

Original Research Article

Phytochemical Screening and Cytotoxicity of Crude Extracts of *Vatica diospyroides* Symington Type LS

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Abstract

Purpose: To investigate the phytochemistry and cytotoxic activity of the leaf, branch and fruit extracts of *Vatica diospyroides* Symington type LS, an endangered medicinal plant in the peninsula of Thailand.

Methods: Extracts of the mature leaves, branches and fruit (both cotyledon and pericarp) of five-year old tree were obtained using various organic solvents. Thin-layer chromatography (TLC) and phytochemical screening were employed to identify the chemical constituents. Cytotoxicity was characterized by 50 % inhibition (IC_{50}) of human breast cancer cell lines (MCF-7 and MDA-MB-468) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: The extracts of the plant parts differed in chemical composition. Terpenoids and anthraquinones were present in all extracts, while cardiac glycosides and tannins were isolated from the branches and leaves, respectively. The acetone and methanol extracts of the fruit cotyledons were highly cytotoxic against MDA-MB-468 cell line (IC_{50} = 3.1 μ g/ml for both extracts), whereas acetone and dichloromethane extracts of the fruit pericarps were highly active against MCF-7 cell line (IC_{50} = 18.2 and 21.0 μ g/ml, respectively). The other extracts were less active.

Conclusion: This work revealed the potentials of *V. diospyroides* fruit as a cytotoxic agent against human breast cancer cell lines. However, further studies, including identification and purification of the active compounds, will need to be pursued.

Keywords: Breast cancer, Crude extract, Dipterocarpaceae, MCF-7, MDA-MB-468, *Vatica diospyroides*

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INTRODUCTION

The family Dipterocarpaceae has many medicinal plants containing valuable curative compounds [1], such as genera *Dipterocarpus*, *Shorea* and *Vatica* [2]. In Thai ethnobotanical medicine, flowers and stems of the genus *Vatica* have been used as functional ingredients in cardiac and blood tonic remedies. Modern drug discovery research has shown that their stems

contain resveratrol derivatives, namely Vaticaphenol A and Vaticanol series [3-5]. These resveratrol derivatives have been found to play an important role in cardiovascular treatment [6], and to be cytotoxic against various human cancer cell lines [3,5]. Resveratrol derivatives for human cancer chemoprevention are being tested in clinical research [7]. Therefore, the stems of plants in the genus *Vatica* not only provide traditional medicines like Thai cardiovascular

tonics, but are currently supplied worldwide as cardioprotective and anticancer agents.

The stem of *V. diospyroides* Symington, an endemic medicinal and fragrant dipterocarp of Thailand peninsula, has strong anticancer activity [3,5]. However, other plant parts such as leaves have only been documented as having secondary compounds unlike the stem [8]. However, extracting the valuable phytochemical constituents of *V. diospyroides* from leaves, branches and fruits has not been reported to date.

Cancer is a leading cause of death worldwide and accounts for 7.6 million deaths especially in low-and middle-income countries. Invasive cancers such as those of lung, stomach, liver, colon/rectum, and breast, cause about 50 % of all deaths [9]. Nowadays, plant secondary metabolites are accepted for use as anti-tumor agents, inhibiting the growth of human cancer cells. Most of the secondary compounds found in common medicinal plants are alkaloids, flavonoids and tannins [10]. The anti-cancer compounds, vinblastine and vincristine in common clinical use, are produced from genus *Catharanthus*, known in Thai as Phaeng phuai farang, and are sold in the U.S. market [1]. In a previous work [3], human oral epidermoid (KB), colon cancer (Col2), and breast cancer (BC1) cell lines were used in bioassay-guided fractionation of resveratrol tetramer, purified from the extract of *V. diospyroides* stem.

We had reported earlier that *V. diospyroides* can be classified into two distinct types of tree, namely the SS and LS forms [11]. It is reasonable to expect that these distinct types may have different activities against human cancer cells. The LS type is dominant in the population of Nong Thung Thong non-hunting area of Thailand, which may be the best genetic source of *V. diospyroides*. The present study aims to identify types of active constituents of various parts of the LS type of this plant and the cytotoxic activity of their extracts against human breast cancer cell lines (MCF-7 and MDA-MB-468).

EXPERIMENTAL

Plant materials and preparation of extracts

Samples of the leaf, branch and fruit of *V. diospyroides* Symington type LS were collected from Nong Thung Thong non-hunting area, Kiansa, Suratthani Province, Thailand. Voucher specimens (Collector number T. Srisawat 001) were deposited in the Herbarium of Queen Sirikit

Botanic Garden (QBG), Maerim, Chiang Mai, Thailand. The samples were authenticated by Dr. Charun Maknoi of QBG.

The plant samples were cut into small pieces and completely air-dried, and stored in glass containers until extraction. About 270 g of the dried plant material was extracted for five days with n-hexane (C₆H₁₄), dichloromethane (CH₂Cl₂), methanol (CH₃OH), or acetone ((CH₃)₂CO). The extracts were filtered through 2 layers of cotton fabric. The extract of each sample was evaporated at room temperature, under reduced pressure, to the dry residue and stored in sterile vial pending phytochemical and cytotoxic screening.

Phytochemical screening

Tests for phytochemical constituents - Alkaloids, terpenoids, anthraquinones, flavonoids, saponins, and tannins - followed the methods described previously [8].

Thin layer chromatography (TLC)

Thin layer chromatography was applied to the extracts. The chromatograms were developed and dried on the silica gel TLC plates, and prior to spraying with vanillin-sulphuric acid, the spots visually observed under UV₂₅₄ lamp to characterize various chemical components. The developing solvents used were ethyl acetate:n-hexane (80:20 and 65:35), ethyl acetate:dichloromethane (85:15), dichloromethane:methanol (80:20), dichloromethane:n-hexane (85:15) or 100% dichloromethane mixtures.

In vitro cytotoxicity assay

Cytotoxic activity was evaluated against human breast cancer cell lines. Cell cultures of MCF-7 (HTB-22TM) and MDA-MB-468 (HTB-132TM) were kindly provided by Assistant Professor Dr. Potchanapond Graidist. MCF-7 and MDA-MB-468 were maintained in RPMI1640 and high glucose DMEM medium, respectively, supplemented with 10 % fetal bovine serum and 100 units/ml Penicillin-Streptomycin, in a humidified atmosphere with 5 % CO₂ at 37 °C. The cells were seeded in 96-well plates at a density of 2x10⁴ cells (in 100 µl of medium) per well. The crude extracts were diluted to the desired concentration (80, 40, 20, 10 and 5 µg/ml) in medium. The cultures were incubated for 72 h with the test extracts.

MTT assay was employed to determine cell viability. After 72 h of incubation, 100 µl of 0.5

mg/ml MTT reagent was added into each well, and incubated for 30 min in a CO₂ incubator. MTT solution was then discarded and 100 µl dimethyl sulfoxide (DMSO) was added into each well to solubilize the cells and dissolve the color substance. For complete solubilization, the plates were vigorously agitated for 5 min at room temperature, and then read for optical density at 570 nm using a plate reader. A calibration curve based on optical density was constructed from which growth inhibition was determined. The 50 % growth inhibition concentrations (IC₅₀) of the extracts were calculated from fitted response curves. The cytotoxic activities of all the extracts against breast cancer cell lines were labeled according to the National Cancer Institute (NCI, USA) criteria (highly inhibiting activity means IC₅₀ ≤ 20 µg/ml) [12].

RESULTS

Phytochemical constituents

Phytochemical data (Table 1) show distinct patterns of chemical compositions in constituents of the extracts. The patterns of composition differed considerably in their quantitative values. The results of phytochemical evaluations are shown in Table 1. All the plant parts investigated were rich in terpenoids and anthraquinones whereas, alkaloids were absent. The n-hexane and methanol leaf extracts contained flavonoids, while only the n-hexane branch extract contained flavonoids was able to extract flavonoids from branches. Tannins and cardiac glycosides were present only in leaves and branches while terpenoids, anthraquinones and saponins were present in both pericarp and cotyledon extracts. Leaves and fruit contained saponins while branches did not.

Chromatographical data

Table 2 presents the Rate of flow (R_f) values observed under UV₂₅₄. The n-hexane leaf extract revealed 3 spots, and the branch extract 4 spots

also, the dichloromethane branch extract presented 4 spots. Details of these and spots are contained in Table 2.

In vitro cytotoxicity

The criteria used to categorize the activity of *V. diospyroides* extracts against human breast cancer cell lines (MCF-7 and MDA-MB-468), based on IC₅₀ values, were modified from those of NCI and Geran *et al* [12] as follows: IC₅₀ ≤ 20 µg/ml = highly active, IC₅₀ 21 - 200 µg/ml = moderately active, IC₅₀ 201 - 500 µg/ml = weakly active and IC₅₀ > 501 µg/ml = inactive. The cytotoxicity data for the fruit extracts against human breast cancer cell lines are displayed in Table 3.

Both acetone and methanol extracts of cotyledon were highly active (IC₅₀ = 3.1 µg/ml) against MDA-MB-468, but inactive and moderately active against MCF-7 (IC₅₀ 661.3 and 90.6 µg/ml, respectively). In contrast, both acetone and dichloromethane pericarp extracts were highly active against MCF-7 (IC₅₀ 21.0 and 18.2 µg/ml, respectively) but only moderately active against MDA-MB-468 (IC₅₀ 76.1 and 77.8 µg/ml, respectively). The hexane and methanol branch extracts were moderately active (IC₅₀ 69.3 and 94.2 µg/ml, respectively), whereas the dichloromethane branch extract was inactive. All leaf extracts were inactive with undetectable IC₅₀ levels.

DISCUSSION

Various active phytochemical compounds were found in the fruit, branch and leaf extracts of *V. diospyroides* Symington type LS. Terpenoids and anthraquinones were the major constituents found in all the extracts. These compounds have previously been reported as principal components of resveratrol derivatives in various extracts of medicinal herbs [13]. In general, the resveratrol compounds found in the stems of dipterocarpaceae are semipolar and polar [2],

Table 1 Phytochemical constituents of various solvent extracts of *Vatica diospyroides* type LS

Constituent	Leaf			Branch			Pericarp		Cotyledon	
	H	D	M	H	D	M	A	D	A	M
Terpenoids	+	-	+	+	+	-	-	+	+	+
Alkaloids	-	-	-	-	-	-	-	-	-	-
Anthraquinones	+	-	+	+	+	+	+	+	+	+
Cardiac glycosides	-	-	-	+	-	+	-	-	-	-
Flavonoids	+	-	+	+	-	-	-	-	-	-
Tannins	-	-	+	-	-	-	-	-	-	-
Saponins	+	+	+	-	-	-	+	+	+	-

Extract abbreviations: H = n-hexane; D = dichloromethane; M = methanol; A = acetone.

+ = presence; - = absence

Table 2: TLC results (R_f value) for extracts of *Vatica diospyroides* type LS parts observed at UV₂₅₄

Solvent ratio	Leaf			Branch			Pericarp		Cotyledon	
	H	D	M	H	D	M	A	D	A	M
Dichloromethane (85%): n-hexane (15%)	-	0.03	-	0.15	-	-	-	-	-	-
	-	0.36	-	0.37	-	-	-	-	-	-
	-	0.15	-	0.57	-	-	-	-	-	-
	-	0.93	-	0.84	-	-	-	-	-	-
	0.63	-	-	-	0.63	-	-	-	-	-
Ethyl acetate (65%): n-hexane (35%)	0.81	-	-	-	0.70	-	-	-	-	-
	0.86	-	-	-	0.78	-	-	-	-	-
	-	-	-	-	0.87	-	-	-	-	-
Dichloromethane (80%): Methanol (20%)	-	-	-	-	-	-	-	-	0.12	0.12
	-	-	-	-	-	-	-	-	0.20	0.20
	-	-	-	-	-	-	-	-	0.32	0.32
Dichloromethane (100%)	-	-	-	-	-	-	0.45*	0.45*	-	-
	-	-	-	-	-	-	0.71*	0.71*	-	-
Ethyl acetate (85%): Dichloromethane (15%)	-	-	-	-	-	0.39	-	-	-	-
	-	-	-	-	-	0.76	-	-	-	-
	-	-	-	-	-	0.81	-	-	-	-
Ethyl acetate (80%): n-hexane (20%)	-	-	0.88	-	-	-	-	-	-	-

Note: *Dichloromethane and acetone extracts of pericarp showed 2 blue spots after staining with CAM (Ceric ammonium molybdate) reagent. **Extract abbreviations:** H = n-hexane; D = dichloromethane; M = methanol; A = acetone. Negative sign (-) indicates that no investigation was carried out.

Table 3: Cytotoxic activity (IC_{50}) of fruit extracts of *Vatica diospyroides* type LS

Fruit part	Extract	Cytotoxic activity (IC_{50} , $\mu\text{g/ml}$)	
		MCF-7	MDA-MB-468
Cotyledon	Acetone	661.3	3.1**
	Methanol	90.6	3.1**
Pericarp	Acetone	21.0*	76.1
	Dichloromethane	18.2*	77.8

* and ** symbolize IC_{50} of $\leq 20 \mu\text{g/ml}$, considered highly cytotoxic against human breast cancer MCF-7 and MDA-MB-468 cell lines, respectively

and extracting these from the leaves, branches and fruit of *V. diospyroides* require polar or highly polar solvents. Our results also indicate acetone and methanol are the best solvents for extraction, in terms of quantitative and cytotoxic properties of the extracts.

On the other hand, alkaloids were absent from all the extracts. The non-existence of alkaloids in *V. diospyroides* is a deviation from several other medicinal plants [10]. Alkaloids are commonly found in most medicinal plants, and they have various useful bioactivities which favours their use as active ingredients in anti-inflammatory medications [14]. In addition, they are widely used for treating various cancers [1] and malaria [15]. Previously, the cardioprotective effect of alkaloids from other plants in dipterocarpaceae family has been described [6]. Thus, if indeed *V. diospyroides* extracts has prophylactic cardiovascular effect, this is likely to be due to non-alkaloidal compounds.

Interestingly, cardiac glycosides were only present in the branch extract. Cardiac glycosides have been found in a number of plants used in blood tonics for treating heart failure via blood

pressure regulation [16]. This may lend some support for the use of *V. diospyroides* stem extract in classic Thai blood tonic. Tannin, however, was present only in the leaf extract while saponin was absent only from only the branch extracts. Flavonoids, which were present both in leaf and branch extracts, are antioxidants with cardiovascular prevention and anticancer properties [1,2,17].

The anticancer activities of terpenoids, saponins, cardiac glycosides and anthraquinones have been reported for various plant extracts [10,18,19]. Terpenoids and saponins are biologically active against various human cancer cell lines [19]. Similarly, these compounds are potent against human breast cancer cell lines. However, these secondary metabolites exert their anticancer activity through different mechanisms. Carvalho *et al* [20] reported that plant extracts can decrease cell proteins, alter cell morphology, and fragment nucleosomal DNA of cancer cells, resulting in cell apoptosis. Therefore cardiac glycosides may induce immunogenic cancer cell death.

The human breast carcinoma cell lines used in this study - MCF-7 and MDA-MB-468 - are in common laboratory use. A high cytotoxic activity against MCF-7 was observed for acetone and dichloromethane extracts of pericarp, whereas the acetone and methanol extracts of cotyledon were highly active against MDA-MB-468. Thus each extract is a different cocktail of ingredients with potential activity that is specific to a particular cell line. This is not unexpected since MCF-7 is a luminal cancer cell line representing low tumor grade. In contrast, the basal-like carcinoma cell line, MDA-MB-468, represents a high tumor grade, and is triple negative for the immunophenotypic markers ER/PGR/HER2. MDA-MB-468 was expectedly more sensitive to the extracts and culture conditions than MCF-7. High cell proliferation of MDA-MB-468 corresponds to a poor prognosis.

Fruit extracts of other medicinal plants, e.g., some compounds produced from the seed kernels of the Thai medicinal plant, *Entada rheedei* Sprengel, have been evaluated against various human cancer cell lines [17]. However, the fruit extracts of *V. diospyroides* have not previously been described and reported in Thai ethnopharmacology, and the active components have not been identified. The current study is the first to demonstrate that the highly active constituents of the fruit extracts inhibit breast cancer cells.

No normal human cells were tested in this study, and cultured immortalized cell lines will not behave as they would *in vivo*. While *in vitro* toxicology may sound attractive, there is a view that it is currently unrealistic. Our findings have indicated that fruit extracts of *V. diospyroides* may be worth further investigation with a view to isolating the active compounds.

CONCLUSION

This work demonstrates that *V. diospyroides* fruit extracts have pharmacological potential. The pericarp and cotyledon extracts of this plant inhibit the growth of MCF-7 and MDA-MB-468, on a highly active level. Further studies are planned to identify and purify the *in vitro* active compounds for subsequent *in vivo* evaluation.

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