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The fractionated *Toona sinensis* leaf extract induces apoptosis of human ovarian cancer cells and inhibits tumor growth in a murine xenograft model

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Abstract

Objective. Aqueous extract from the leaves of *Toona sinensis* Roem. has been shown to have an anti-proliferative effect on human lung cancer cells. In this study, we analyzed the anti-cancer activity/effect of different extraction fractions of the extract from *T. sinensis* leaves on ovarian cancer cells.

Methods. Cell viability was determined by XTT cell proliferation assay and cell survival assay. Apoptotic effect was detected by morphological analysis and immunoblotting. Cell cycle effect was evaluated by flow cytometry analysis and immunoblotting. In vivo therapeutic effect was evaluated by the subcutaneous inoculation of SKOV3 cells in nude mice (Foxnlnu/Foxnlnu) model.

Results. TSL2 of *T. sinensis* was more cytotoxic than other fractions and exhibited selectivity for ovarian cancer cell lines. TSL2 arrested SKOV3 ovarian cancer cells at the G2/M phase and induced cancer cells go through apoptotic pathway. Ex vivo xenograft study of nude mice showed that intraperitoneal injection of TSL2 was able to suppress the proliferation of ovarian cancer cells without significant nephrotoxicity, liver toxicity, or bone marrow suppression.

Keywords: *Toona sinensis*; Ovarian cancer; Apoptosis; Cell cycle

Introduction

Toona sinensis Roem. or *Cedrela sinensis*, commonly known as Chinese mahogany cedar or Chinese *Toona,* is a perennial deciduous tree of the family Meliaceae [1–3]. Almost every part of *T. sinensis*, including seeds, bark, root bark, petioles, and leaves, has a medicinal effect [1]. Leaves of *T. sinensis* have anti-inflammatory, antidoting, and worm-killing effects and are useful for treating enteritis, dysentery, carbuncles, boils, dermatitis, scabies, and tinea blanca, as well as for improving body health. In addition, aqueous extracts of leaves of *T. sinensis* have been used as a folk medicine for lowering blood pressure associated with diabetes. They have also been reported to enhance glucose uptake and lipolysis in 3T3-L1 adipocytes [4,5]. Poon et al. also showed that crude *T. sinensis* is able to inhibit Leydig cell steroidogenesis [6].

One recent publication has also showed that methyl gallate from *T. sinensis* can protect against hydrogen-peroxide-induced oxidative stress and DNA damage in MDCK cells [7]. The anti-cancer effects of *T. sinensis* for the most part remain unclear. Aqueous extract from the leaves of *T. sinensis* can inhibit the

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proliferation of human lung adenocarcinoma cells A549 by inhibiting the expression of cyclins D1 and E [8].

In this study, we explored the anti-cancer activity of different extraction fractions of leaf extracts from *T. sinensis* and found that TSL2, a specific fraction of the leaf extracts from *T. sinensis*, has a very potent anti-ovarian cancer activity, the mechanism of which we also addressed.

Materials and methods

Preparation and fractionation of leaf extracts of T. sinensis

The leaves used in this preparation were obtained from *T. sinensis* Roem. grown in Tuku (Yunlin County, Taiwan) and were picked and washed briskly with water. Reverse osmosis water (RO water) was added to the leaves at a proportion of 4 l of RO water to 1 kg of leaves. The mixture was heated to a boil and kept boiling for 30 min and then cooled down slowly without further boiling for 2 h at room temperature. The leaves were then removed, and the remaining liquid was concentrated over low heat and was filtered with a sieve (70-mesh). At this point, we began the fractionation. The filtered concentrate was lyophilized with a Virtis apparatus to obtain a crude extract (TSL1). Through this procedure, 100 g of leaves yielded approximately 5–6 g of lyophilized TSL1 powder. The powder was then dissolved in 99.5% ethanol and was centrifuged at 3000 rpm at 4°C (Beckman AvantiTM J-30I) for 12 min to give a supernatant portion and a precipitate portion. The supernatant portion was further lyophilized with a Virtis apparatus to obtain the lyophilized powder, TSL2. The precipitate portion was further lyophilized using a Virtis apparatus and then dissolved in 50% ethanol. The 50% ethanol solution was centrifuged at 4°C and at 3000 rpm for 12 min to give a supernatant portion and a precipitate portion. The supernatant portion was further lyophilized with a Virtis apparatus to obtain an extract in the form of lyophilized powder, TSL3. The precipitate portion was lyophilized with a Virtis apparatus and then dissolved in 25% ethanol. The 25% ethanol solution was centrifuged at 4°C and at 3000 rpm for 12 min to give a supernatant portion and a precipitate portion. The supernatant portion was lyophilized to obtain the powder form, TSL4. Finally, the precipitate portion was dissolved in RO water and centrifuged at 4°C and at 3000 rpm for 12 min to give a supernatant portion, which was then lyophilized to a powder form, TSL5.

Reagents and cell culture

Ovarian cancer cell lines SKOV3 and PA-1, cervical cancer cell lines HeLa and HeLa S3, and endometrial cancer cell line RL95-2 were purchased from the American Type Culture Collection and grown in DMEM-F12 supplemented with 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B. SKOV3 cells at various cell cycle phases were retrieved by the following procedures. First, we grew the SKOV3 cells for 2 more weeks after the cells became 100% confluence on T75 flask, and then the cells were dissociated by trypsinization and replated on 10 cm dishes. The replated SKOV3 cells were harvested at different time points to determine their cell cycle distribution patterns or treated with TSL2 the different time points after replating. Twenty-four hour after being replated, SKOV3 cells were treated with 0.4 μg/ml Nocodazole for 10 h to retrieve M-phase-enriched SKOV3 cells [9]. The cell cycle distribution of the SKOV3 cells was determined by EPICS flow cytometer (Beckman Counter).

XTT cell proliferation assay

Because only viable cells can metabolize tetrazolium salt XTT into a formazan dye, it was used in the colorimetric assay to determine cell proliferation and viability. SKOV3 cells were plated out at a density of 5000 cells/well in 96-well microtiter plates the day before treatment with different extraction fractions began. After 72 h of being treated with different extraction fractions, the cytotoxicity of TSL2 was determined using XTT colorimetric cell proliferation assay (Roche Molecular Biochemicals). Briefly, the culture medium was removed, and 100 μl of fresh culture medium and a pre-formulated 50 μl XTT mixed reagent (XTT reagent:electronically coupled reagent = 50:1) were added. The culture plate was incubated at 37°C for 4 h. Light absorbance values (OD = OD490 − OD650) were read at wavelengths of 490 nm and 650 nm using an ELISA reader for calculating the 50% inhibitory concentration (IC50), i.e., the cell concentration at which the light absorbance value of the experimental group is one half of that of the control group [10].

Morphology analysis and cell survival assay

Various cancer cell lines, grown in 6 cm petri dishes, were treated with TSL2 at different dosages (0, 10, 100, 1000 μg/ml) for 24 h, and the morphological changes were observed and photographed under an inverted microscope (Nikon TS100). For cell survival assay, the cells were treated with TSL2 at a concentration of 1, 10, 100 μg/ml for 24 h. After trypsinization and mixing with trypan blue at the ratio of 1:1, the surviving cells were counted under an inverted microscope [11].

FACS analysis

SKOV3 cells (2×10^5 cells/35 mm dish) were treated with TSL2 at different dosages for 24 h, harvested by centrifugation, washed with ice-cold PBS, and then resuspended with ice-cold 70% ethanol for 1 h. The ethanol-treated cells were spun down and stained with 40 μg/ml propidium iodide (PI) for 30 min. The DNA content was then measured by an EPICS flow cytometer (Beckman Coulter) [12].

Immunoblotting analysis

After TSL2 treatment, the cells were washed twice with PBS and lysed in EBC buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM β-mercaptoethanol, 50 mM NaF, and 1 mM Na_3VO_4) followed by centrifugation. The supernatant was collected and measured by the Bio-Rad protein assay. The detailed immunoblotting procedure was followed accordingly [11]. The proteins were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Nude mice assay

SKOV3 cells (2×10^6 cells in 0.1 ml of PBS) were injected subcutaneously at one site of the right flank of 6-week-old female nude mice (Foxnlnu/Foxnlnu).

Table 1
Cell survival assay showing the percentage of various surviving cancer cell lines after treatment with TSL2

Cell lines	Dose (μg/ml)	Survival (%)
SKOVA	1	79.0 ± 8.8
	10	55.4 ± 9.2
	100	25.9 ± 7.3
PA-1	1	67.5 ± 4.2
	10	50.4 ± 6.5
	100	15.5 ± 9.7
HeLa	1	102.4 ± 5.5
	10	101.6 ± 8.2
	100	70.4 ± 8.7
HeLa S3	1	100.6 ± 12.6
	10	100.3 ± 7.5
	100	101.0 ± 8.4
RL95-2	1	100.5 ± 5.1
	10	100.4 ± 5.3
	100	103.1 ± 9.0

The cells were treated with 1, 10, 100 μg/mL TSL2 for 24 h, and the surviving cells were determined and presented as a percentage of the untreated cells as control (the index in the control group was 100%). The experiment was repeated three times and presented as mean ± SD. One significant difference was that TSL2 was mostly cytotoxic to ovarian cell lines, PA-1 and SKOV3.

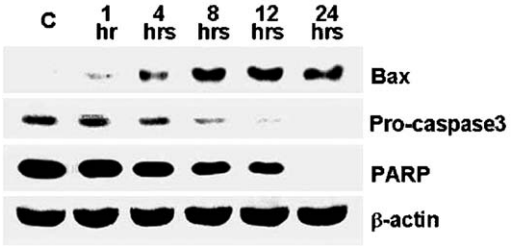


Fig. 1. Immunoblotting analysis showing the expression of the pro-apoptotic proteins in SKOV3 cells upon treatment with TSL2 at the dosage of 500 μg/ml for different time periods. (C) Untreated control; (1, 4, 8, 12, 24) treated with TSL2 for 1, 4, 8, 12, and 24 h individually.

When the subcutaneous tumors became distinctively visible (approximately 3 × 3 mm in size), usually at about 2 weeks after inoculation, the mice were randomly divided into three groups (ten mice per group) and then treated with TSL2 intraperitoneally for 7 weeks. The control group, high dose group and low dose group were given PBS, 6.7 μg/g body weight of TSL2 in PBS (a dose equals to one-fourth of the IC50 for SKOV3 cells), and 0.67 μg/g body weight of TSL2 in PBS (a dose equals to one-tenth of the dosage for high dose group), individually. Mice were injected 5 days per week, and tumor size was measured twice a week for 7 weeks. Tumor volumes were calculated by the formula of ([1/2] × [longest dimension] × [shortest dimension]2), as described previously [12]. Complete blood count of the nude mice blood was determined by Sysmex X1-2100, and the plasma BUN, Cr, AST, and ALT levels were determined by Beckman LX20.

Results

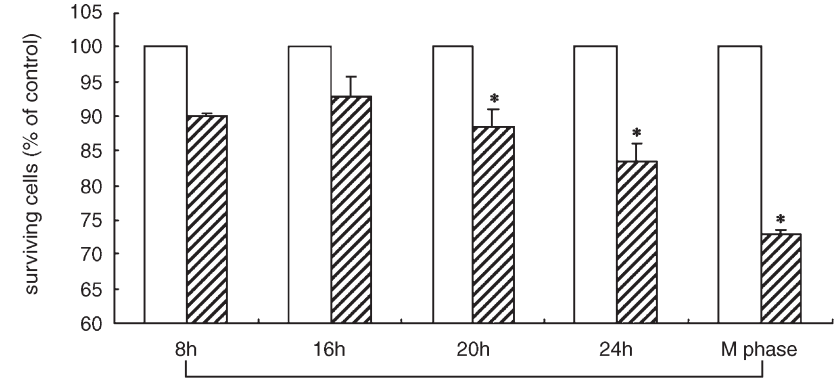
Cytotoxicity of different extraction fractions from T. sinensis leaves on various cancer cell lines

Using ovarian cancer cell line SKOV3, XTT cell proliferation assay was performed to screen the cytotoxicity of different extraction fractions from the leave extracts of *T. sinensis* on cancer cells. Fraction 2 of the leaf extracts of *T. sinensis* (TSL2) was found to have the highest dose-dependent cytotoxicity with IC50 of 26 μg/ml (data not shown). The IC50 is more than 100 μg/ml for other extraction fractions from *T. sinensis* leaves.

One previous report showed that *T. sinensis* leaf extracts were cytotoxic to human lung adenocarcinoma cells A549. In this study, we further explored the cytotoxicity of TSL2, which had the highest anti-ovarian cancer cell activity among different extraction fractions from the leave extracts of *T. sinensis*, on different cancer cells derived from ovarian cancer (PA-1 and SKOV3), cervical cancer (HeLa and HeLa S3), and endometrial cancer (RL95-2). The growth inhibition for various cancer cell lines, determined by cell survival assay, is shown in Table 1. Twenty-four hours after treatment with 1, 10, and 100 μg/ml of TSL2, we found that TSL2 had the most potent activities against ovarian cancer cells PA-1 and SKOV3, with IC50 of 11.0 and 26.4 μg/ml, respectively. In contrast, under the same dosage and time period, TSL2 had a very low cytotoxicity on cervical cancer cells HeLa and HeLa S3 as well as endometrial cancer cells RL95-2. Therefore, we chose SKOV3, which was derived from the most common type ovarian cancer, epithelial ovarian cancer, to further study the antitumor mechanism of TSL2 in vitro and in vivo.

Induction of pro-apoptotic and anti-apoptotic genes upon treatment with TSL2 on SKOV3 cells

To dissect the detailed mechanism for TSL2-induced apoptotic cell death, the pro-apoptotic and anti-apoptotic genes were explored for their possible involvement in the cytotoxicity of *T. sinensis*. TSL2 was able to induce the expression of Bax at 1 h after treatment and the proteolytic cleavage of procaspase 3 and PARP at the dosage of 500 μg/ml (Fig. 1).



% G0/G1	92.5	69.4	46.6	46.9	33.1
%S	7.2	21.6	45.5	18.3	20.5
%G2+M	0.3	5.1	7.9	34.8	46.4
	G1		S	G2/M	

Fig. 2. TSL2 was more cytotoxic to M-phase-enriched SKOV3 cells than cells at other phases. Various cell-cycle-phase-enriched SKOV3 cells (see Materials and methods) were treated with TSL2 at the dosage of 100 μg/ml, and the surviving cells were determined by cell survival assay at 4 h after treatment. The experiment was repeated three times and presented as mean ± SD. *Significantly different from the control group ($P < 0.05$).

Table 2
TSL2 arrested SKOV3 cells at G2/M phase

Dosage	Percentage (mean ± SD) of ovarian cancer cells at each cell cycle phase after treatment with TSL2 for 24 h		
	G1	S	G2M
0 μg/ml	59.0 ± 0.2	27.3 ± 0.9	13.8 ± 0.7
10 μg/ml	53.7 ± 1.3 *	32.1 ± 1.4	14.2 ± 1.1
100 μg/ml	54.7 ± 1.6 *	20.9 ± 0.9 *	25.0 ± 2.5 *

SKOV3 cells were treated with TSL2 at different dosages for 24 h and cell cycle distribution was determined by flow cymmetry. The experiment was repeated four times and presented as mean ± SD.
* Significantly different from the control group ($P < 0.05$).

The cytotoxicity of TSL2 on SKOV3 cells at different cell cycle phases

Cell-cycle-phase-enriched SKOV3 cells were achieved by density arrest (please see Materials and methods for detail) and were treated with TSL2 for 4 h to assess the cytotoxicity of TSL2 on SKOV3 cells at different phases. Interestingly, TSL2 had an enhanced cytotoxicity on SKOV3 cells enriched at M phase (Fig. 2).

The effects of TSL2 on cell cycle distribution and cell-cycle-related proteins

To study the effect of TSL2 on cell cycle distribution, unsynchronized SKOV3 cells were treated with TSL2 for 24 h and analyzed by fluorescence-activated cell sorter (FACS). Interestingly, SKOV3 cells enriched at G2/M phase were increased after TSL2 treatment in a dose-dependent manner (Table 2).

We also analyzed, by immunoblotting, the gene expression of several cell cycle regulatory proteins, including Chk1, Chk2, Cdc25c, and Cdc2 in SKOV3 cells upon TSL2 treatment. At 1 h and the later time points after treatment, TSL2 was found to have significantly enhanced the expression of phosphorylated Chk1 and Chk2 (p-Chk1 and p-Chk2) in SKOV3 cells, which then phosphorylated and inactivated Cdc25C phosphatase, a key activator of Cdc2/cyclin B that controls M-phase entry in eukaryotic cells (Fig. 3). The inactivated Cdc25C was unable to dephosphorylate Cdc2 and caused the increase of phosphorylated Cdc2, an inactive form of Cdc2, in SKOV3 cells (Fig. 3).

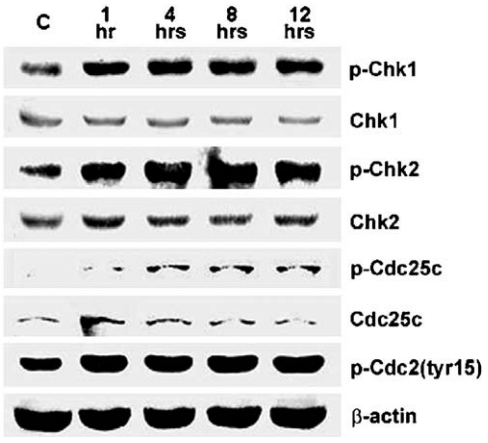


Fig. 3. Expression of cell cycle checkpoint proteins upon TSL-2 treatment. SKOV3 cells were treated with TSL2 at a dosage of 500 μg/ml for different time periods and then harvested for immunoblotting analysis. (C) Untreated control; (1, 4, 8, 12, 24) treated with TSL2 for 1, 4, 8, 12, and 24 h individually.

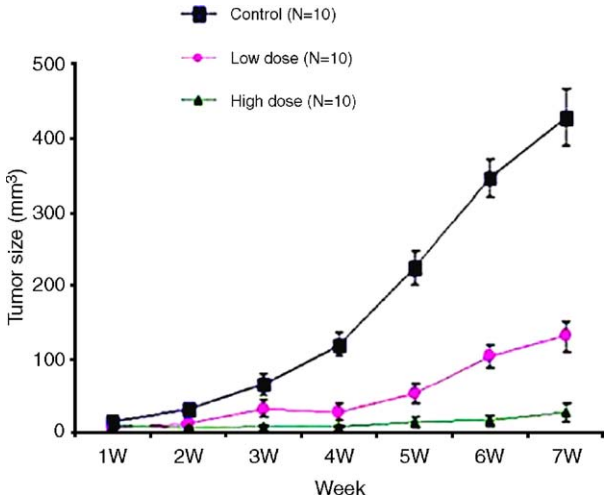


Fig. 4. TSL2 suppressed the ex vivo ovarian cancer cell growth in a dose-dependent manner. Two million SKOV3 ovarian cancer cells were injected subcutaneously into the nude mice, and, after the tumor mass became noticeable, TSL2 was given by intraperitoneal injection 5 days per week for a total duration of 7 weeks at the dosage of 0.67 μg/g body weight (low dose) and 6.7 μg/g body weight (high dose). The tumor volumes were measured for each group and presented as mean ± SD.

Tumoricidal effect of TSL2 on ovarian cancer cells using nude mice model

To verify whether TSL2 had an anti-cancer effect in vivo, we used a nude mice xenograft model (see Materials and methods for the detailed information). After the subcutaneous tumors had developed to at least 3 mm, which took about 2 weeks, the nude mice were given intraperitoneal injections of either TSL2 (low dose group and high dose group) or PBS (control group). After 7 weeks of TSL2 treatment, the tumor growth was significantly suppressed by TSL2 in a dosage-dependent manner, while the tumors continued to grow in the control group (Fig. 4). Moreover, after intraperitoneal injection of TSL2 for 7 weeks, no significant impairment of hematopoiesis or renal function was observed in the mice (Table 3). Although liver enzymes AST and ALT were elevated in the high dose group, no statistical significance was reached (Table 3).

Discussion

In this study, we showed that fraction 2 of *T. sinensis* (TSL2) had significant cytotoxic effect on ovarian cancer cells both in vitro and in xenograft studies. Furthermore, we illustrated the molecular mechanisms for the anti-cancer effects of TSL2 on SKOV3 ovarian cancer cells.

Table 3
(A) Body weight (BW) profile, (B) complete blood count and biochemical profile for the nude mice after treatment with TSL2 for 7 weeks

	Control (N = 10)		Low dose (N = 10)		High dose (N = 10)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
(A)						
BW (g) mean ± SD	27.8 ± 1.9	26.2 ± 2.3	27.8 ± 1.9	26.2 ± 2.3	27.8 ± 1.9	26.2 ± 2.3
	Control (N = 10)	Low dose (N = 10)	High dose (N = 10)			
(B)						
Mean ± SD						
WBC ($\times 10^3/\mu l$)	3.1 ± 0.5	3.6 ± 0.3	3.2 ± 0.8			
RBC ($\times 10^6/\mu l$)	9.0 ± 0.4	9.4 ± 0.4	9.5 ± 0.2			
HGB (g/dl)	12.2 ± 0.5	12.7 ± 0.5	12.9 ± 1.1			
HCT (%)	40.3 ± 2.2	40.6 ± 1.3	41.7 ± 2.1			
MCV (fl)	44.2 ± 1.6	43.4 ± 1.9	44.9 ± 1.6			
MCH (pg)	13.4 ± 0.2	13.3 ± 0.5	13.6 ± 0.2			
MCHC (%)	30.6 ± 0.8	30.7 ± 0.7	60.5 ± 0.7			
PLT ($\times 10^3/\mu l$)	617.47 ± 108.8	687.4 ± 30.4	646.6 ± 25.8			
BUN (mg/dl)	28.0 ± 3.7	25.8 ± 2.6	26.9 ± 4.8			
Creatinine (mg/dl)	0.43 ± 0.11	0.47 ± 0.04	0.47 ± 0.16			
AST (IU/l)	94.0 ± 22.2	96.2 ± 24.7	107 ± 8.7			
ALT (IU/l)	30.3 ± 17.6	34.1 ± 7.3	33.8 ± 14.9			

The data were presented as mean ± SD. No significant difference was noticed between each group, thought AST and ALT showed an elevated tendency after high dose TSL treatment.

Poly(ADP-ribose) polymerase (PARP) is a multifunctional protein and is involved in several crucial cellular processes: DNA replication, transcription, DNA repair, genome stability, and apoptosis [13–16]. On the other hand, caspases cause PARP cleavage and inactivation during apoptosis [17–19]. Our study shows that TSL2 decreased the expression of PARP and arrested the repairing function of PARP (Fig. 1).

The expression of pro-apoptotic proteins Bax was enhanced by at 1 h and the later time points after TSL2 treatment (Fig. 1). In our study, although procaspase 3 expression was decreased at 1 h and the later time points after TSL2 treatment, the P12 and P17 subunits of caspase 3 did not appear after TSL2 treatment in SKOV3 cells (Fig. 1). A similar observation has been reported by Seo and Surh, who in one study treated human promyelocytic leukemia HL-60 cells with Eupatilin [20]. However, the reason for this observation is currently unclear.

Checkpoint controls ensure chromosomal integrity through the cell cycle. Chk1 and Chk2 are effector kinases in the G2-phase checkpoint activated by damaged or unreplicated DNA [21,22]. Chk1 and Chk2 inhibit Cdc2 by phosphorylation and inactivation of Cdc25, the phosphatase that normally activates Cdc2 [23,24]. Cdc25 is a protein phosphatase responsible for dephosphorylating and activating Cdc2, a crucial step in regulating the entry of all eukaryotic cells into the M-phase of the cell cycle [25–27]. In this study, we showed that TSL2 enhanced the expression of the kinases of both Chk1 and Chk2, which phosphorylated/inactivated Cdc25C and inhibited the activation of Cdc2 (Fig. 3). These results were consistent with our findings that the treatment of TSL2 enriched the cell population of G2 phase in SKOV3 cells (Table 2). Our study clearly demonstrated that growth of transplanted human ovarian cancer cells in nude mice was inhibited after intraperitoneal injection of TSL2 (Fig. 4). We also found injection of TSL2 into the nude mine to have no significant toxicity to the bone marrow, kidneys, or liver (Table 3).

In summary, we demonstrated that TSL2, a fractionated extract from the leaves of *T. sinensis*, arrested ovarian cancer cell growth at the G2 phase and also caused significant cytotoxicity to cancer cells at the G2/M phase. In vivo xenograft study showed that TSL2 suppressed the growth of ovarian cancer cells without significant nephrotoxicity, liver toxicity, or bone marrow suppression. Our results indicate that TSL2 could someday be developed into a promising anti-ovarian cancer drug and that it is worthy of further study.

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