Anticancer Activity of Oldenlandia Diffusa & Viola Philippica Car

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Abstract: *Oldenlandia diffusa* (OD, Bai Hua She She Cao) and *Viola philippica Car.* (VPC, Zi Hua Di Ding) are both commonly used traditional Chinese medicine (TCM). Although studies have demonstrated their anticancer activities on cancer cells both *in vitro* and *in vivo*, no systematic reports were carried out. The objective of this study was to examine their cytotoxic activities *in vitro.* We used the ethanol extracts of these two herbs and examined their cytotoxic effect on four cancer cell lines and two non-cancer cell lines using an MTT cytotoxicity assay. The results showed that the ethanol extracts of OD and VPC effectively inhibited the growth of all the cancer cell lines, particularly MiaPacA-2 cancer cells. Significantly less cytotoxicity was observed in non-cancer cell line NIH3T3. We also tested the drug sensitivity of OD and VPC in a P-glycoprotein (P-gp) overexpressing multidrug resistant cell line KB-C2. Our results showed that OD and VPC can potently inhibit the growth of KB-C2 cells. Therefore, this study has revealed the remarkable anticancer activity of these two TCMs. To the best of our knowledge, this is the first report that shows potential anticancer activity in multidrug resistant cells in extracts of OD and VPC using scientific methods of evaluation.

Keywords: Hedyotis diffusa, Herba violae, cytotoxicity, anticancer, ethanol extract, traditional Chinese medicine (TCM).

INTRODUCTION

Oldenlandia diffusa (OD, Bai Hua She She Cao, Hedyotis diffusa) and *Viola philippica Car* (VPC, Zi Hua Di Ding, Herba violae) are two of the most commonly used herbs to treat cancers in traditional Chinese medicine (TCM) [1]. They have been used as TCM for treating diseases for centuries and were studied by a world famous medical scientist in Ming Dynasty in China, Shi-Zhen Li, in his most famous monumental work "Compendium of Materia Medica" [2]. Recently, both clinical and laboratory evidences have shown efficacy of these two herbs in the treatment of cancers of different tissue origin [3-10]. Herbal medicines in TCM were normally prepared by water instead of organic solvent extraction. The tradition in China is to boil the herbs, and then orally administer the suspension (decoction) to the patient once cooled to a proper temperature. Zhao [3] reported two cases of successful treatment of nasopharyngeal carcinoma using the herb OD. Li and Huang [4] successfully treated 53 patients with non-small cell lung carcinoma using OD extracts in combination with recommended chemotherapy, compared to 33 patients who were treated with recommended chemotherapy alone. The combination plan was more effective, with less side-effects and increased quality of life. Similar results of the combined therapy plan (OD extract with the recommended chemotherapy) were reported by Zhang *et al*. [5] in a cohort (cohort study) of 36 patients with liver cancer and by Huang *et al.* [6] in 40 patients with acute non-lymphocyte leukemia. More recent clinical investigations have also shown positive effects using these herbs on various types of cancers, mostly in late stage of disease development [7-9]. In addition, the anticancer effects of these herbs were reported in other studies by using different cell lines and animal models. Shan *et al.* [10] reported that aqueous extracts of the herbs enhanced the potency of both natural killer cells and phagocytic macrophages in exterminating cancer cells from the body. The inhibitory effect on cancer cell growth of the aqueous extracts of these herbs was also reported in both *in vivo* and *in vitro* studies [11, 12].

In this study, we investigated the cytotoxicity of the crude extracts of these two Chinese herbs, OD and VPC, in four different cancer cell lines and two non-cancer cell lines. In addition, we also studied the cytotoxicity of OD and VPC in a drug resistant human epidermoid carcinoma cell line, compared with its parental cell line, KB-3-1.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM], bovine serum and penicillin/streptomycin were

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purchased from Hyclone (Logan, UT). Phosphate buffered saline (PBS) was purchased from Invitrogen GIBCO (Grand Island, NY). Colchicine, paclitaxel, vinblastine, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). One Cuisinart DCG-12BC Coffee Grinder was purchased from Target Corporation. A Branson 2510 Ultra sonicator, a Yamato RE500 Rotary Evaporator attached with OAKTON Aspirator Pump WP-15 and a LABCONCO Freeze Dry/Shell Freeze System (model LYPH-LOCK 12) had been previously obtained. Other routine laboratory reagents were obtained from commercial sources of analytical grade. All other chemicals used were purchased from Sigma (St. Louis, MO).

Herbal Extraction

Dried herbs of OD and VPC cultivated in China were purchased from Zhaoqing City Medicine Company Chinese Medicinal Slices Factory [Guangdong province, China]. The dry whole plants of these two Chinese herbs were ground and sieved. A 100 g portion of each of the powdered herbs was extracted with 300 ml 70% ethanol with help of sonication for 6 hours (h) at room temperature. The extraction suspension was filtered, concentrated under reduced pressure below 40°C and then frozen at -20°C overnight and lyophilized for 24 h. The yields were 2.35% for OD and 3.08% for VPC, respectively. The powdered extracts (representing the OD and VPC, respectively) were stored at -20°C until used for various cell culture treatments. When applicable, 1.0 g of each extract was dissolved in DMSO and the stock concentration was 1.0 g/ml. These stock solutions of OD and VPC were further diluted in PBS (pH 7.4) to different concentrations ranging from 1 µg/ml to 1000 µg/ml. Cytotoxicity of the ethanol extract was analyzed with the MTT assay in four different cancer cell lines and two non-cancer cell lines.

Cell Lines and Cell Culture

Non-small cell lung cancer cell lines, A549 and NCI-H460, the human pancreatic cell line MiaPacA-2 and human embryonic kidney cell line HEK293 were obtained from the American Type Culture Collection (Rockville, MD). NIH3T3 cells, which the mouse derived BALB-murine sarcoma virus (MSV) [13] was a clonal BALB-MSV nonproducer transformant [14], was generously provided by Dr. Gary Kruh (University of Illinois at Chicago, Chicago, IL). Drug resistant human epidermoid carcinoma cell line KB-C2 was a gift from Dr. Akiyama (Kagoshima, Japan) [15]. As a parental cell line of KB-C2, the human epidermoid carcinoma cell line KB-3-1 was provided by Dr. Gottesman (NIH, MD). All cell lines were grown as adherent monolayers in flasks with DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin under standard cell culturing conditions in a humidified incubator containing 5% CO_2 at 37 °C.

MTT Cytotoxicity Assay for Cell Survival

The sensitivity of cultured cells to the two herbal extracts was analyzed using an MTT colorimetric assay [16]. The assay detects the reduction of MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide) by mitochondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitochondria and hence cell viability. Cells were

Table 1: *In Vitro* Cytotoxicity of OD and VPC Against Various Human Cancer Cell Lines and Non-Cancer Cell Lines in 70% Ethanol Extract of Whole Roots

Cell Line	IC_{50} ± SD (µg/ml)[a]	
	OD	VPC
H460	188.29±13.97	169.03±10.31
A549	203.58±17.19	225.02±13.9
MiaPacA-2	90.69±3.72	93.67±4.59
KB-3-1	132.97±9.74	139.63±7.57
HEK293	164.13±16.27	297.54±25.39*
NIH/3T3	901.9±75.07**	609.96±37.83**

[a]IC_{50}: concentration that inhibited cell survival by 50% (means±SD).
Cell survival was determined by MTT assay as described in "Materials and Methods".
Data are means±SD of at least three independent experiments performed in triplicate.
*P <0.05; ** P <0.01.

seeded in 96-well plates in triplicate at 4000 to 5000 cells/well. After incubation in DMEM supplemented with 10% FBS at 37°C for 24 h, various concentrations of herbal ethanol extracts [OD or VPC] were added and incubated with the cells continuously for 72 h. After incubation of cells with the OD or VPC extracts for 72 h, 20 µl of 4 mg/ml MTT in PBS was added to each well and the plates were incubated at 37°C for 3 h, allowing viable cells to develop the yellow-colored MTT into dark-blue formazan crystals. Subsequently, the medium was gently removed without agitating the adhesive monolayer of cells, and 100 µl of DMSO was added into each well to dissolve the formazan crystals. The plates were well shaken for 5 min, and the absorbance of control and extract-treated cells was measured spectrophotometrically at 570 nm using an OPSYS microplate reader from DYNEX Technologies Inc. (Chantilly, VA). We first used this MTT assay to determine the cytotoxicity of OD and VPC to four different cancer cell lines, H460, A549, MiaPacA-2, KB-3-1, and two non-cancer cell lines, HEK293 and NIH/3T3. Then, in order to determine if OD and VPC have a cytotoxic effect on drug resistant cell lines, we further used this MTT assay to test the sensitivity of drug resistant human epidermoid carcinoma cell line KB-C2, together with its parental cell line KB-3-1.

Statistical Analysis

All experiments were repeated at least three times and the differences were determined using Student's *t*-test. The statistical significance was determined at $P< 0.05$.

RESULTS

OD and VPC have Cytotoxic Effect on Cancer Cells

To investigate the effect of OD and VPC in various cancer cell lines, we have examined the anti-cancer activity and selectivity of OD and VPC on cancer using cell-based assays. For this purpose, a set of established human cancer cell lines were used, including the epidermoid carcinoma cell line KB-3-1, the human non-small cell lung cancer cell line H460, the human lung adenocarcinoma epithelial cell line A549, and the human pancreatic cell line MiaPacA-2. Table **1**, Figures **1A**, **B** and **2**, show the IC_{50} values of OD and VPC on all cancer cell lines used in this study. The concentrations of growth inhibition at 50% (IC_{50} values) in all cancer cell lines ranged from 90.69 to 225.02 µg/ml after 72 h treatment. These results suggest that the ethanol extracts of both OD and VPC exhibited strong antiproliferative activity against all cancer cell lines tested.

Selective Cytotoxicity of OD and VPC on Cancer Cells

As shown in Table **1**, Figures **1A**, **B** and **2**, both OD and VPC showed significant differences in cytotoxicity between cancer cell lines. Both of the ethanol extracts of OD and VPC specifically and strongly inhibited the

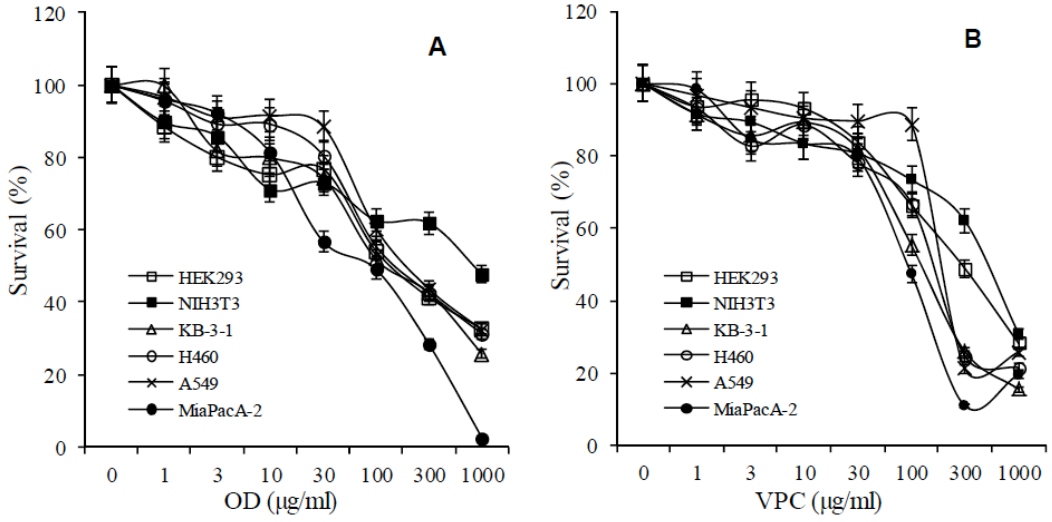

Figure 1: The survival curves of different cell lines at different concentrations of OD and VPC.

Six cell lines, H460, A549, MiaPacA-2, KB-3-1, HEK293 and NIH3T3, were used for this experiment. After seeding and culturing cells for 24 h, different concentrations of OD and VPC were added into each of the cells above. Three days later after culturing, cell survival was determined by MTT assay as described in "Materials and Methods". Data points are the mean±SD of triplicate determinations. Experiments were performed at least three independent times, and a representative experiment is shown.

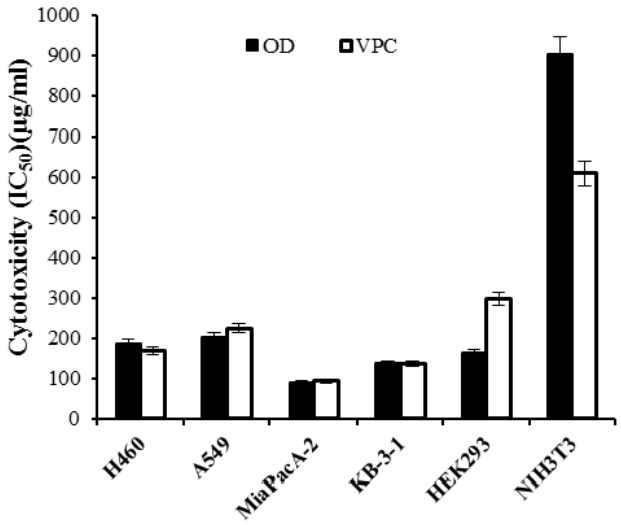

Figure 2: The cytotoxicity of OD and VPC in various cell lines. Same experiment as Figure **1**. Data points are the mean±SD of triplicate determinations, showing the IC_{50} values of OD and VPC to six different cell lines.

growth of the human pancreatic cell line MiaPacA-2 (90.69 ± 3.72 μg/ml for OD, 93.67±4.59 μg/ml for VPC) and significantly inhibited the growth of other cancer cells, H460 (188.29±13.97 μg/ml for OD, 169.03±10.31 μg/ml for VPC), A549 (203.58±17.19 μg/ml for OD, 225.02±13.9 μg/ml for VPC), and KB-3-1(132.7±9.74 μg/ml for OD, 139.63±7.57 μg/ml for VPC). OD showed slightly more potent cytotoxic effect than VPC against all the cancer cell lines tested.

OD and VPC Showed Selective Cytotoxicity on Non-Cancer Cells

As shown in Table **2**, Figures **1A**, **B** and **2**. the concentrations of growth inhibition at 50% (IC_{50}) ranged from 164.13 μg/ml to 901.90 μg/ml after 72 h treatment. The extracts had very limited cytotoxicity (<20% inhibition) on the non-cancer cells HEK293 at the concentration of 50 μg/ml and NIH/3T3 at 100 μg/ml. OD & VPC both had significantly less effects on the non-cancer cell line NIH/3T3 (OD: 901.9±75.07, VPC: 609.96±37.83 μg/ml) than those in cancer cell lines (*P* <0.01), but compared to their cytotoxicity in cancer cells, OD & VPC showed only slightly less effects on the human embryonic kidney cell line HEK293 over a wide range of concentrations (OD: 164.13±16.27, VPC: 297.54±25.39 μg/ml). OD had significantly less cytotoxicity than VPC on NIH/3T3 cells (P<0.01) but significantly more cytotoxicity than VPC on HEK293 cells.

OD and VPC have Potent Effect Against Multidrug Resistant KB-C2 Cells

To determine if OD and VPC are potent to drug resistant cells, we used a P-gp overexpressing drug resistant human epidermoid carcinoma cell line, KB-C2, together with its parental KB-3-1 cell line. As shown in Table **2** and Figure **3C** and **D**, KB-C2 cell line shows only a 1.23-fold resistance (*P*>0.05) to VPC compared to the parental KB-3-1 cells (Table **2**) while it does not confer resistance to OD compared to KB-3-1 cells. Instead, the KB-C2 cells even showed relatively more sensitivity to OD than KB-3-1 cells (0.59-fold resistance to OD compared to the KB-3-1 cells. *P*<0.05). These results suggested that OD may not be a substrate of P-gp. VPC is also not a good substrate of P-gp because KB-C2 cells showed a very low resistance to OD (Table **2**, Figure **3C** and **D**), whereas the KB-C2 cells showed resistance to known P-glycoprotein (P-gp) substrates such as colchicine (736.31-fold), paclitaxel (553.85-fold) and vinblastine (9.25-fold) (Table **2**, Figure **3A** and **B**). Further investigation is needed to determine the value of these two extracts in other drug resistant cell lines.

Table 2: Drug Resistance Profile of KB-C2 Cells

Compound or herb extract	IC_{50}±SD[a]		Resistance fold[b]
	KB-3-1	KB-C2	
Colchicine[c]	7.08±1.48	5213.04±1777.14	736.31**
Paclitaxel[c]	8.26±1.47	4574.82±873.79	553.85**
Vinblastine[c]	50.88±2.83	470.68±213.72	9.25*
OD[d]	138.28±8.37	81.07±6.85	0.59*
VPC[d]	137.57±14.68	169.8±16.52	1.23

[a]IC_{50}: concentration that inhibited cell survival by 50% (means±SD).
[b]Fold-resistance was the value of that IC_{50} value for colchicine, paclitaxel, vinblastine, OD or VPC of KB-C2 to KB-3-1.
[c]The unit of these drugs: nM.
[d]The unit of herbal extracts: μg/ml.
***P<0.001*. * *P<0.05*. The experiments were repeated at least three times.

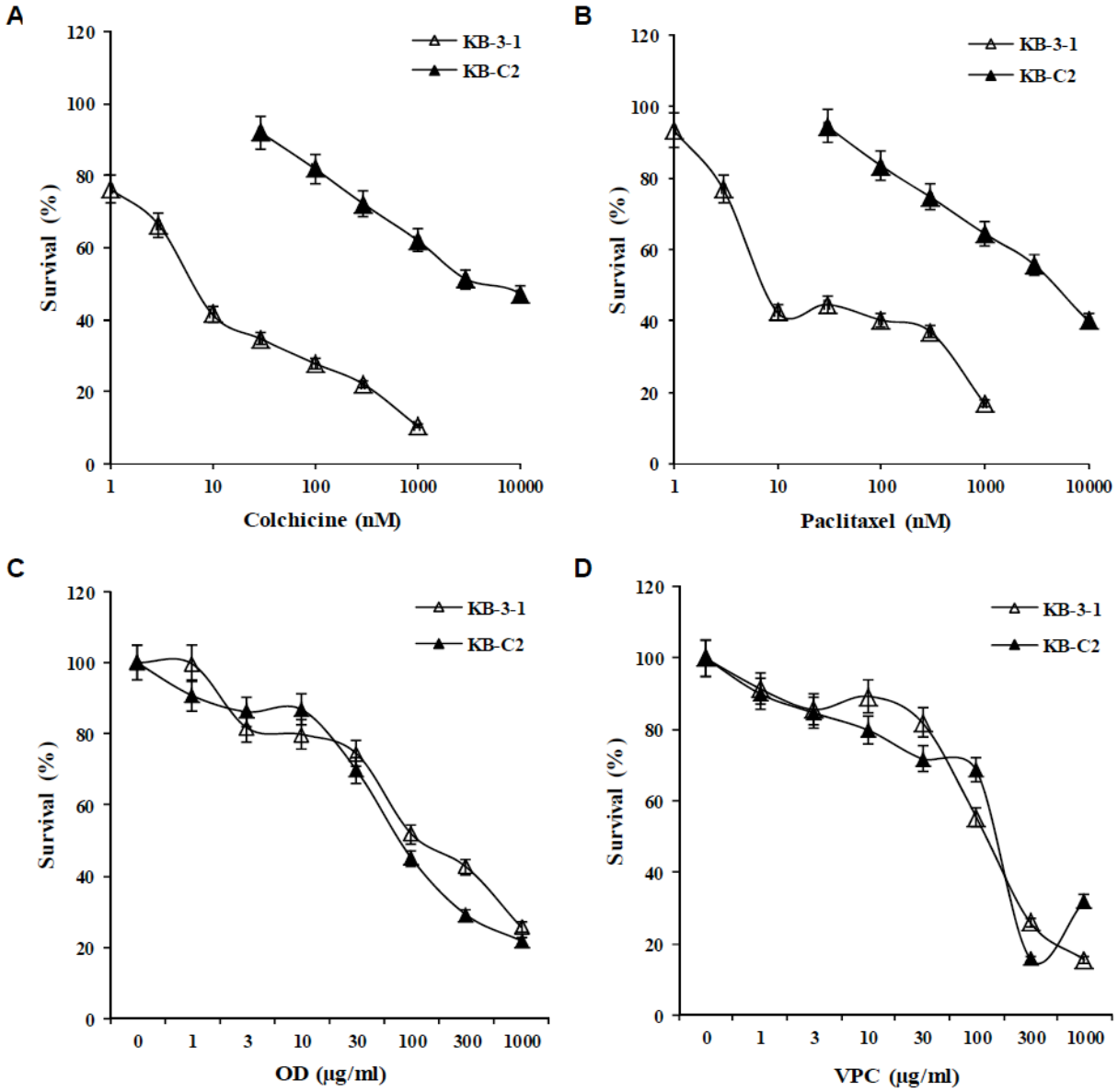

Figure 3: The survival curves of KB-3-1 and KB-C2 to OD, VPC, colchicine and paclitaxel. The sensitivity of KB-3-1 and KB-C2 cells to colchicine, paclitaxel, OD and VPC. Cells were seeded and cultured for 24 h and further incubated for 72 h. The absorbance was determined at 570 nm by an OPSYS microplate Reader. The representative figures show the sensitivity of KB-3-1 and KB-C2 cells to colchicine, paclitaxel, OD and VPC.

DISCUSSION

Chemotherapy is currently one of the gold standards in cancer treatment. However, chemotherapeutic drugs are highly toxic and have serious side effects. Scientists have been developing various new strategies to control and treat human cancers [17]. Most of them are hoping to extract promising compounds from natural origin. Plant-derived medicines have been used for centuries and more than half of the known anticancer drugs have originated from herbal plants [18-21]. Natural herbal products such as a variety of different flavonoids have anti-cancer activity [22, 23]. One study reported anti-cancer activity *in vitro* using a natural herbal extract containing many kinds of flavonoids [24]. More and more plant extracts are found to have cytotoxic effects on cancer cells [25-28]. Natural products are also the leading molecules for many of the drugs that are in use today [29]. The phytochemicals present in some herbal products and plants may have the potential to act as preventive or therapeutic agents against various human cancers [14]. The increased popularity of herbal remedies for cancer therapy perhaps can be attributed to the belief that herbal drugs provide benefit over that of allopathic medicines while being less toxic [30]; especially in China, there is a long history and great

valuable experience in using natural plants as medicine [31, 32]. Although the conventional therapies have devastating side effects, it cannot be denied that there must be a continuous need to search for new herbal cures of cancer [33].

OD and VPC are both commonly and extensively used in most of the Chinese herbal pharmaceuticals and nutraceuticals [1, 2]. OD is an annual herb growing in fertile sandy loam or humus loam as a wild plant and well described in Guangxi Chinese Traditional Medicine Records [34]. It has anticancer and anti-inflammatory immune function in clinical treatment [3, 4, 6, 11, 30]. In the long history of Chinese medical practice, OD has been used to treat nasopharyngeal carcinoma [3], non-small cell lung carcinoma [4, 5], acute non-lymphoid leukemia [6], gastrointestinal carcinomas [7, 9], and hepatocellular carcinoma [8]. VPC is a perennial herb growing in well-draining sandy clay loam as a wild plant and has major functions as anti-inflammatory, resolving toxin and dispersing swelling in Chinese medical history [1, 2]. Many experiments were conducted to study these two herbs on cancer cells [35-38]. OD extract, for instance, was shown to have cytotoxic effects in ovarian cancer cells [35], human breast cancer cells [36], hepatocellular carcinoma HepG2 cells [37], and leukaemic cells [38]. Some mechanisms were reported on *in vitro* studies of both OD & VPC [10, 39-42], especially VPC. The present investigation was to evaluate the antiproliferative potential possessed by the 70% ethanol extract of the whole plant of OD and VPC against various human cell lines, including four cancer cell lines and two non-cancer cell lines.

In this study, we demonstrated that the ethanol extracts of OD and VPC were toxic to several cancer cell lines. The concentrations of growth inhibition at 50% (IC_{50}) ranging from 90.69 to 225.02 μg/ml after 72-h incubation, showing the ethanol extracts of OD and VPC exhibited potent antiproliferative activity against all cancer cell lines tested (Table **1**, Figures **1**, **2**). This revealed that both OD and VPC have broad cytotoxic effect on cancer cells and showed selective cytotoxicity among different cell lines. Interestingly, although VPC is well known for its major functions as anti-inflammatory and resolving toxin but not as an antitumor agent, it also showed potent cytotoxicity in the cell lines tested, with comparable effects to OD. Both ethanol extracts of OD and VPC specifically inhibited the growth of the human pancreatic cell line MiaPacA-2 and significantly inhibited the growth of the other cancer cells. OD and VPC both had a lesser effect on the non-cancer cell line NIH/3T3 but showed only a slightly less effect on the human embryonic kidney cell line HEK293 over a wide range of concentrations. OD had significantly less cytotoxicity than VPC in NIH/3T3 but significantly more potent cytotoxicity than VPC in HEK293 (Table **1**, Figures **1A**, **B** and **2**). We used one P-gp overexpressing drug resistant human epidermoid carcinoma cell line, KB-C2, together with KB-3-1, and used P-gp subtrates, colchicine, paclitaxel and vinblastine, compared to OD and VPC. As shown in Table **2** and Figure **3C** and **D**, KB-C2 cell line showed high resistance to colchicine, paclitaxel and vinblastine compared to the parental KB-3-1 cell line. KB-C2 cells did not confer resistance to VPC (1.23-fold resistance compared to the control cells KB-3-1, but $P > 0.05$) (Table **2**, Figure **3C** and **D**). Interestingly, KB-C2 cells were even more sensitive to OD (0.59-fold resistance compared to the control cells KB-3-1, $P<0.05$). These results suggest that OD and VPC may be good for the treatment of drug resistant cancer cell lines. Further study is needed to use other resistant cell lines in order to verify these results.

To conclude, the ethanol extracts of OD and VPC effectively inhibited the growth of all the four cancer cell lines, showing especially potent cytotoxicity in cancer cells MiaPacA-2. OD and VPC have less cytotoxicity in non-cancer cell lines NIH/3T3 and HEK293 cells. VPC also showed relatively less cytotoxic effect in KB-C2 compared to KB-3-1 cells while OD is a more potent inhibitor to KB-C2 cells than to KB-3-1 cells. Hence, this study has revealed remarkable anticancer potential of OD and VPC and they deserve further investigations. Future studies should be aimed to separate and isolate the active ingredients from the ethanol fraction of OD and VPC and test the anti-cancer activity of the active ingredients. In addition, study using an *in vivo* tumor xenograft model for these two extracts as well as their active components will also be a promising modality.

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