.22 μm pore size) before use. For cell culture experiments, the pH of BJ was adjusted to 7.4 and subsequently diluted in culture media until the desired concentrations are achieved.

2.2. Quali-quantitative evaluation of flavonoids in bergamot juice

The juice was analyzed without any pre-treatment: the juice was centrifuged and then filtered on Acrodisc filter 0.22 μm. The sample was analyzed in triplicate by RP-HPLC. Since the pH of the BJ is roughly 3.5–3.8, all the mobile phases have been adjusted to the appropriate pH value (pH 3) with formic acid in order to suppress the ionization of the phenolic groups [14].

LC analyses were carried out using a Shimadzu Prominence LC-20A system (Shimadzu, Milan, Italy), including a CBM-20A controller, two LC-20 AD dual-plunger parallel-flow pumps, a DGU-20A3 on-line degasser, and a CTO-20A column oven. Data were acquired and processed by LCsolution Version 1.21 SP1 software (Shimadzu). An SPD-M20A UV detector and an LCMS-2020, through ESI interface (Shimadzu), were employed for quantification and characterization of bioactive molecules, respectively. MS data acquisition was performed by the LCMSsolution Ver. 3.30 software (Shimadzu).

LC analyses were carried out on an Ascentis Express C18, 150 × 4.6 mm I.D. with a particle size of 2.7 μm (Supelco, Bellefonte, PA). The injection volume was 2 μl: mobile phase consisted of water/formic acid (99.9:0.1, v/v; solvent A) and acetonitrile/formic acid (99.9:0.1, v/v; solvent B). The step-wise gradient profile was as follows: 0 min, 5% B, 40 min, 25% B, 60 min, 100% B, 70 min, 100% B, 73 min, 5%, and 80 min 5% B. Flow-rate was 0.7 ml/min. Data were acquired using a photodiode array detector in the range 190–400 nm and the chromatograms were extracted at 283 nm and 325 nm. Time constant was 0.64 s and sample frequency 1.5625 Hz. Data acquisition was performed by Shimadzu LCsolution software ver 3.3.

MS acquisition was performed using ESI in negative mode. ESI conditions: mass spectral range, m/z 100–700; interval, 0.5 s; scan speed, 938 amu/s; nebulizing gas (N2) flow, 1.5 l/min; ESI temperature, 350 °C; heat block, 300 °C; DL (desolvation line) temperature, 300 °C; DL, voltage, −34 V; probe voltage, +4.5 kV; Qarray voltage, 1.0 V and detection gain, 1.05 kV.

To quantify the flavonoid content in the BJ, calibration curves have been constructed by using each single standard. Five different concentrations of each component in the range between 100 and 0.2 mg/l (stock solution of 1000 mg/l in methanol) were analyzed for five consecutive times by HPLC under the same chromatographic conditions optimized for the sample. Limit of detection (LOD) and limit of quantification (LOQ) values were also calculated as reported [14].

2.3. Cell lines, proliferation assay and cytotoxicity study

SK-N-SH and LAN-1 human NB cell lines were cultured as described for other NB cell lines [15] at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. At confluence, cells were routinely harvested using 0.05% trypsin–0.02% EDTA and replaced in 75 cm^2 tissue culture flasks. All chemicals were from Gibco (Paisley, Scotland and Milan, Italy).

Cell proliferation was quantified by the MTT assay [16] with modifications. Cells were plated into 96-well plates at a density of 15 × 10^3 cells/ml (LAN-1) or 6 × 10^3 cells/ml (SK-N-SH) and cultured for 24 h before administration of growing percentage of BJ (from 1% to 10%). Each concentration was eightfold tested and at least four independent experiments were carried out. The control cells received only fresh medium. After incubation for 24, 48 and 72 h, the plates were centrifuged to collect the floating cells in the media, and the supernatants in each well were replaced with 100 μl fresh medium without phenol red containing 0.5 mg/ml of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; Milan, Italy). The plates were returned in the incubator for 4 h and gently shaken occasionally. Then, the medium was removed and 100 μl of ethanol/dimethyl sulfoxide (DMSO) 1:1 lysis buffer was added to solubilize the formed formazan crystals (MTT metabolic product) that were spectrophotometrically quantified with a microplate spectrophotometer (Bio-Rad Laboratories, Milan, Italy). Absorbance of

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