

Original Research Article

Vatica diospyroides Symington type LS Root Extract Induces Antiproliferation of KB, MCF-7 and NCI-H187 Cell Lines

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Abstract

Purpose: To investigate the therapeutic efficacy of *V. diospyroides* Symington type LS root extract as a chemopreventive agent against various cancer cell lines.

Methods: Acetone root extract was evaluated for *in vitro* cytotoxicity against KB (oral cavity cancer), MCF-7 (breast cancer), and NCI-H187 (small cell lung cancer), using Resazurin microplate assay (REMA). Toxicity against a representative normal cells, Vero (African green monkey kidney), was assessed using green fluorescence protein (GFP)-based assay.

Results: *V. diospyroides* root extract showed significant cytotoxic effects on KB and MCF-7 cell lines in a dose-dependent manner with IC_{50} of 35.05 ± 1.45 and 36.63 ± 3.40 $\mu\text{g/mL}$, respectively. NCI-H187 was not significantly inhibited ($\leq 19.39\%$ inhibition) at the concentrations tested. IC_{50} against Vero cells was outside the concentration range of 0.2 - 50 $\mu\text{g/mL}$.

Conclusion: These results indicate that the root extract of *V. diospyroides* has *in vitro* cytotoxic effect on human oral cavity cancer and breast cancer cells. No toxic effect on normal cells was observed. Thus, the extract may provide bioactive substances for human cancer therapy.

Keywords: Breast cancer, Oral cavity cancer, Lung cancer, Cytotoxicity, Vero cells, *Vatica diospyroides*

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INTRODUCTION

In recent years, the development of cancer therapies is pursued with emphasis on chemo/radiation therapy and surgery. However, the survival rates and expected survival times are very low with most diagnosed cancers [1]. Since chemotherapy tends to suffer cancer patients from serious side effects [2], compelling and urgent needs motivate discovery of new therapies and drugs with efficacy, safety and low

cost [3]. The contribution of modern drug discovery could be the identification of novel natural active ingredients, to serve as the basis for chemical modifications and massive screening of their therapeutic actions by scientists. Thus, extraction and *in vitro* investigation of effective compounds from medicinal plants seems a first realistic approach to such contributions. Many medicinal plant parts used by Thai folk medicine have been explored for their anti-cancer properties [4]. Interestingly,

the stem of *Vatica diospyroides* Symington was the first plant from which a well-known class of anticancer agents, namely resveratrol tetramer such of vatdiospyroidol, was isolated and identified [5]. Moreover, the root extract of *V. diospyroides* has therapeutic property against breast cancer *in vitro* [6]. Two most useful methods for testing the efficacy of chemopreventives are 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Resazurin microplate (REMA) assays which are recommended for cytotoxic testing [7]. The therapeutic potential of plant extracts on various cancer cell lines can be tested with these assays.

In this study, we tested the efficacy of *V. diospyroides* root extract on three human cancer cell lines (KB, MCF-7 and NCI-H187), and on a normal mammalian cell line (Vero) using REMA and Green Fluorescence Protein (GFP) assays, respectively. We determined the extract concentration causing 50 % inhibition of cell proliferation *in vitro* to quantify efficacy and toxicity, as early indications of potential in human cancer therapy.

EXPERIMENTAL

Plant material and extract preparation

Excising of roots from full-grown *V. diospyroides* type LS (20-year old tree) and preparation of acetone extract of the root followed the method described previously [6]. The roots were collected in October 2012, at the Nong Thung Thong non-hunting area, Suratthani Province, Southern Thailand. The sample (Collector number T. Srisawat 003) was authenticated by Dr. Charun Maknoi at the Herbarium of Queen Sirikit Botanic Garden (QBG), Maerim, Chiang Mai, Thailand and then deposited in the QBG. The root was chopped into small pieces prior to air dried and macerated in acetone for five days. The solution was evaporated to dryness and the extract was then stored in cool and dark conditions prior to evaluating cytotoxicity with the African green monkey kidney (Vero cell, ATCC CCL-81), epidermoid carcinoma of oral cavity (KB cell line, ATCC CCL-17), breast adenocarcinoma (MCF-7, ATCC HTB-22), and small cell lung carcinoma (NCI-H187, ATCC CRL-5804) cell lines.

Resazurin microplate assay (REMA)

This assay was performed using the method of Brien *et al* [8]. Cells at a logarithmic growth phase of KB, MCF-7 and NCI-H187 were harvested and diluted to 2.2×10^4 (for KB) and

3.3×10^4 cells/mL (for MCF-7 and NCI-H187), in fresh medium. Consecutively, 5 μ L of the root extract diluted in 5 % DMSO, and 45 μ L of cell suspension were added to 384-well plates, and incubated at 37 °C in 5 % CO₂ incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 μ L of 62.5 μ g/mL resazurin solution was added to each well, and the plates were then incubated at 37 °C for 4 h. Fluorescence signal was measured using SpectraMax M5 multi-detection micro-plate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm, respectively. Percentage of inhibition of cell growth was calculated using Eq 1.

$$\text{Inhibition (\%)} = \{1 - (\text{FUT}/\text{FUC})\} \times 100 \dots\dots\dots (1)$$

where FUT and FUC are the mean fluorescent unit readings from treated and untreated conditions, respectively.

Dose response curves were plotted from 6 concentrations of 3-fold serially diluted test compounds, and the sample concentrations that inhibit cell growth by 50 % (IC₅₀) were estimated using the SOFTMax Pro software (Molecular Devices, USA). Ellipticine, doxorubicin and tamoxifen were used individually as positive controls, and 0.5 % DMSO and water were used as a negative control.

Green fluorescent protein (GFP) assay

Cytotoxicity with normal cells was evaluated by the method of Hunt *et al* [9]. The GFP-expressing variant of Vero cell line was generated in-house, by stably transfecting the African green monkey kidney cell line (Vero) with pEGFP-N1 plasmid (Clontech). The cell line was maintained in minimal essential medium supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.8 mg/ml geneticin, at 37 °C in a humidified incubator with 5 % CO₂. The assay was carried out by adding 45 μ L of cell suspension at 3.3×10^4 cells/mL to each well of 384-well plates containing 5 μ L of the root extract previously diluted in 0.5 % DMSO, and then incubated for 4 days in 37 °C incubator with 5 % CO₂. Fluorescence signals were measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode, with excitation and emission wavelengths of 485 and 535 nm. Background fluorescence at day 0 was subtracted from fluorescence signal at day 4. The percentage of cytotoxicity was calculated, and the IC₅₀ values estimated from equation fit of the response curve, as described

in REMA assay. Ellipticine and 0.5 % DMSO were used as positive and negative controls, respectively.

Data analysis

The data are shown as mean \pm standard deviation from three-independent replicates. One-way ANOVA was performed using SPSS software (version 11.0). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Cytotoxic activities of *V. diospyroides* root extract against three cancer cell lines are shown in Table 1. Various concentrations of the extract (0.21 - 50 $\mu\text{g/mL}$) were characterized using REMA assay compared to ellipticine, doxorubicin and tamoxifen as positive controls. The criterion of cytotoxic activity for the crude extract is an inhibition $\geq 50\%$ for REMA, and cell growth $\leq 50\%$ in the GFP assay. Extract concentration at 50 $\mu\text{g/mL}$ induced over 50 % growth inhibition of KB and MCF-7 cell lines (67.87 and 62.77 %, $p < 0.0001$) indicating cytotoxic activity against both. The dose responses to the extract and IC_{50}

values are shown in Table 1 and Figure 1. The IC_{50} values were 35.05 ± 1.45 and 36.63 ± 3.40 $\mu\text{g/mL}$ for KB and MCF-7, respectively, indicating high cytotoxicity of the extract on both cell lines. At concentrations lower than IC_{50} the extract induced less than 25 % inhibition, and these would be unsuitable treatment concentrations for KB and MCF-7. On the other hand, the root extract induced lower 50 % growth inhibition on NCI-H187 cell line, representing weak cytotoxic effects on the cells, even at 50 $\mu\text{g/mL}$ extract concentration which showed 19.39 % inhibition (Table 1).

In a successful manner, as a representative of normal mammalian cells Vero cell line was used, and the High cell growth ($> 62\%$) persisted with extract concentration at 50 $\mu\text{g/mL}$, while at lower extract concentrations tested (0.21 - 16.67 $\mu\text{g/mL}$) no significant inhibition was found (100 % cell growth) (Table 2). Although there was significantly different cell growth ($p = 0.001$) between 50 $\mu\text{g/mL}$ and the other dose levels, in all cases cell growth exceeded 50 %, indicating no toxic effects on these normal cells.

Table 1: Cytotoxic activities of acetone root extract of *V. diospyroides* type LS, against KB, MCF-7 and NCI-H187 cell lines, determined by REMA

Cell line	Extract concentration ($\mu\text{g/mL}$)	Inhibition (%) [*]	Activity	IC_{50} ($\mu\text{g/mL}$)
KB	50.00	67.87 \pm 2.06 ^a	Active	35.05 \pm 1.45
	16.67	24.06 \pm 3.77 ^b	Inactive	N/A
	5.56	7.45 \pm 4.38 ^c	Inactive	N/A
	1.85	2.77 \pm 5.53 ^c	Inactive	N/A
	0.62	-2.88 \pm 7.01 ^c	Inactive	N/A
	0.21	-4.83 \pm 8.52 ^c	Inactive	N/A
MCF-7	50.00	62.77 \pm 3.39 ^a	Active	36.63 \pm 3.40
	16.67	25.28 \pm 4.40 ^b	Inactive	N/A
	5.56	16.35 \pm 10.64 ^b	Inactive	N/A
	1.85	13.23 \pm 6.77 ^b	Inactive	N/A
	0.62	11.74 \pm 6.36 ^b	Inactive	N/A
	0.21	8.28 \pm 5.34 ^b	Inactive	N/A
NCI-H187**	50.00	19.39 nd	Inactive	-
	16.67	13.91 nd	Inactive	-
	5.56	-9.43 nd	Inactive	-
	1.85	-4.55 nd	Inactive	-
	0.62	-7.73 nd	Inactive	-
	0.21	-5.70 nd	Inactive	-

^{*}Inhibition exceeding 50 % was considered cytotoxic against a cancer cell line, otherwise the extract is considered inactive and IC_{50} level cannot be estimated (N/A). Three-independent experiments were performed except for NCI-H187** that only had two independent trials, neither showing cytotoxic activity. Different superscripts within a column indicate statistically significant differences in cell line inhibition (mean \pm SD), as analyzed by Tukey's test at $p < 0.05$; nd not statistically different from control)

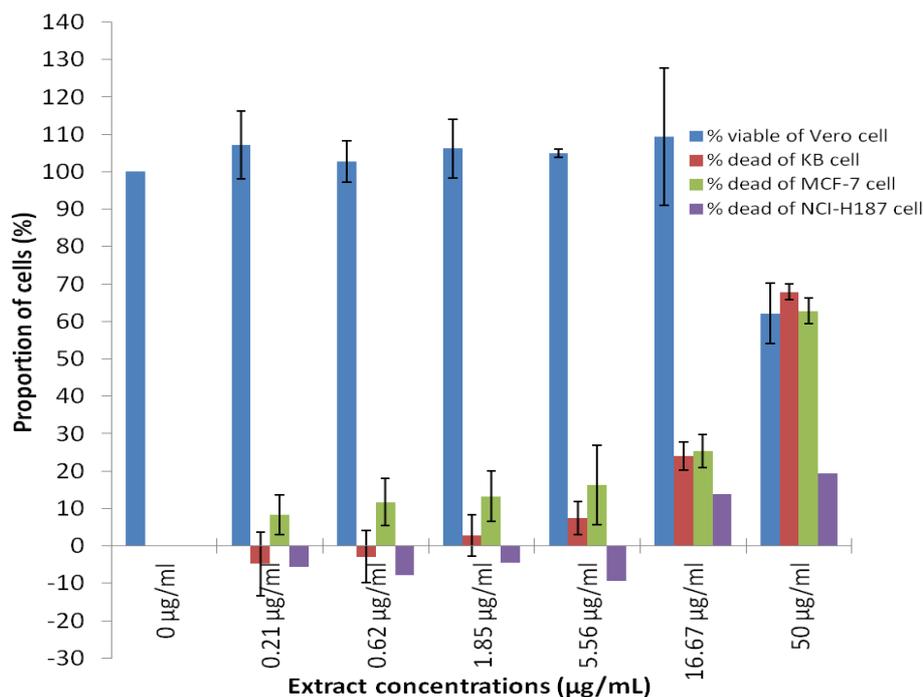


Figure 1: Proportions of Vero cell compared to cancer KB, MCF-7 and NCI-H187 cell lines response to various concentrations of *V. diospyroides* type LS root extract

Table 2: Effect of acetone root extract of *V. diospyroides* type LS on Vero cells, chosen as *in vitro* representative of normal (non-cancer) cells

Cell line	Extract concentration (µg/mL)	% Cell growth*	Activity	IC ₅₀ (µg/mL)
Vero cell	50.00	62.15±8.0 ^b	Non-cytotoxic	N/A
	16.67	109.39±18.35 ^a	Non-cytotoxic	N/A
	5.56	104.94±1.03 ^a	Non-cytotoxic	N/A
	1.85	106.19±7.81 ^a	Non-cytotoxic	N/A
	0.62	102.75±5.57 ^a	Non-cytotoxic	N/A
	0.21	107.20±9.12 ^a	Non-cytotoxic	N/A

*Cell growth less than 50 % would indicate cytotoxicity against Vero cells, otherwise the extract was considered inactive and the IC₅₀ level could not be determined (N/A). Three independent replicate experiments were performed; Different superscripts within a column indicate statistically significant differences in Vero cell growth (mean ± SD), as analyzed by Tukey's test at $p < 0.05$

DISCUSSION

The results confirm that acetone root extract of *V. diospyroides* has cytotoxic properties similar to those reported previously for various cancer cell lines [5,6]. These activities might be due to some bioactive compounds such as alkaloids and terpenoids found in the acetone extract of *V. diospyroides* type LS root [6]. These substances exhibit anti-proliferation and anti-metastatic effects against several cancers, both of *in vitro* and *in vivo* [10,11]. In addition, human oral epidermoid (KB), colon cancer (Col2), and breast cancer (BC1) cell lines were previously tested in a bioassay-guided fractionation of resveratrol tetramer, purified from the stem extract of *V. diospyroides* [5]. Resveratrol induces apoptotic death mode of KB and MCF-7 cells [12,13], so

the cytotoxicity of the root extract on KB and MCF-7 in the current study might be due to some resveratrol derivatives existing in *V. diospyroides*. Interestingly, the antiproliferation efficacy of root extract on MCF-7 determined with REMA assay is similar to the MTT assay result in a previous study, which gave IC₅₀ of 36.63 µg/mL [6]. These methods have been compared by Cui *et al* [7], who observed no significant difference between them. Therefore, either method can be selected for testing cytotoxic effects of plant compounds on cancer cell lines.

However, the extract had no cytotoxic action on NCI-H187. The NCI-H187 is more resistant to various drugs and chemotherapeutic agents than KB or MCF-7 [14,15]. KB and MCF-7 cell lines are good models for studying properties of

chemotherapeutic agents such as resveratrols that may not have high potency [12,13]. In contrast, the inhibition of NCI-H187 is strongly induced by other types of compounds, such as flavonoid derivatives [16]. These substances are not present in *V. diospyroides* root extract [6]. Therefore the limited cytotoxicity of the root extract on NCI-H187 might be due to lack of flavonoids in acetone extract of root. It is to be noted, that *in vitro* toxicology is in very limited use by the pharmaceutical companies, and its predictive value appears to be poor in general. Animal experiments remain absolutely necessary, but still fall short in predicting human toxicology. In addition, any cultured cell line is inherently abnormal, as it has been immortalized, and it is not part of the synergistic system of a live body. Therefore *in vivo* both the anticancer effects and the toxic effects against healthy cells can dramatically differ from *in vitro* effects of a compound. However, *in vitro* studies remain the unavoidable experimental option to initially screen for candidates.

CONCLUSION

The root extract of *V. diospyroides* may provide anticancer agents, and inhibit *in vitro* the growth of oral cavity and breast cancer cells. The extract is not cytotoxic against a lung cancer cell line or of normal mammalian cells. It requires further investigation to identify new bioactive compounds from this root extract, with potential benefits for the treatment of malignant tumors.

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