In vitro Cytotoxic Activity of Vatica diospyroides Symington Type LS Root Extract on Breast Cancer Cell Lines MCF-7 and MDA-MB-468

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Vatica diospyroides Symington is the valuable source of important curative compounds in human cardiovascular and cancer prevention. In order to investigate root extracts of V. diospyroides, their composition by chromatography and for their cytotoxic activity against breast cancer cell lines in vitro, Thin-layer Chromatography (TLC) was used to separate the chemical constituents and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the 50% inhibition (IC₅₀) concentrations against human breast cancer cell lines MCF-7 and MDA-MB-468. Specific morphological changes of the cancer cells were observed to further elucidate the induction of apoptosis. The chromatography data indicated that different solvents extracted different numbers of components, so that the extracts varied in their chemical compositions. The acetone extract of roots had the highest yield and was highly active whereas the methanol extract was active against the MDA-MB-468 cell line (IC₅₀ = 12.82 and 28.41 μL⁻¹, respectively). The other root extracts were active moderately, or inactive, against MCF-7 and MDA-MB-468. Cell shrinking, membrane blebbing, ballooning and chromatin condensation were detected under phase contrast microscopic observations, in the treated cancer cells, as indications of effects that lead to apoptosis. Our results indicate that V. diospyroides root extracts can be highly cytotoxic against breast cancer cell lines. This encourages identifying and purifying an active compound in the root extracts, in a further study.

Key words: Apoptosis, breast cancer, cytotoxicity, root extract, Vatica diospyroides

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INTRODUCTION

Breast cancer is currently the second leading cause of death for women dying of cancer (Lester and Cotran, 1999) both in the developed and developing countries, and the incidence rates are greatly increasing worldwide (WHO, 2013). Over the last 4 years, in less developed countries, the cancer survival rates have been very low because adequate diagnosis and effective drug treatments are lacking and the situation is exacerbated by increasing treatment costs. Thus, there is high motivation to discover new effective low-cost natural drugs, extracted from various organisms such as fungi (Balde et al., 2010), marine sponges (Chairman et al., 2012) and higher plants (Kinghorn et al., 2011). Plant extracts play an important role in folk medicines, as an indication of their therapeutic properties against various diseases. At present, extracts of higher plants are widely studied for their human cancer healing activity. However, by using the crude extracts, an in vitro inhibition of cancer cell growth provides a first screen before making higher investments on the risky path towards an accepted drug. Actual pharmaceutical drug development would likely involve creating many variations of the active molecule and would pursue safety, efficacy and selection of targeted cancer types and formulations and delivery to the tumor, as well as demonstration in clinical trials, in stages of increasing costs. The scopes of this study and our potential follow-up studies are necessarily more modest but could spur such larger scale development if successful.

Most plants in the family dipterocarpaceae contain resveratrol that are effective cancer chemopreventive agents, such of Vatica phellodendron and Vatica alba series purified from stem extract of plant in the genus Vatica (Kinghorn et al., 2011; Aggarwal et al., 2004; Seo et al., 1999). Stem and flower of Vatica dysopyroides Symington type LS, a critically endangered fragrant species that is known in the Nong Thung Thong non-hunting area of Thailand, are used by traditional Thai medicine in cardiovascular and blood tonic recipes. The stem and fruit of this plant have been reported to have highly effective anti-tumor (Seo et al., 1999) and cytotoxicity (Srisawat et al., 2013) properties while its root has never been investigated to date. Roots of many medicinal plants, such as Taraxacum officinale and Tectaria singaporea, have been a rich source of highly bioactive constituents demonstrated against melanoma (Chatterjee et al., 2011) and MCF-7 (Amini et al., 2008) cell lines. Interestingly, the highly cancer therapeutic agent resveratrol is commonly found in the roots of Veratrum grandiflorum O. Loes (Aggarwal et al., 2004). Therefore, the root extracts of V. dysopyroides that have so far not been given attention, are worth inspecting for their anticancer potential.

In the present study, we have pre-screened chromatographically for chemical constituents and investigated the cytotoxic activity of V. dysopyroides type LS root extracts, in vitro against breast cancer cell lines MCF-7 and MDA-MB-468. We have also observed cell morphological changes, indicative of the apoptotic mechanisms of cancer cells. This is the first time that the root extracts of V. dysopyroides type LS have been assessed for their cytotoxic properties. Isolating and identifying the active compounds in the root extracts is the topic of a potential further study while the current study aims to assess whether such activity is present.

MATERIALS AND METHODS

Plant materials and preparation of extract: The roots of V. dysopyroides type LS were excised from a twenty-year-old tree on October 2012, at the Nong Thung Thong non-hunting area, Suratthani Province, Southern Thailand. Voucher specimens (Collector number T. Srisawat 003) were deposited in the Herbarium of Queen Sirikit Botanic Garden (QBG), Maerum, Chang Mai, Thailand and also authenticated by Dr. Charun Maknoi at the QBG.

To remove soil and debris, the excised roots were washed and cleaned with tap water. The sample was then cut into small pieces and completely air-dried under shadow. Dried pieces of root (about 300 g) were extracted in steps with dichloromethane (CH₂Cl₂), acetone ((CH₃)₂CO) and methanol (CH₃OH), in this sequence, each extraction taking five days. The solvent of each extraction was then removed to new container, filtered by cotton fabric and evaporated at room temperature using a rotor evaporator (Heidolph Rotary Evaporator, D-91126, Germany) under reduced pressure and the dry remainder was stored in cool and dark conditions.

Thin layer chromatography (TLC): Thin Layer Chromatography (TLC) was applied to the extracts, following the method described previously (Srisawat et al., 2013). The developing solvents used were dichloromethane (85)-ethyl acetate (15), ethyl acetate (75)-acetone (25) and ethyl acetate (85)-dichloromethane (15) mixtures.
**In vitro cytotoxicity assay:** Cell cultures of MCF-7 (HTB-22™) and MDA-MB-468 (HTB-132™) were purchased from the American Type Culture Collection (ATCC, USA) and maintained at the Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Thailand. The culture conditions of cell lines, the desired concentration of extracts used and the MTT assay followed the methods described previously (Srisawat et al., 2013). The cytotoxic activities of all extracts against breast cancer cell lines MCF-7 and MDA-MB-468 were determined according to the NCI (labeled for active level as IC<sub>50</sub> of ≤30 µg mL<sup>-1</sup>) and Geren et al. (1972) (labeled for activity levels as follows: IC<sub>50</sub>≤20 µg mL<sup>-1</sup> = highly active, IC<sub>50</sub> 21-200 µg mL<sup>-1</sup> = moderately active, IC<sub>50</sub> 201-500 µg mL<sup>-1</sup> = weakly active and IC<sub>50</sub> > 501 µg mL<sup>-1</sup> = inactive).

**Cell morphological features related apoptosis:** Cell morphological changes were observed after treating with *V. diospyroides* root extract within 72 h of incubation. Cell shrinking, membrane blebbing (protrusion of membrane), ballooning and chromatin condensation, were observed as indicators of the cancer cells approaching treatment induced apoptosis. On the other hand, cytoplasm vacuolation, formation of double membrane vesicles and cell swelling or rupturing were considered indications of necrotic or non-specific cell death (Darzynkiewicz et al., 1992) that might not necessarily be induced by the treatment.

**RESULTS**

**Yields of extractions and chromatography data:** The extraction yields from sequential use of different solvents are shown in Table 1. The highest yield (6.64% per gram dry weight) was obtained with acetone, in second place of the extraction sequence.

Table 2 presents the Rate of flow (R<sub>r</sub>) values observed under UV<sub>254</sub>, along with other details of chromatography results. The dichloromethane extract of root had 5 spots whereas the acetone and methanol extracts presented 4 spots with different R<sub>r</sub> values.

**In vitro cytotoxicity (MTT assay):** The cell lines MCF-7 and MDA-MB-468 were used for a comparative cytotoxicity study. The cytotoxicity data for the root extracts against these cancer cell lines are listed in Table 3. The acetone extract of root was highly active (IC<sub>50</sub> = 12.82 µg mL<sup>-1</sup>) against MDA-MB-468 (Fig. 1a) while the methanol and dichloromethane extracts were active and moderately cytotoxic (IC<sub>50</sub> = 28.41 and 43.09 µg mL<sup>-1</sup>, respectively). Against the MCF-7 cell line, the acetone and methanol extracts were moderately active (IC<sub>50</sub> = 32.74 and 77.32 µg mL<sup>-1</sup>, respectively) (Fig. 1b) whereas, the dichloromethane extract was inactive.

**Cell morphological observations indicative of treatment induced apoptosis:** Under phase contrast microscopic observation, acetone extract treated cells (>80%) were enlarged, ballooned, blebbled, nuclear shrank or fragmented, within 72 h of culture with treatment, indicating apoptotic mechanisms of cell death. Slight cell vacuolation and swelling were also found in the cell cultures, suggesting that all cancer cell deaths were
Fig. 1(a-b): Cytotoxic activity of acetone extract, of *V. diospyroides* type LS root, showed consistent dose dependent behavior. The responses of breast cancer cell lines (a) MDA-MB-468 and (b) MCF-7 at 72 h of incubation. The dose responses were determined with the MTT assay and the IC<sub>50</sub> values were subsequently calculated according to the NCI and Geren *et al.* (1972) methods.

not only treatment induced apoptosis but might include non-specific necrosis also. The morphological changes appeared dose dependent, seemingly correlated with the cytotoxicity assay.

**DISCUSSION**

We found an efficacious extract from the root of *Vatica diospyroides* Type LS, such that inhibited the cell proliferation and induced apoptosis in two human breast cancer cell lines (MCF-7 and MDA-MB-468). *V. diospyroides* was chosen for this study, because the flower and stem of this plant are used in cardiovascular and blood tonics in Thai ethnopharmacology (Srisawat *et al.*, 2013) and are also used as therapeutic agents in alternative medicine for human cancer prevention (Kinghorn *et al.*, 2011). In the present study, root of *V. diospyroides* was extracted sequentially by three solvents and these extracts were found to have different chemical compositions (as seen in R<sub>t</sub> values) and had different efficacies as breast cancer inhibitors in an *in vitro* cytotoxicity assay. Because the different components have different affinities to the solid matrix and the solvent molecules, they travel different distances during chromatography. Moreover, our prior preliminary studies have observed some bioactive constituents in the dichloromethane, acetone and methanolic extracts of the root, including terpenoids, alkaloids, flavonoids and saponins, with previously described methods (Kamba and Hassan, 2010). Interestingly, terpenoids and alkaloids were only present in the acetone extract of root whereas flavonoids and saponins were found in the methanolic and acetone extracts. The phytochemical constituents, such as terpenoids, alkaloids, flavonoids and saponins, present in medicinal plants, have exhibited anti-inflammatory and other biological activities (Barbosa-Filho *et al.*, 2006), such as curing malaria (Francois *et al.*, 1997) and cancer inhibition (Kinghorn *et al.*, 2000). This corroboration suggests that medicinal plant root extracts can support alternative medicine, with safety and low toxicity on normal cells and high toxicity on pathogens (Vijayarathna and Sasidharan, 2012). Extracting an effective compound from *V. diospyroides* root requires a polar or highly polar solvent, because the active compounds found in plants of Dipterocarpaceae are semipolar and polar. Acetone or methanol is therefore suitable as extraction solvent. Previous work has also shown high cytotoxicity against cancer cell lines KB and HeLa, of a new bioactive compound (dimeric resveratrol) extracted with acetone from the root of *Shorea roxburghii* (also dipterocarp species) (Patcharamun *et al.*, 2011). Roots of other medicinal plants have been supported by *in vitro* and *in vivo* studies for inhibiting cancer cell proliferation (Arican *et al.*, 2012; Zick *et al.*, 2011; Badisa *et al.*, 2000). These observations suggest that *V. diospyroides* roots provide a rich source of compounds with potential efficacy, serving further development into human cancer drugs.

Two breast cancer cell lines, MCF-7 and MDA-MB-468, were chosen to investigate the cytotoxic effects of *V. diospyroides* root extracts in this study. The acetone extract of root had cytotoxicity against both cancer cell lines in a dose dependent manner, at the end of 72 hours of incubation. It nearly satisfied the “active inhibition” criterion of NCI (IC<sub>50</sub> of ~ 30 µg mL<sup>-1</sup>), with the range 12-32 µg mL<sup>-1</sup> for both cell lines; the “highly active” level of 20 µg mL<sup>-1</sup>, by Geren *et al.* (1972) criterion, was satisfied for one cell line only. Resveratrol derivatives are known to induce apoptosis and the compound is commonly found in medicinal plant roots (Aggarwal *et al.*, 2004), so the extracts of *V. diospyroides* root could be promoting apoptosis due to resveratrol. The
morphological characteristics of treated cells were coherent with apoptotic death, showing cell blebbing, shrinking, ballooning and nuclear condensing. However, slight necrotic vacuolization or swelling of cells was observed. Cell cytotoxic activities were expressed in concentration units, in a cell line specific manner. At 72 h of incubation, in MDA-MB-468, the acetone and methanol extracts of root at concentrations up to 20 μg mL⁻¹ reduced the cancer cell growth. In contrast, with the MCF-7 cell line a two-fold concentration (40 μg mL⁻¹) was needed for the same level of cytotoxicity. MCF-7 is a luminal cancer cell line representing a low tumor grade while MDA-MB-468 is from a basal-like carcinoma with a high tumor grade. Since the IC₅₀ of MDA-MB-468 cell line was lower than that of MCF-7, the former is more sensitive to the extract than the latter (Srisawat et al., 2013). The terpenoids and saponins in the extracts of medicinal plant roots can cause apoptosis of cancer cell lines (Zhang and Li, 2007). Both types of constituents were present in the acetone and/or methanol extracts of V. diospyroides root and prior work has shown that also the alkaloids in acetone extracts of roots can have potent anticaner activity (Kinghorn et al., 2000). The current study interestingly indicates that the acetone extract of root is highly toxic for both cancer cell lines which may be due to specific active compounds in the extract (Srisawat et al., 2013; Patcharamun et al., 2011). The phytochemical constituents have also been reported to cause apoptosis by fragmenting nucleosome DNA, decreasing cell proteins and altering cell morphology (Carvalho et al., 2012). The present study has revealed potent cytotoxic effects of acetone root extract resembling those obtained in our previous study with acetone extract of V. diospyroides fruit and the same breast cancer cell lines (Srisawat et al., 2013). Continued discovery of anticancer drugs from the roots of medicinal plants is thus encouraged, with the hope of saving human lives (Tripathi and Tripathi, 2003).

CONCLUSION

In conclusion, we have shown firstly that a root extract of V. diospyroides induced apoptosis in breast cancer cell lines MDA-MB-468 and MCF-7, in vitro. The cytotoxicity determinations may be slightly biased by necrotic cell deaths, increasing the apparent activity against cell lines. Isolation and characterization of the active compound(s), should be pursued in further experiments, either to provide natural concentrated anticancer agents, or to support modification in further drug development.

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