

## Anti-proliferative and antioxidant activities of *Saposhnikovia divaricata*

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**Abstract.** The dry root of *Saposhnikovia divaricata* (Turcz.) Schischk. (SD, syn. *Ledebouriella divaricata* (Turcz.); Umbelliferae), Siler, a perennial herb of the carrot family, is also known as Fang Feng in traditional Chinese herbal medicine. It is a herbal ingredient included in many poly-herb formulae. This study investigated the *in vitro* anti-proliferative, antioxidant and anti-inflammatory activities of the SD extract (1 g/10 ml 70% ethanol). IC<sub>50</sub> (50% inhibition) is estimated at 1/300, 1/1400, 1/250 and 1/600 dilutions, for the K562, HL60, MCF7 and MDA-MB-468 cell lines, respectively. The combination of non-cytotoxic concentrations of SD with chemotherapeutic drugs such as camptothecin or paclitaxel showed additive anti-proliferative effects on K562, HL60 and MCF7 cells, and antagonistic effects on MDA-MB-468 cells. At a dilution of 1/2000, SD induced a differentiation of 17.5±2.5% in HL60 cells along the granulocyte lineage compared to 2.8±0.8% in the untreated controls, but not along the monocyte/macrophage lineage. At non-cytotoxic 1/10000, 1/5000 and 1/2000 dilutions, the SD extract did not affect nitric oxide (NO) production by non-stimulated RAW 264.7 cells, but dose-dependently and significantly reduced NO production by lipopolysaccharide (LPS)-activated RAW 264.7 cells. RT-PCR analyses showed that SD at a dilution of 1/2000 did not affect TNF $\alpha$ , IL-1 $\beta$ , iNOS and COX-2 mRNA expression in RAW 264.7 cells compared to the unstimulated controls, but significantly reduced ( $p < 0.05$ ) iNOS and its mRNA expression in LPS-activated cells. It is concluded that the SD ethanol extract possesses strong anti-proliferative properties against several human tumor cell lines, a mild granulocyte differentiation inducing property on HL60 cells, and potent antioxidant, anti-inflammatory and protective properties on LPS-activated RAW 264.7 cells. Further research is required in order to

identify the major ingredients present in the *Saposhnikovia divaricata* root and rhizome showing the observed activities.

### Introduction

The dry root of *Saposhnikovia divaricata* (Turcz.) Schischk. (SD, syn. *Ledebouriella divaricata* (Turcz.); Umbelliferae), Siler, a perennial herb that belongs to the carrot family, is also known as Fang Feng in traditional Chinese herbal medicine, and Bofu in Japan. It is a herbal ingredient included in many poly-herb formulae used to dispel 'wind', induce diaphoresis, alleviate rheumatic conditions and relieve spasm. It has also been reported to possess analgesic, antipyretic and antibiotic properties (1). Active ingredients in the dry root include essential oils, mannitol, 'bitter' glycoside, chromones, coumarins, and polyacetylenes (2-4). Despite its long historical usage in Chinese medicine, relatively little scientific research has been conducted on this herb. Several studies have reported the anticancer and antioxidant properties of this herb (3-7). This study investigated the *in vitro* anti-proliferative, antioxidant and anti-inflammatory activities of the ethanolic extract of SD.

### Materials and methods

**Plant material and preparation of the extract.** SD dry roots were purchased from Chinese herbal stores in Vancouver. Voucher samples are stored in the authors' laboratory. The dry root pieces were pulverized in a blender into fine powder. For cell culture studies, 1 g of the powder was extracted with 10 ml 70% ethanol for two hours at 55°C. The suspension was centrifuged at 5,000 x g for 10 min and the supernatant was used as stock and further diluted to 1/200-1/10000 for cell culture studies.

**Chemicals and reagents.** Unless specified, all chemicals were of reagent grade purchased from Sigma-Aldrich of Canada (Oakville, Ontario, Canada). The chemotherapeutic agents, camptothecin (CAM, Cat. No. C9911) and paclitaxel (PTX, Cat. No. T7402) were dissolved in dimethyl sulfoxide (DMSO) as 1 mM stock and further diluted to the desired concentrations with culture medium. Other chemicals used were 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox, Cat. No. 39,192-1), 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS, Cat. No. A1888),

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lipopolysaccharide (LPS, Cat. No. L2630), phorbol-12-myristate-13-acetate (PMA, Cat. No. P8139), dimethyl formamide (DMF), nitro-blue tetrazolium (NBT, Cat. No. N6876).

*High performance liquid chromatography (HPLC) profiling.* Ethanol extracts of SD (70%) were prepared according to the prescribed method. British Columbia's Institute of Technology Forensic Science Center performed the HPLC fingerprint. HPLC analysis was carried out using an Agilent 1100 HPLC equipped with a diode array detector and readings were taken at 280 nm.

*Antioxidant activity - Trolox equivalent antioxidant capacity (TEAC).* The test is based on the reduction of ABTS radical cation by antioxidants (8,9). Trolox (25 mM) was prepared in ethanol for use as a stock standard. Fresh working standards were prepared daily by dilution with distilled water. Stock solution of ABTS radical cation was prepared by mixing ABTS (7 mM) with 2.45 mM potassium persulfate in water. The mixture was kept for 12-24 h at ambient temperature in the dark until the reaction was complete and the absorbance became stable. To prepare an ABTS<sup>•+</sup> working solution, the ABTS<sup>•+</sup> stock solution was diluted with water to an absorbance of  $0.700 \pm 0.02$  at 734 nm. A stock solution of Trolox (1 mM) was prepared with water. For the photometric assay, 1 ml ABTS<sup>•+</sup> working solution and 10  $\mu$ l Trolox or test solution were mixed for 45 sec and measured immediately after 1 min at 734 nm. The antioxidant activity of the test substances was calculated by determining the decrease in absorbance using the following equation: Percentage antioxidant activity =  $\{(E[\text{ABTS}^{\bullet+}] - E[\text{standard}])/E[\text{ABTS}^{\bullet+}]\} \times 100$ . Trolox equivalents were estimated by the linear interpolation of the antioxidant activity from the Trolox standards.

*Cell lines and culture conditions.* Human mammary adenocarcinoma MCF7 and MDA-MB-468, human promyelomonocytic HL60 cells, and the murine RAW 264.7 macrophage/monocyte cell line were purchased from the American Type Culture Collection (Rockville, MD, USA). The human leukemia cell line, K562, was a gift from Dr A.J. Tingle, BC's Child and Family Research Institute. MDA-MB-468 cells were cultured in L15 medium, HL60 cells were cultured in Iscove's medium, and MCF7, K562 and RAW264.7 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50  $\mu$ g/ml gentamycin. The cultures were maintained in a humidified 5% CO<sub>2</sub> incubator at 37°C except for MDA-MB-468, which was maintained in an incubator with air only. Cells were subcultured every 3-4 days to maintain logarithmic growth.

*Cell proliferation assay.* For testing, tumor cells were cultured in 96-well plates. Starting cell numbers were  $2.5 \times 10^4$  cells per well per 200  $\mu$ l for K562, and  $10^4$  cells for MCF7 and MDA-MB-468. For HL60 cells, the starting cell number was  $10^5$  cells per well per ml in 24-well plates. After the cells had stabilized overnight, triplicate (duplicate for HL60) samples of cells were treated with culture medium containing different concentrations of SD alone, or in combination with

different concentrations of the cancer chemotherapeutic agents, CAM or PTX. Cell counting was set at 2 days after treatment for K562, 3 days for HL60 and 4 days for MCF7 and MDA-MB-468. Cell numbers were determined by hemocytometer counting and viability was assessed by the trypan blue dye exclusion test. Cell counts in the samples treated with the test compounds were normalized to a percentage of the control and the means and standard errors (SEM) were calculated from at least three independent experiments (10).

The combined effect of SD and the anti-neoplastic agents was expressed as combination index (CI) based on median effect calculation (11). This method involved plotting dose-effect curves for each agent (SD, PTX or CAM) and the multiply diluted fixed ratio combination of agents. A combination index is determined with the equation:  $CI = (D)a / (Dx)a + (D)b / (Dx)b + a[(D)a(D)b / (Dx)a(Dx)b]$  where  $(Dx)a$  is the dose of agent *a* required to produce a 50% inhibitory (IC<sub>50</sub>) effect alone, and  $(D)a$  is the dose of agent *a* required to produce the same IC<sub>50</sub> in combination with  $(D)b$ . Similarly,  $(Dx)b$  is the dose of agent *b* required to produce the same effect in combination with agent  $(D)a$ . If the agents are mutually exclusive (e.g. similar mode of action), then *a* is 0 (i.e. CI is the sum of 2 terms); if the agents are mutually non-exclusive (e.g. independent mode of action), *a* is 1 (i.e. CI is the sum of 3 terms). For simplicity, however, the third term of the Chou and Talalay equation is usually omitted, and thus, the mutually exclusive assumption or classic isobologram is indicated. The CI is additive if it is equal or close to 1, synergistic if it is <1 and antagonistic if it is >1 (12). For this study, a dose combination of SD with CAM or PTX with a CI of 0.9-1.1 was considered additive, a CI below 0.9 was synergistic and a CI above 1.1 was antagonistic.

#### *Cell differentiation assay*

*Differentiation into granulocyte lineage by NBT reduction assay.* Duplicate 50  $\mu$ l aliquots of HL60 cells treated with the different dilutions of SD were incubated with an equal volume of phenol red free RPMI containing 200 ng/ml of PMA and 0.2% NBT at 37°C for 45 min in a flat bottom 96-well culture plate. For each preparation, 200 cells were examined microscopically in order to determine the proportion of cells containing blue-black formazan granules, indicative of the ability of HL60 to generate the superoxide anion during an induced respiratory burst. In addition, cytocentrifuge smears of control and treated cells were stained with Giemsa and analyzed by microscopy in order to allow the observation of granulocytic features i.e. a multilobular nucleus and prominent cellular indentation. A positive control of differentiation to the granulocytes was achieved by the addition of 100 mM DMF. The results were expressed as a percentage of positive cells (13,14).

*Differentiation into macrophage/monocyte lineage by non-specific esterase (NSE) activity assay.* Standard NSE staining (15) was performed on duplicate microscope slides from samples prepared by a cytocentrifuge. Two hundred cells were examined microscopically in order to determine the proportion of positive cells (brownish red cytoplasm). Cells treated with 50 ng/ml PMA were used as the positive control.

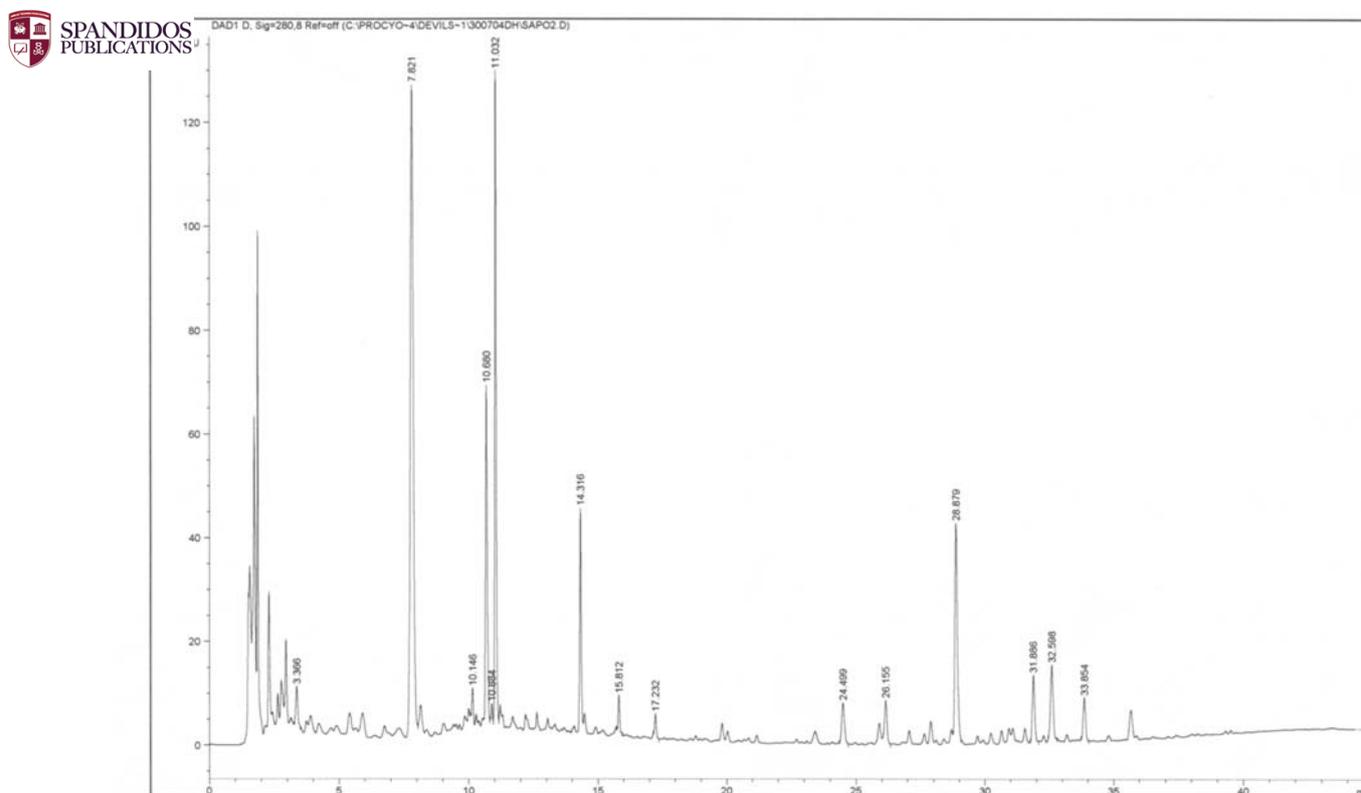


Figure 1. High performance liquid chromatography profile of *Saposhnikovia divaricata* extract at 280 nm.

**Inhibition of LPS-activated nitric oxide (NO) production by RAW264.7 cells.** RAW 264.7 cells were cultured in 24-well plates ( $1 \times 10^6$  cells/ml/well, NUNC culture plates) with phenol red free RPMI medium supplemented with 5% FBS. On the following day varying dilutions of SD were added alone, or in combination with  $1 \mu\text{g/ml}$  LPS, in fresh medium to the RAW264.7 cells. After 48-h incubation at  $37^\circ\text{C}$ , the amount of NO released into the culture supernatant was determined with Griess reagent and compared to sodium nitrite standards by absorbance at 550 nm. Cell proliferation and viability at the end of the experiment were determined by staining the cells with  $50 \mu\text{g/ml}$  neutral red for one hour (16,17).

**RT-PCR.** Three ml of RAW 264.7 cells at  $2 \times 10^6$  cells/ml were cultured in 6-well culture plates in DMEM supplemented with 10% FBS, 2 mM L-glutamine and  $50 \mu\text{g/ml}$  gentamycin at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  in a humidified incubator. After 48 h, the cells were stimulated with 1/2000 and 1/5000 dilutions of SD alone or in combination with  $1 \mu\text{g/ml}$  LPS in fresh culture medium. After 5 h, RNA was isolated with TRIzol reagent (Invitrogen, Burlington, Ontario, Canada). One microgram total RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase according to the manufacturer's protocol (No. 18064, Invitrogen) at  $42^\circ\text{C}$  for 50 min. PCR was performed with 100 ng of the cDNA template in  $25 \mu\text{l}$  volumes containing 1X reaction buffer, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTPs, 0.5U Platinum Tag DNA polymerase (No. 10966, Invitrogen), and 0.4  $\mu\text{M}$  of the following primers: IL-1 $\beta$ , 5'- TTT ACG GAC CCC AAA AGA TG -3' and 5'- AGA AGG TGC TCA TGT CCT CA -3'; TNF- $\alpha$ , 5'- TCT

CAT CAG TTC TAT GGC CC -3' and 5'- GGG AGT AGA CAA GGT ACA AC -3'; iNOS, 5'- CCC TTC CGA AGT TTC TGG CAG CAG -3' and 5'- GGC TGT CAG AGA GCC TCG TGG CTT TGG -3'; COX-2, 5'- CCC CCA CAG TCA AAG ACA CT -3' and 5'- CCC CAA AGA TAG CAT CTG GA -3'; and G3PDH, 5'- TGA AGG TCG GTG TGA ACG GAT TTG GC -3' and 5'- CAT GTA GGC CAT GAG GTC CAC CAC -3' (18-20).

After an initial denaturation for 2 min at  $94^\circ\text{C}$ , 30-35 cycles of amplifications ( $55^\circ\text{C}$  for 40 sec,  $72^\circ\text{C}$  for 1 min, and  $94^\circ\text{C}$  for 1 min) were performed followed by a 7-min extension at  $68^\circ\text{C}$  in the DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT, USA). A  $10 \mu\text{l}$  aliquot from each PCR reaction was electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. PCR products of mRNA expression were semi-quantitatively measured using the GelDoc system with a CCD camera (BioRad, Molecular Analyst), and the relative IL-1 $\beta$ , TNF- $\alpha$ , iNOS and COX-2 signals were normalized against the housekeeping gene, G3PDH. Data were expressed as a ratio of G3PDH.

**Statistical analysis.** Data are presented as the means  $\pm$  SEM from at least three independent experiments. The Student's two-tailed unpaired t-test was used in a statistical comparison of two groups. Differences were considered significant when  $p < 0.05$ .

## Results

**HPLC profile.** Fig. 1 shows the HPLC profile of the SD extract at 280 nm. It is clear that the presence of multiple

Table I. IC<sub>50</sub> estimation of the SD extract, CAM or PTX and their combined anti-proliferative effects on cancer cell lines.

Agent	IC <sub>50</sub> (mean ± SEM)				% Reduction vs single agent; p-value
	K562	HL60	MCF7	MDA-MB-468	
<b>A</b>					
SD dilution	1/(300±30)	1/(1400±200)	1/(250±10)	1/(600±30)	
CAM μM	0.28±0.02	0.28±0.07	-	-	
PTX nM	-	-	6±0.39	4.82±0.13	
<b>B<sup>a</sup></b>					
SD 1/1000 + CAM	0.097±0.014	-	-	-	75 p<0.01
SD 1/500 + CAM	0.13±0.03	-	-	-	54 p<0.001
SD 1/2000 + CAM	-	0.064±0.027	-	-	78.5 p<0.05
SD 1/500 + PTX	-	-	4.93±0.39	-	17.8 NS
SD 1/300 + PTX	-	-	1.53±0.54	-	74.5 p<0.001
SD 1/1000 + PTX	-	-	-	2.32±0.24	51.9 p<0.001
<b>C<sup>b</sup></b>					
SD + CAM 0.05 μM	1/(300±30)	-	-	-	NS
SD + CAM 0.1 μM	1/(400±40)	-	-	-	NS
SD + CAM 0.05 μM	-	1/(1650±250)	-	-	NS
SD + CAM 0.1 μM	-	1/(1450±150)	-	-	NS
SD + PTX 1 nM	-	-	1/(350±40)	-	NS
SD + PTX 1 nM	-	-	-	1/(400±10)	NS
SD + PTX 2.5 nM	-	-	-	1/(450±40)	NS

SD, *Saposhnikovia divaricata*; CAM, camptothecin; PTX, paclitaxel; B<sup>a</sup>, the concentration of SD was fixed, as indicated, in combination with varying concentrations of CAM (0.05 to 0.5 mM), or PTX (1 to 10 nM); C<sup>b</sup>, the concentration of CAM or PTX was fixed, as indicated, in combination with varying dilutions of SD between 1/200-1/1000, 1/1000-1/10,000, 1/100-1/2000 and 1/100-1/2000 dilutions for K562, HL60, MCF7 and MDA-MB468 cells, respectively.

peaks in the HPLC profile suggests that there are numerous compounds present in the ethanolic SD extract.

**Anti-proliferative activity.** Table IA shows that the SD extract has differential anti-proliferative activities on the leukemia and breast cell lines tested. Among these cell lines, HL60 was the most susceptible to SD while MCF7 the least. IC<sub>50</sub> (50%) inhibition was estimated at 1/300, 1/1400, 1/250 and 1/600 dilutions, for K562, HL60, MCF7 and MDA-MB-468 cell lines, respectively. IC<sub>50</sub> of CAM alone on K562 and HL60 cells was 0.28±0.02 μM and 0.28±0.07 μM, respectively. IC<sub>50</sub> of PTX alone on MCF7 and MDA-MB-468 cells was 6±0.39 and 4.82±0.13 nM, respectively.

Table IB shows the IC<sub>50</sub> values of CAM or PTX in combination with fixed dilutions of SD. It was noted that 1/1000 and 1/500 dilutions of SD effectively reduced the IC<sub>50</sub> of CAM to 0.097±0.014 and 0.13±0.02 μM, respectively, from the IC<sub>50</sub> of 0.28±0.02 μM when CAM was used alone on K562 cells. This represents a 75 and 54% reduction in the CAM alone IC<sub>50</sub> dosage on these cells. Similarly, SD at a dilution of 1/2000 (IC<sub>50</sub> at a dilution of 1/1400) reduced the IC<sub>50</sub> of CAM to 0.064±0.027 μM (78.7% reduction) from 0.28±0.067 μM when CAM was added alone in HL60 cells.

Table IB shows that the IC<sub>50</sub> values of PTX were reduced when non-cytotoxic doses of SD combined with PTX were added to the breast carcinoma cell lines. SD at 1/500 and 1/300 dilutions combined with PTX effectively reduced the IC<sub>50</sub> of PTX from 6±0.39, when PTX was added alone, to 4.93±0.39 and 1.53±0.54 nM, respectively, representing a 17.8 and 74.5% reduction on IC<sub>50</sub> values in the MCF7 cells. The IC<sub>50</sub> of PTX was at 2.32±0.24 nM on MDA-MB-468 cells. It was reduced by 51.9%, when added in combination with a 1/1000 dilution of SD. In contrast, Table IC shows that non-cytotoxic doses of either CAM or PTX when combined with SD did not effectively reduce the IC<sub>50</sub> values of SD in the four cell lines. However, in the MDA-MB-468 cells, the inclusion of 1 and 2.5 nM PTX increased the IC<sub>50</sub> values of SD from a dilution of 1/(600±30) with SD added alone to 1/400 and 1/450, respectively, thus supporting the 'antagonistic' effects observed by CI estimation.

Table IIA shows that for K562 cells, only a 1/1000 dilution of SD combined with 0.1 μM CAM has an 'additive' effect with CI = 1.06, while a 1/1000 dilution of SD combined with a lower concentration of CAM at 0.05 μM has an 'antagonistic' anti-proliferative effect with CI = 1.26. Combining a higher concentration of SD at a 1/500 dilution with either

SPANDIDOS PUBLICATIONS Combination index estimation by median effect plots extract alone or in combination with CAM or PTX.

SD dilution	Combination index	
	CAM 0.05 $\mu$ M	CAM 0.1 $\mu$ M
<b>A. K562</b>		
1/1000 dilution	1.26	1.06
1/500 dilution	1.38	1.18
<b>B. HL60</b>		
1/2000 dilution	1.07	1.2
	PTX 1 nM	PTX 2.5 nM
<b>C. MCF7</b>		
1/500 dilution		1.56
1/300 dilution		0.99
<b>D. MDA-MB-468</b>		
1/1000 dilution	2.07	1.29

SD, *Saposhnikovia divaricata*; CAM, camptothecin; PTX, paclitaxel.

0.05 or 0.1  $\mu$ M CAM is 'antagonistic' with a CI of 1.38 and 1.18, respectively. Table IIB shows that a 1/2000 dilution of SD combined with 0.05  $\mu$ M CAM has an 'additive' anti-proliferative effect on HL60 cells with a CI of 1.07. The combination of SD at a 1/2000 dilution with 0.05  $\mu$ M CAM produced an 'antagonistic' effect with a CI of 1.2. Table IIC shows that for MCF7 cells, a 1/500 dilution of SD and 2.5 nM of PTX is 'antagonistic' with CI = 1.56. However, a higher concentration of SD at a 1/300 dilution and 2.5 nM of PTX is 'additive' in their combined anti-proliferative effect with CI = 0.99. Table IID shows that for MDA-MB-468 cells, a 1/1000 dilution of SD combined with 1 or 2.5 nM PTX has CI of 2.07 and 1.29, respectively and thus is 'antagonistic' in its combined anti-proliferative effect.

**HL60 cell differentiation.** Fig. 2 shows that the SD extract at a dilution of 1/2000 induced a 17 $\pm$ 2.5% HL60 cell differentiation along the granulocyte lineage, significantly higher than the 2.8 $\pm$ 0.8% in the control (p<0.05). It did not induce HL60 cell differentiation along the monocyte/macrophage lineage.

**Antioxidant activities.** Cell free TEAC assay by the ABTS hydroxyl radical scavenging method compared to Trolox standards showed that the undiluted SD ethanolic extract has substantial antioxidant activity equivalent to 6.9 and 7.6  $\mu$ M Trolox, respectively, for two separate batches of SD dry roots.

Fig. 3A shows the effect of SD on LPS-activated NO production by RAW 264.7 cells. The basal level of NO expressed by the nitrite concentration in the culture medium was <2  $\mu$ M. SD at non-cytotoxic 1/10000, 1/5000, 1/2000 and 1/1000 dilutions to RAW 264.7 cells did not induce NO

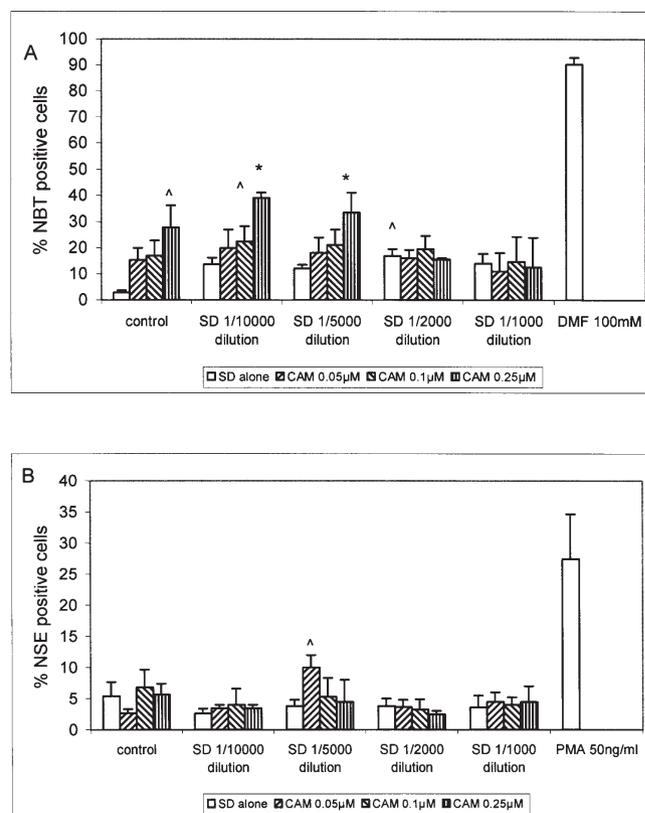


Figure 2. Effects of the *Saposhnikovia divaricata* (SD) extract on the differentiation of HL60 leukemia cells. HL60 cells (1 ml) ( $1 \times 10^5$ /ml) were transferred to each well of 24-well culture plates. After overnight culture, fresh media containing 1/1000, 1/2000, 1/5000 and 1/10000 dilutions of SD were added and the cells were cultured for an additional 3 days and then assessed for their nitroblue tetrazolium (NBT) reduction capacity and non-specific esterase (NSE) activity. Panel A shows the effects of the SD extract on HL60 cell differentiation along the granulocyte lineage by increased presence of NBT-positive cells. Panel B shows the effects of the SD extract on HL60 cell differentiation along the monocyte/macrophage lineage by the increased presence of NSE positive cells. Dimethyl formamide (100 mM) and phorbol-12-myristate-13-acetate (50 ng/ml) are inducers of granulocyte and monocyte/macrophage differentiation, respectively. Camptothecin is a potent anti-neoplastic agent. <sup>#</sup>p<0.05, <sup>\*</sup>p<0.01, <sup>\*</sup>p<0.001.

production in the non-stimulated cells. However, at these dilutions SD significantly reduced NO production by LPS-activated RAW 264.7 cells in a dose-dependent manner, from 47.7 $\pm$ 3.6  $\mu$ M in the cells activated by LPS alone to 31.6 $\pm$ 9.6, 30.6 $\pm$ 6, 22.9 $\pm$ 3.6 and 9.1 $\pm$ 5.3  $\mu$ M, respectively (p-value at not significant, <0.05, < 0.001, <0.001, respectively).

Viability assay by neutral red incorporation showed that SD at 1/10,000, 1/5000 and 1/2000 dilutions was not cytotoxic to the RAW 264.7 cells, although it significantly protected cell viability in the LPS-activated RAW 264.7 cells (p<0.05). SD at a dilution of 1/1000 was not cytotoxic, although it reduced cell viability in the LPS-activated cells (p<0.05) (Fig. 3B).

**RT-PCR.** Fig. 4 shows that non-cytotoxic 1/5000 and 1/2000 dilutions of SD did not affect TNF $\alpha$ , IL-1 $\beta$ , iNOS and COX-2 mRNA expression in RAW 264.7 cells compared to the un-

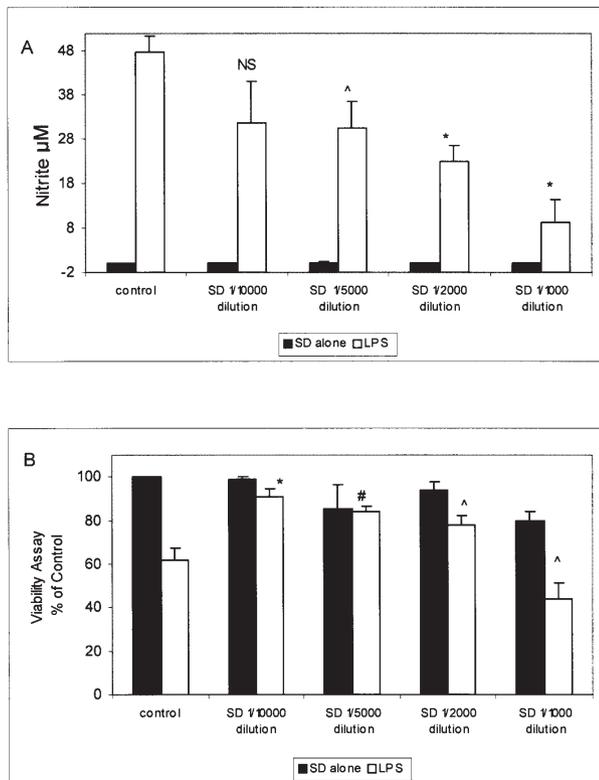


Figure 3. Antioxidant properties of the *Saposhnikovia divaricata* (SD) extract. (A) Effects of the SD extract on nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated dilutions of the SD extract alone or in combination with LPS (1 µg/ml) for 48 h. The culture supernatant was collected and analyzed for NO production as nitrite with Griess reagent. (B) Neutral red dye viability assay on RAW 264.7 cells shows that the dilutions of the SD extract used are not cytotoxic on these cells. <sup>^</sup>p<0.05, <sup>#</sup>p<0.01, <sup>\*</sup>p<0.001.

stimulated controls, nor did they affect TNF $\alpha$  and IL-1 $\beta$  mRNA expression in LPS-activated cells. SD at a dilution of 1/2000 significantly reduced LPS-activated iNOS mRNA expression in LPS-activated cells (p<0.05). COX-2 mRNA expression in the LPS-activated cells was not significantly inhibited by SD at 1/5000 and 1/2000 dilutions.

## Discussion

The HPLC profile at 280nm shows that more than 16 peaks of different sizes are present in the SD ethanol extract. Due to the differences in extraction methods, and the mobile phases used, a comparison between chromatograms of other reported studies with the present one is difficult. Reported active ingredients present in the SD extract include chromones, polyacetlenes, coumarins and polysaccharides, depending upon the methods of extraction (21,22). Coumarins at high concentrations of >100 µg/ml have been reported to show *in vitro* anti-tumor activities (23). Furanocoumarins (imperatorins and deltoin) isolated from SD showed antioxidant activities through the inhibition of inducible NO synthase (iNOS) activity in LPS-activated RAW 264.7 cells (5). Polyacetlenes (panaxynol and falcariindiol) extracted from SD inhibited iNOS expression in LPS-activated C6 glioma cells and rat primary astrocytes (6,7). Panaxynol also inhibited growth, cell cycle progression and cyclin E mRNA expression in six tumor cell lines (4). In particular, in the K562 cells, the IC<sub>50</sub> of the SD ethanol extract and panaxynol was, 67±5.3 µg/ml and 62.5±5.6 µM, respectively (which is equivalent to 15.3 µg/ml with the formula weight of panaxynol estimated at 244). In this study, the IC<sub>50</sub> of the SD extract in the K562 cells was at a dilution of 1/300. With a recovery

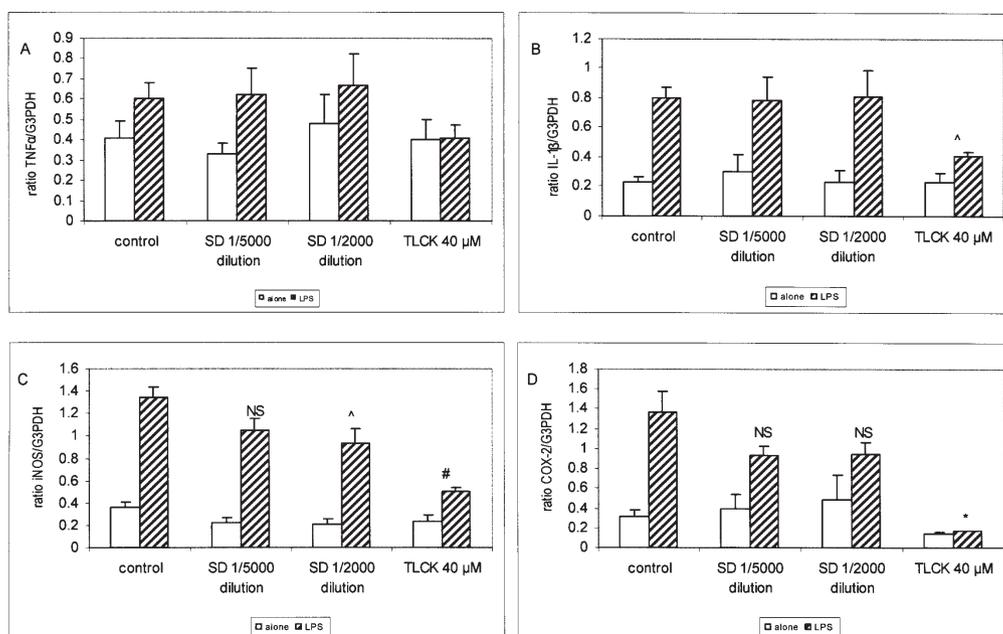


Figure 4. RT-PCR analyses of the effects of the *Saposhnikovia divaricata* (SD) extract on TNF $\alpha$ , IL1 $\beta$ , inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) mRNA expression in RAW 264.7 cells with and without LPS (1 µg/ml) activation. (A) Expression of TNF $\alpha$  relative to G3PDH. (B) Expression of IL1 $\beta$  relative to G3PDH. (C) Expression of iNOS relative to G3PDH. (D) Expression of COX-2 relative to G3PDH. Each bar represents the densitometric analysis of the gel photograph of the gene product normalized to that of the housekeeping gene, G3PDH. Results are means  $\pm$  SEM of more than three separate experiments. TLCK (40 µM) is a known inhibitor of iNOS mRNA transcription via the NF $\kappa$ B pathway. Student's t-test analyses show that SD at a dilution of 1/2000 significantly attenuated iNOS mRNA expression in LPS-activated cells. <sup>^</sup>p<0.05, <sup>#</sup>p<0.01, <sup>\*</sup>p<0.001.

 SPANDIDOS PUBLICATIONS. lids from the 70% ethanol extract of SD at ~15%, starting extraction at 0.1 g/ml of 70% ethanol, a 1/300 dilution would be equivalent to 50  $\mu$ g/ml in the culture medium, thus comparable to the IC<sub>50</sub> at 67  $\mu$ g/ml observed by Kuo *et al* (4).

Our *in vitro* study showing the ethanolic SD extract with potent anti-proliferative activities on human leukemia and breast carcinoma cell lines adds to the list of tumor cell lines, which have previously been reported to be inhibited by the SD extract, including K562, Raji, Wish, HeLa, Calu-1 and Vero, as well as the pure panaxynol compound (4). Despite the fact SD has been shown to have anti-proliferative activities on tumor cells, and is often included as a component herb in poly-herbal decoction taken by many, including cancer patients in Asia, North America and Europe, *in vitro* and *in vivo* information on the pharmacological interactions between SD and conventional therapeutic drugs is still limited. The crude water or ethanol extract of SD did not inhibit cytochrome P450 3A activity although furanocoumarin purified from SD showed such activity (24,25).

It has been reported that panaxynol, an ingredient in SD, blocks gene expression of cyclin E, a key regulatory event leading to the G1/S boundary (4). The chemotherapeutic agent, camptothecin, blocks topoisomerase I activity in the S phase, while paclitaxel blocks mitosis by interfering with tubulin stabilization. Therefore, we further studied combinations of non-cytotoxic dosages of SD with CAM or PTX in order to see if they could produce additive, synergistic or antagonistic anti-proliferative effects on the four tumor cell lines tested here. CI estimation based on median effect plots by Chou and Talalay demonstrated that the effects are cell line-dependent, and are also dependent on the doses of both the drug and SD. With the doses of SD and CAM and PTX tested, 'additive' combined anti-proliferative effects were observed in the K562, HL60 and MCF7 cells. In addition, non-cytotoxic doses of SD effectively reduced the IC<sub>50</sub> values of CAM in K562 and HL60, and PTX in MCF7 and MDA-MB-468 cells from the values when these drugs were tested alone on these cells. This observation is of potential clinical significance as it suggests that the co-administration of non-cytotoxic doses of SD with lower doses of chemotherapeutic drugs such as CAM and PTX, could achieve a similar anti-proliferative effect as those administered alone at higher doses, which can cause toxic side-effects. Herbs, including food vegetables, are generally considered a good source of antioxidants and serve as chemopreventive agents against oxidative stress leading to cancer and heart diseases. This study shows that the SD ethanol extract demonstrated significant antioxidant activities in hydroxyl radical scavenging by the cell free TEAC assay. In the *in vitro* RAW 264.7 cell model, non-cytotoxic concentrations between 1/2000 to 1/10000 dilutions of the SD extract attenuated NO production by LPS-activated RAW 264.7 cells and protected these cells from LPS-induced cytotoxicity.

Since SD has been reported to have anti-pyretic effects, we therefore assessed its effect on the mRNA expression of the pro-inflammatory mediators, TNF $\alpha$ , IL-1 $\beta$ , iNOS and COX-2 gene expression, in LPS-activated RAW 264.7 cells by RT-PCR. Our data show that the SD extract at a dilution of 1/2000 did not affect the mRNA expression of the TNF $\alpha$  and IL-1 $\beta$  cytokines. However, the expression of iNOS and

COX-2 mRNA was significantly and non-significantly attenuated in the LPS-activated RAW 264.7 cells, respectively. Thus the inhibition of iNOS mRNA expression correlated with the NO production detected in the RAW 264.7 cells. We were unable to test doses higher than the 1/1000 dilution of the SD 70% ethanol extract due to its toxicity on RAW 264.7 cells. It is therefore possible that SD has significant anti-inflammatory activity when tested *in vivo*.

Although direct comparisons are not available, our data on the antioxidant activities of the SD ethanol extract agree with the reported iNOS synthase inhibitory effects of the SD-derived furanocoumarins (imperatorin and deltoin), poly-acetylenes (panaxynol and faltarindiol), and faltarindiol on RAW 264.7, C6 glioma cells, and rat primary astrocytes, respectively (5-7). The mechanism of inhibition was attributed to the attenuation of IKK and JAK activation leading to the blockade of the nuclear translocation of NF- $\kappa$ B and Stat-1, consequently eliminating the induction of iNOS. Derivatives of coumarins have been reported to affect 5-lipoxygenase and cyclooxygenase activity differently (26). Although our study did not identify which component(s) in the SD extract contribute to the attenuation of COX-2 mRNA expression in RAW 264.7 cells, the observation substantiates the traditional usage of SD for anti-inflammatory applications.

In conclusion, our present *in vitro* study of the SD ethanol extract showed that the herb possesses strong anti-proliferative properties against several human tumor cell lines, and also showed additive effects when combined with paclitaxel and camptothecin at certain concentrations. It also demonstrated a mild granulocyte differentiation inducing property on HL60 cells. These activities add to the potential use of SD for cancer therapy in addition to its traditional usage in traditional Chinese medicine. The significant antioxidant and anti-inflammatory activities observed in the SD extract also lend support to its inclusion in many traditional Chinese herbal formulations.

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