

***In vitro* anti-proliferative and anti-inflammatory activity of leaf and fruit extracts from *Vaccinium bracteatum* Thunb.**

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Abstract: The aim of this study was to evaluate *in vitro* anti-proliferative (tested on MCF-7, MDA-MB-231, and MCF-10A cell lines) and anti-inflammatory (evaluated as inhibition of prostaglandin E₂ synthesis catalyzed by cyclooxygenase-2) effect of various extracts from *Vaccinium bracteatum* leaves and fruits. The highest anti-proliferative effect possessed leaf dichloromethane extract with IC₅₀ values ranging from 93 to 198 µg/mL. In the case of cyclooxygenase-2 inhibition, *n*-hexane, dichloromethane, and ethanol fruit extracts showed the best activity with IC₅₀ values = 2.0, 5.4, and 12.7 µg/mL, respectively. These results indicate that *V. bracteatum* leaves and fruits could be useful source of anti-cancer and anti-inflammatory compounds.

Keywords: anti-proliferative activity, anti-inflammatory activity, breast cancer, cyclooxygenase, *Vaccinium bracteatum*, xCELLigence system

INTRODUCTION

Vaccinium bracteatum Thunb. (Ericaceae) is an evergreen shrub or small tree growing in the hilly areas of East Asia (Ruizheng & Stevens, 2005). Its leaves are used to stain cooked rice (Ruizheng & Stevens, 2005; Wang *et al.*, 2008) and pigment from leaves is also offered by some Chinese companies as natural dye for proteins, hairs, starch and white vinegar. Moreover, *V. bracteatum* is included in the traditional Chinese herbal medicine (Wang *et al.*, 2010) and according to Duke & Ayensu (1985) it is used for the treatment of cancer in a folk medicine. In the last years several papers referred about the anti-diabetic (Wang *et al.*, 2010), tyrosinase inhibiting (Kim *et al.*, 2012), and antioxidative (Wang *et al.*, 2011) effects of leaf extracts. Furthermore, some phytochemical works about *V. bracteatum* leaf constituents were previously published (Tu *et al.*, 1997; Li *et al.*, 2008; Zhang *et al.*, 2009; Wang *et al.*, 2011).

According to our knowledge, there is no report about the effect of *V. bracteatum* on the proliferation of cancer cells in the literature. Therefore, we tested the anti-proliferative activity of four leaf and four fruit extracts on the three breast cell lines. Additionally, we evaluated the inhibitory activity of these extracts toward cyclooxygenase-2 (COX-2) which plays major role in the biosynthesis of pro-inflammatory mediators prostaglandins. COX-2 activity is beside of the sites of inflammation increased also in various cancer types such as breast and colorectal cancer (Charlier & Michaux, 2003).

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), Ham's Nutrient Mixture F12, horse serum, arachidonic acid, indomethacin, L-epinephrine, hematin porcine, human recombinant COX-2, disodium ethylenediamine tetraacetate (Na₂EDTA), dimethylsulphoxide (DMSO), TRIS, and formic acid were obtained from Sigma-Aldrich (MO, USA). Dichloromethane, *n*-hexane, and ethanol were purchased from Penta Chemicals (Czech Republic). PGE₂ EIA kit was purchased from Enzo Life Sciences (MI, USA). Fetal bovine serum (FBS) and gentamycin sulphate were purchased from Invitrogen (CA, USA). E-plates for xCELLigence system were obtained from Roche (Germany).

Plant material extraction

Leaves and fruits of *V. bracteatum* were collected by Prof. Ji-Dong Lou from farmlands and gardens in Zhejiang province, China. Dried leaves and fruits (10 g) were ground into the flour and successively soaked in the *n*-hexane, dichloromethane, ethanol, and water for 24 h in each solvent in the dark. Afterward extracts were filtered and solvents were evaporated under vacuum at 40°C using a rotary evaporator. Obtained extracts were kept in 4°C until tested.

Cell cultures

Human breast carcinoma MCF7 and MDA-MB-321 cells

were purchased from European Collection of Cell Cultures (UK). Cells were propagated in three subcultivations and aliquots were frozen and kept in the liquid nitrogen. Every set of experiments (lasted 3-9 weeks) was performed with new batch of stored cells. The cells were kept in DMEM with phenol red and NaHCO₃. The culture medium contained 10% heat-inactivated FBS, 10 mM HEPES and gentamicin sulphate (10 mg/ml). T-75 cm² culture flasks, humidified atmosphere containing 5% CO₂ and 37°C were used for the cell grow.

Human mammary epithelial MCF-10A cells were derived from fibrocystic mammary disease, which were spontaneously immortalized, were purchased from American Tissue Culture Collection (VA, USA). Cells were propagated and stored as was described for MCF7 and MDA-MB-321 cells. The cells were kept in DMEM/Ham's Nutrient Mixture F12 with 15 mM HEPES and sodium bicarbonate, without L-glutamine. The culture medium contained 5% heat-inactivated horse serum, epidermal growth factor, hydrocortisone, insulin, and penicillin/streptomycin. Growth conditions were same as for two previous cell lines. All used cell lines were checked for the absence of mycoplasma by Generi Biotech (Czech Republic).

Cell proliferation assay

Cell proliferation was examined by using the RTCA MP instrument (Roche, Germany). Cell index values were recorded and normalized by RTCA Software 1.2 (Roche, Mannheim, Germany). Micro-electrodes integrated at the bottom of 96-wells measured electrical impedance giving quantitative information about the number and viability of the cells.

Culture medium (90 µl) was added into each well and plates were inserted to the device for background measurement. Then, 100 µl of cell suspension was added in quadruplicate to the appropriate wells. The plates were left in a laminar box at room temperature for 30 min and then inserted to the device. In the first phase, the growing impedance (corresponding to cell proliferation) was measured every hour for 24 hours. After 24 hours, 10 µl of *V. bracteatum* extracts (dissolved in DMSO) was added into each well, plates were inserted back to device and impedance was measured every hour for 48 hours. The obtained results were expressed as percentage of untreated controls. IC₅₀ values were calculated from values recorded 72 h after treatment by extracts. At least three concentrations were used for the construction of inhibition curves. IC₅₀ values were determined by regression analysis using Microsoft Excel.

Cyclooxygenase-2 assay

The assay was performed according to a procedure described by Reininger & Bauer (2006) with human recombinant COX-2. COX-2 (0.5 unit/reaction) was mixed with 180 µL of 100 mM TRIS buffer (pH 8.0), 5

µM porcine hematin, 18 mM L-epinephrine, and 50 µM Na₂EDTA. Afterward, tested substances dissolved in DMSO (10 µL) or pure DMSO (in case of blank wells) were added. After 5 min incubation at room temperature the reaction was initiated by addition of 5 µL of 10 µM arachidonic and moved to the water bath set up at 37°C and incubated 20 min. Finally, 10 µL of 10% formic acid stopped the reaction. Quantification of PGE₂ (main product of the reaction) was achieved by PGE₂ ELISA kit after the dilution of samples in ratio 1:15. The measurement of the absorbance corresponding to PGE₂ concentration was accomplished by a microplate reader Tecan Infinite M200 (Tecan Group, Switzerland) at 405 nm. The results were stated as percentage inhibition of PGE₂ synthesis in comparison with untreated samples (blanks). IC₅₀ values were calculated from three different concentrations. The experiment was repeated two times with at least two replicates in each experiment. IC₅₀ values were calculated by regression analysis using Microsoft Excel.

RESULTS

Anti-proliferative activity of *V. bracteatum* extracts

The extracts obtained by the successive maceration with *n*-hexane (HE), dichloromethane (DE), ethanol (EE) and water (WE) from leaves and fruits were examined for their ability to regulate the growth of two breast cancer cell lines MCF-7 and MDA-MB-231 and one non-cancerous breast epithelial cell line MCF-10A. System xCELLigence was used for real-time analysis of cell proliferation during 72 h incubation. This system, measuring impedance, allowed continual monitoring of cells growth without risk of interference of color extracts with dyes used in classical colorimetric tests of cell viability. Results obtained in MCF7 cells demonstrated that several extracts from *V. bracteatum* were able to inhibit cell proliferation. Leaf DE was the most effective followed by HE and EE from leaves and DE from the fruits (IC₅₀ values are summarized in table 1). The rest of extracts was inactive in the inhibition of MCF-7 at the highest tested concentration (500 µg/mL) and therefore, were not further tested on other two cell lines.

Table 1: IC₅₀ values (µg/ml) for the anti-proliferative activity after 72 h incubation with various *V. bracteatum* extracts

Leaf extracts	Cell line		
	MCF7	MDA-MB-231	MCF10A
IC ₅₀ ± SD (µg/ml)			
HE	206.75±6.60	298.98±85.17	105.97±17.41
DE	93.37±5.03	197.57±34.57	<90*
EE	478.00±26.34	>500	259.89±7.41
Fruit extracts			
DE	265.50±43.01	>500	367.40±7.93

* IC₅₀ lies deep bellow the lowest tested concentration

Fig. 1 demonstrates the effect of DE on proliferation of MDA-MB-231 during the time period. Addition of extracts (dissolved in DMSO) rapidly reduced cell number (expressed as cell index) after 24 h cultivation. Control cells (treated with pure DMSO only) recovered after one hour from the shock caused by the addition of solvent and their proliferation continued whereas proliferation of cells treated by DE was inhibited in dose-dependent manner. The higher concentrations of DE reduced the number of living cells indicating the cytotoxicity of the extracts at these concentrations.

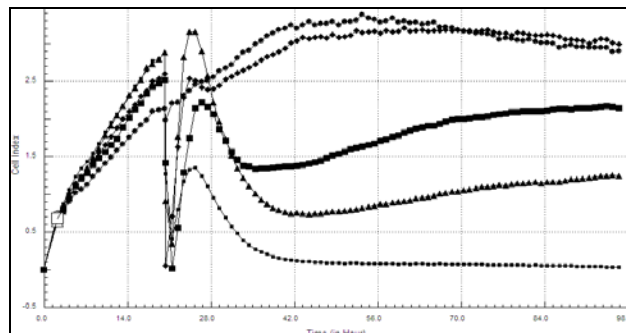


Fig. 1: Dose and time depended effect of leaf DE on proliferation of MDA-MB-231 breast cancer cell line. Y axis: cell index corresponding to the number of living cells; X axis: incubation time in hours; circles - control well with DMEM; diamonds - control well with DMSO (blank); big squares - DE 125 µg/ml; triangles - DE 250 µg/ml; small squares - DE 500 µg/ml.

Anti-inflammatory activity of *V. bracteatum* extracts

HE, DE, and EE from *V. bracteatum* fruits showed very strong inhibitory effect on COX-2 while extracts from the leaves possessed weaker activity (IC_{50} values are summarized in table 2) and water extracts from both plant parts were inactive in the highest tested concentration (100 µg/ml).

Table 2: IC_{50} values (µg/ml) for COX-2 inhibition by various *V. bracteatum* extracts

	$IC_{50} \pm SD$ (µg/ml)
Leaf extracts	
HE	21.37±11.49
DE	58.32±0.35
EE	64.05±14.49
Fruit extracts	
HE	2.02±1.13
DE	5.43±3.00
EE	12.73±4.62
Reference inhibitor	
Indomethacin	0.67±0.21

DISCUSSION

Anti-proliferative activity

Since the anti-proliferative and pro-apoptotic effect of

ursolic acid (Kassi *et al.*, 2009; Shan *et al.*, 2011; Wang *et al.*, 2012), quercetin (Choi *et al.*, 2001; Chou *et al.*, 2010), and apigenin (Yin *et al.*, 2001) in MCF-7 cells was described, it is possible that these compounds previously identified in *V. bracteatum* leaves (Tu *et al.*, 1997; Wang *et al.*, 2011) are responsible for its anti-proliferative effect.

Anti-inflammatory activity

While acute inflammation is natural defense reaction, chronic inflammation may result in arthritis, cancer, cardiovascular diseases, and diabetes (Aggarwal *et al.*, 2006). NSAIDs (nonsteroidal anti-inflammatory drugs) provide their effects by inhibition of the COX, and, consequently, the conversion of arachidonic acid into prostaglandins. Although they are not without shortcomings, they remain a first choice in the treatment of various inflammatory diseases (Fiorucci *et al.*, 2001).

Since fatty acids are common in the plants, the active components could be fatty acids in the less polar extracts (HE and DE). Fatty acids such as α -linolenic and linoleic acid are known as competitive inhibitors of COX-2 (Huff *et al.*, 1995 Johnson *et al.*, 1995; Ringbom *et al.*, 2001). Since we used successive extraction, non-polar constituents such as fatty acids were expected mainly in the *n*-hexane and dichloromethane extracts while EE should be free of these constituents. Therefore, the high activity of EE indicates the presence of other COX-2 inhibitors in *V. bracteatum* fruits.

CONCLUSIONS

Our results are in the concordance with the ethnopharmacological use of *V. bracteatum* as anti-cancer agent. Recorded activity indicates that the leaves contain anti-proliferative compounds. Since extracts from *V. bracteatum* leaves are used as food dye, they could act as a preparation for the cancer prevention. Further, fruit extracts were found to be strong COX-2 inhibitors *in vitro* and therefore, further research including identification of active compounds should be performed on *V. bracteatum*.

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