

Extracts of the Medicinal Plants *Pao Pereira* and *Rauwolfia vomitoria* Inhibit Ovarian Cancer Stem Cells *In Vitro*

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Abstract

Ovarian cancer has an enrichment of cancer stem cells (CSCs) which contribute to the treatment resistant tumor's high rate of recurrence and metastasis. Here we investigated 2 plant extracts from the medicinal plants *Pao Pereira* (Pao) and *Rauwolfia vomitoria* (Rau) each for their activities against ovarian CSCs. Both Pao and Rau inhibited overall proliferation of human ovarian cancer cell lines with IC₅₀ ranging from 210 to 420 µg/mL and had limited cytotoxicity to normal epithelial cells. Ovarian CSC population was examined using cell surface markers and tumor spheroid formation assays. The results showed that both Pao and Rau treatment significantly reduced the ovarian CSC population. Pao and Rau had similar activities in inhibiting ovarian CSCs, with IC₅₀s of ~120 µg/mL for 24 hours treatment, and ~50 µg/mL for long-term tumor spheroid formation. Nuclear β-catenin levels were decreased, suggesting suppression of Wnt/β-catenin signaling pathway. Taken together, data here showed that Pao and Rau both inhibited ovarian cancer stem cells, probably in preference to the bulk of tumor cells. Further mechanistic studies and *in vivo* investigation validating these findings are warranted, given that inhibition of cancer stem cells holds the promise of comprehensively inhibiting cancer metastasis, drug resistance and recurrence.

Keywords

ovarian cancer, cancer stem cells, *Pao Pereira*, *Rauwolfia vomitoria*, medicinal plant, natural product

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Introduction

Ovarian cancer is the most lethal type of gynecologic cancer, and the fifth leading cause of cancer-related death in the United States. The American Cancer Society estimated that 19 880 women will be diagnosed with ovarian cancer in 2022, and 12 810 will die from it.¹ Due to the lack of reliable screening methods of early detection for average-risk women, approximately 75% of ovarian cancer patients are diagnosed at Stage III or IV, for whom the survival rates are 39% and 17% respectively.^{2,3} Standard of care treatment consists of primary cytoreductive surgery (CRS) followed by adjuvant or neoadjuvant chemotherapy (NACT) with interval CRS followed by adjuvant chemotherapy. Chemotherapy traditionally comprises platinum/taxane doublets, which improves short term survival, but does not benefit the long-term survival due to development of drug resistance and recurrence of the disease.^{4,5} A variety of genome-wide abnormalities contribute to the

chemoresistance of ovarian cancer, including P53 and BRCA1/2 mutations, RB1, NF1 suppression, and MDR1 overexpression.⁶ Several platinum-/taxane- free regimes have been developed that have improved the overall survival of ovarian cancer patients, including VEGF inhibitors,^{7,8} and PARP inhibitors.^{9–11} However, the toxic side effects and the high costs limit their usage.^{12,13} Unfortunately, 70% to 75% of women who successfully complete optimal therapy will experience tumor relapse. Cumulative toxicity, cross-resistance to chemotherapies, and compromised quality of life are additional serious clinical challenges.

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The high metastasis and recurrence rate of ovarian cancer are partially due to an enriched cancer stem cells (CSC) population.^{14,15} CSCs are a small population of cells in the bulk of tumor which are distinguished by their tumorigenic and self-renewal ability.¹⁶ Based on the microenvironment, a stem cell can either divide and generate daughter cells which do not differentiate but keep the full potential of differentiation as the parent stem cell (self-renew), and/or raise daughter cells which will further differentiate.¹⁷ Thus CSCs possess self-renewal ability and have the ability to give rise to all cell types found in a particular bulk of tumor (tumorigenicity).¹⁸ CSCs are highly resistant to current chemo and radiation therapy,¹⁹ and are responsible for tumor metastasis²⁰ and recurrence,²¹ which are the main reasons for the failure of cancer therapy. Therefore, treatments that inhibit cancer stem cells would hold great promise in achieving comprehensive inhibition in tumor growth, metastasis, drug resistance, and recurrence.

Here we will investigate extracts from 2 medicinal plants of the Apocynaceae family, each for their activities in inhibiting ovarian CSCs. One is Pao Pereira (Pao), the Amazonian tree species *Geissospermum vellosii*. The other is *Rauwolfia vomitoria* (Rau), a tropical shrub originating in Africa. This family of plants has been used as folk medicines in South American and Africa to treat a variety of health related conditions, such as hypertension,^{22,23} fever,^{24,25} gastrointestinal diseases,²⁶ liver diseases,²⁷ and cancers.^{28,29} The extracts of Pao and Rau each as a mixture are used as health supplements. Extracts from Pao and Rau are enriched with β -carboline alkaloids and indole alkaloids.³⁰ β -Carboline alkaloids have been reported to demonstrate many bioactivities, including antitumor effects.^{31,32} We previously reported that extracts of Pao and Rau potentiates carboplatin effects against ovarian cancer *in vitro* and *in vivo* by inducing ovarian cancer cell apoptosis.^{33,34} Further, Pao and Rau were found to inhibit CSC population in pancreatic cancer cellular models.^{35,36} Here we will examine their capabilities in inhibiting ovarian cancer stem cells.

Materials and Methods

Cell Lines and Reagents

Human ovarian cancer cell lines OVCAR5, OVCAR8, and SKOV3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in the lab. Human ovarian cancer cell line SHIN3 was donated by Dr P. Eck of the University of Manitoba, Canada. An immortalized human lung epithelial cell line, MRC-5 was provided by Dr. Sitta Sittampalam at the National Center for Advancing Translational Sciences, NIH. An ovarian epithelial cell line HIO80 was provided by Dr. Andrew Godwin at the University of Kansas Medical center. The MRC-5 and HIO80 cells were used as normal cell

comparisons to the cancer cells. All cells were cultured at 37°C in 5% CO₂/95% air in recommended growth media containing 10% fetal bovine serum and 1% antibiotics. The extracts of Pao Pereira (Pao) and *Rauwolfia vomitoria* (Rau) were provided by the Maison Beljanski (New York, NY, USA, formerly the Natural Source International Ltd.) as commercially available products which were manufactured through standardized extracting methods. The extracts were prepared in sterile PBS in 10 mg/mL stock solutions and stored at -20°C.

Cell Viability Assay

Cells were assessed for viability by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay at 48 hours of treatment. Cells in exponential growth phase were exposed to serial dilutions of Pao or Rau for 48 hours. Cells were then changed into fresh media containing MTT and were incubated for 4 hours. Formazan crystal was dissolved in 100 μ L DMSO and absorbance was detected at 570 nm. The colorimetric MTT assay assesses relative proliferation, based on the ability of living, but not dead cells, to reduce MTT to formazan. Cells did not reach plateau phase during the incubation period. The 50% inhibitory concentration (IC₅₀) was defined as the concentration of drug that inhibited cell growth by 50% relative to the untreated control. Pilot experiments for each cell line were performed to optimize cell density and assay duration and to center drug dilution series approximately on the IC₅₀.

Tumor Spheroid Formation Assay

Single-cell suspension was plated into 24 well ultra-low attachment plates (Corning Inc., Corning, NY) at a density of 5000 cells/well in stem cell medium and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The stem cell medium consist of DMEM (Corning Inc., Corning, NY) supplemented with 1X B27 Supplement, 20 ng/ml human basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 100 units/ml penicillin/streptomycin (Invitrogen, Grand Island, NY), and 4 μ g/ml heparin calcium salt (Fisher Scientific, Pittsburg, PA). Spheroids were counted after 4 weeks under the microscope.

Flow Cytometry for Detection of Cancer Stem Cells Surface Markers

Cells were exposed to various concentrations of Pao or Rau for 24 hours or 48 hours. Cells were then washed with PBS 3 times, and re-suspended in binding buffer (PBS supplemented with 0.1% Bovine serum albumin) for 15 minutes. PE-cy7 conjugated anti-CD44 antibody, and APC conjugated anti-CD117 antibody (Biolegend, San Diego, CA)

were added into cell suspension and incubated for 15 min according to the manufacturer's protocol. Cells were washed in PBS 3 times after staining and then analyzed by BD LSR II Flow Cytometer. The data was normalized to cell death (Normalized CSC population = original CSC population detected with flow cytometry \times % cell viability detected with MTT assay).

Western Blot

Cells were lysed with NE-PER™ nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA) containing protease inhibitors and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO), following the manufacture protocol. BCA method was used for protein quantification (Pierce BCA protein assay kit, Waltham, MA). SDS-PAGE and Western blot was performed as routine. Primary antibodies and the secondary antibody were purchased from the Cell Signaling Technology, Beverly, MA. Primary antibodies included rabbit anti- β -catenin (dilution 1:1000), rabbit anti-vinculin (dilution 1:1000), and rabbit anti-Histone-3 (dilution 1:2000). A goat anti-rabbit and anti-mouse polyclonal horseradish peroxidase conjugated secondary antibody (dilution 1:5000,) was used. Blots were established using a chemiluminescence detection kit (Pierce ECL or ECL + western blotting substrate, Thermo Scientific, Waltham, MA).

RNA Isolation, cDNA Synthesis, and Real-time PCR

Total RNA was extracted from cells using TRIZOL reagent according to the protocol of the manufacturer (Invitrogen, Grand Island, NY). cDNA synthesis was performed with 1 μ g of total RNA using Omniscript RT kit according to manufacturer's protocol (Qiagen, Valencia, CA). cDNA was diluted 1:5 in DEPC treated nanopure water and used for further analysis. Real-time PCR was performed using Bio-Rad iQ iCycler detection system with iQ SYBR green supermix (Bio-Rad Laboratories Ltd, Hercules, CA). Reactions were performed in a total volume of 10 μ l, including 5 μ l of 2X iQ SYBR green supermix, 0.4 μ l of primers at 20 pmol/ μ l and 0.4 μ l of cDNA template. All reactions were carried out in 4 repeats for each sample and 3 independent experiments for each treatment condition. GAPDH was used as housekeeping gene for normalization. Primers used in Real-time PCR were according to previous study.³⁰

Statistical Analysis

Statistical analysis was performed using SPSS software for student T-test. A difference was considered significant at the $P < .05$ level.

Results

Pao and Rau Inhibited Ovarian Tumor Spheroids Formation

A panel of human ovarian cancer cell lines (OVCAR5, OVCAR8, SHIN3, and SKOV3), a normal ovarian epithelial cell line (HIO80) and an immortalized fibroblast cell line (MRC-5) were subjected to Pao and Rau treatment for 48 hours. Results showed that both Pao and Rau inhibited viability of the 4 tested ovarian cancer cell lines. IC₅₀ values of Pao ranged from 210 to 420 μ g/mL (Figure 1A), and the IC₅₀s of Rau ranged from 300 to 345 μ g/mL (Figure 1B). The non-cancerous fibroblast cell line MRC-5 and epithelial cell line HIO80 were more resistant to Pao and Rau treatment. IC₅₀ values for MRC-5 cells were 537 μ g/mL for Pao and 566 μ g/mL for Rau. IC50 values for HIO80 cells were 718 μ g/mL for Pao and 596 μ g/mL for Rau, significantly higher than those of cancer cells (Figure 1A and B). These data are consistent with our previous studies that Pao and Rau inhibited proliferation of ovarian cancer cells with less effects on normal cells.³⁷

The ability of Pao and Rau in inhibiting tumor spheroid formation was detected in SKOV3 cells. At the concentration of 50 μ g/mL, both Pao and Rau significantly reduced the number of the SKOV3 tumor spheroids (Figure 2A and B). At the concentration of 200 μ g/mL and above, both Pao and Rau completely eliminated the SKOV3 tumor spheroids (Figure 2A and B). The estimated IC₅₀ values for tumor spheroids inhibition were 54 μ g/mL for Pao and 43 μ g/mL for Rau. In comparison, the IC₅₀ value of Pao to the bulk of SKOV3 cells is 280 μ g/mL and for Rau is 350 μ g/mL (Figure 1A and B). In the bulk SKOV3 cell population, 200 μ g/mL of Pao or Rau inhibited cell viability by 20% to 25%, whereas 100% tumor spheroids were inhibited at this concentration (Figure 2A and B). These results suggest preferential inhibition in ovarian CSCs by Pao and Rau treatments.

Pao and Rau Reduced the Number of Ovarian Cancer Stem Cells

CSC populations can be identified by specific cell surface markers. In ovarian cancer, a sub-population of cells with high expression of surface markers CD44, and CD117 (CD44+CD117+ cells) were reported to possess strong self-renewal ability and the ability to produce differentiated progeny and to generate new tumors in mice that were histologically identical to parent tumors.³⁸ Here, we use these markers as indicative markers for ovarian CSCs, and detected changes in these markers with Pao or Rau treatment. SKOV3 cells were treated with Pao or Rau for 24 hours or 48 hours at 50, 100, and 200 μ g/mL. CD44 and CD117 were examined by immune staining

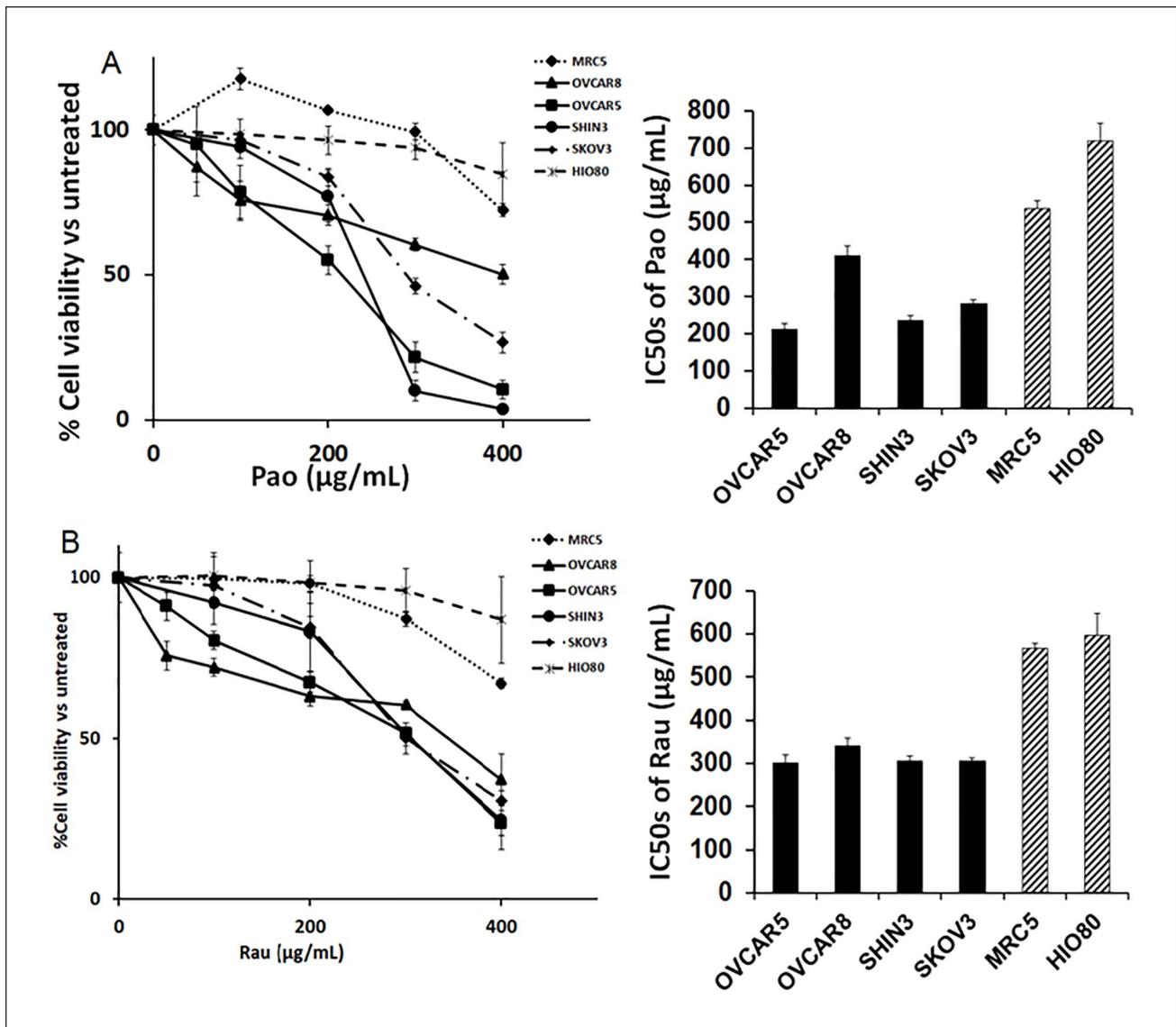


Figure 1. Pao and Rau reduced ovarian cancer cell viability. Human ovarian cancer cells OVCAR5, OVCAR8, SHIN3, and SKOV3 were treated with serial concentrations of Pao and Rau for 48 hours. Cell viability was detected by MTT assay. A normal fibroblast cell line MCR-5 and a normal ovarian epithelial cell line HIO80 were subjected to the same treatment. (A) Dose-response curves (left panel) and IC₅₀ values of Pao (right panel). (B) Dose-response curves (left panel) and IC₅₀ values of Rau (right panel). Data represent means \pm SD of 2 to 3 independent experiments each done in triplicates.

and flow cytometry analysis. Pao and Rau reduced the CD44+CD117+ population at both 24 and 48 hours (Figure 3A–D). The reductions in CD44+CD117+ cells were concentration dependent to both Pao and Rau, and were time dependent to Rau treatment, but were less likely time dependent to Pao treatment. In the control groups, CD44+CD117+ cells consisted of 10% to 14% of the whole population. At 100 μ g/mL, Pao reduced the CD44+CD117+ cells population to 6.83% at 24 hours (Figure 3A) and 6.22% at 48 hours (Figure 3B). Rau reduced the CD44+CD117 cells to 8.33% at 24 hours (Figure 3C) and 4.64% at 48 hours

treatment (Figure 3D). At 200 μ g/mL, Pao reduced the CD44+CD117+ cells to 3.52% at 24 hours treatment (Figure 3A), and to 4.38% at 48 hours (Figure 3B), whereas Rau reduced the CD44+CD117+ cells to 3.58% at 24 hours (Figure 3C), and to 1.98% at 48 hours (Figure 3D).

Pao and Rau Suppressed the Canonical Wnt/ β -Catenin Signaling Pathway

Canonical Wnt/ β -catenin signaling pathway plays an important role in maintaining the self-renewal and spheroid

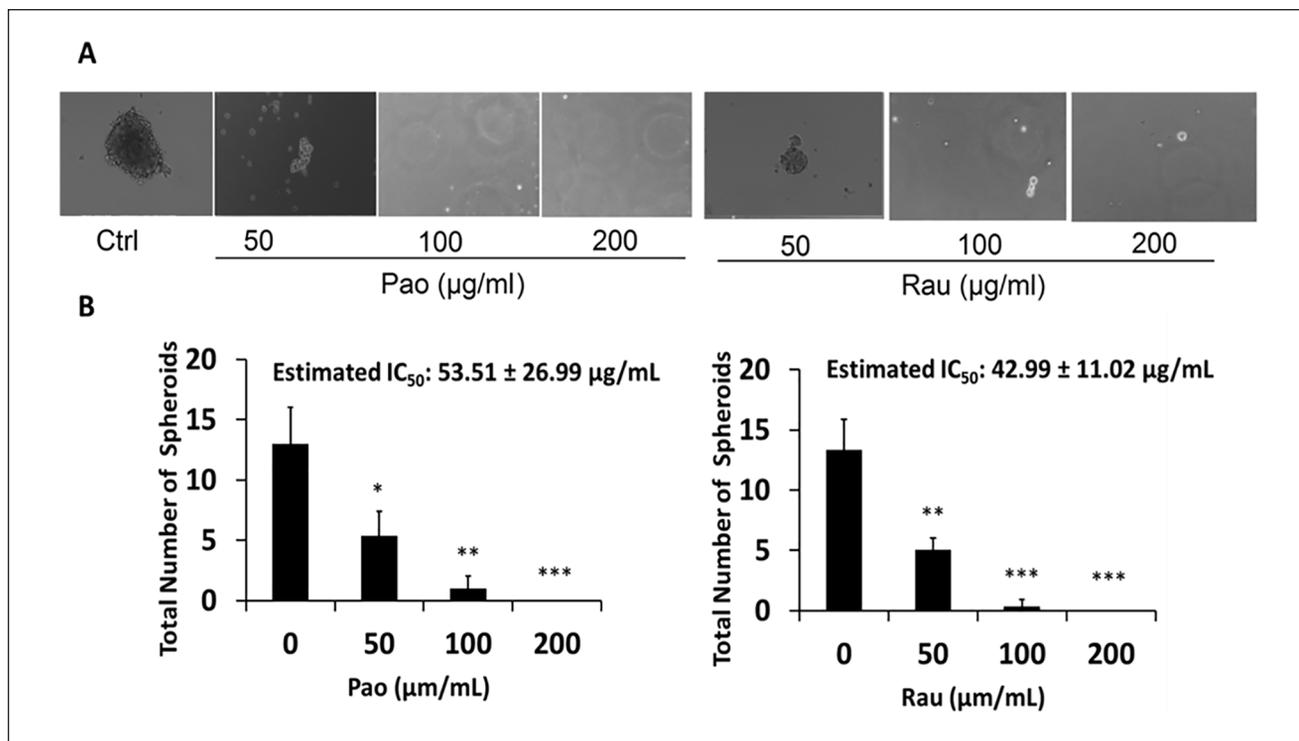


Figure 2. Pao and Rau inhibited ovarian tumor spheroids formation. SKOV3 single-cell suspension was plated into 24 well ultra-low attachment plates at a density of 5000 cells/well in stem cell medium, and treated with Pao or Rau at the indicated concentrations. Tumor spheroids were counted after 4 weeks. (A) Representative images of the spheroids with Pao or Rau treatment. (B) Number of SKOV3 spheroids. Data shows means \pm SD of 3 independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared to untreated control by *t*-tests.

formation capacities of CSCs.^{18,39} Accumulation of β -catenin in the nuclear as a transcriptional factor is a hallmark of Wnt/ β -catenin pathway activation.⁴⁰ Here, the cytoplasmic and nuclear fractions of the SKOV3 cells were examined for β -catenin levels with or without Pao/Rau treatment. Pao 100 $\mu\text{g}/\text{mL}$ at both 24 hours and 48 hours reduced the nuclear β -catenin levels (Figure 4A). The cytoplasmic β -catenin levels were not changed at both 24 hours and 48 hours treatment (Figure 4A). With 100 $\mu\text{g}/\text{mL}$ Rau treatment, the nuclear β -catenin level was slightly decreased at 24 hours, and was robustly decreased at 48 hours (Figure 4A).

To examine other genes and transcription factors that are important for CSC initiation and maintenance, a panel of 10 genes and transcription factors were examined by RT-PCR.⁴¹ Data here showed that the mRNA expressions of these genes were not changed with 24 hours of Pao or Rau treatment (Figure 4D). These data suggest that Pao and Rau inhibit ovarian cancer CSCs mainly through suppression of the Wnt/ β -catenin pathway.

Discussion

Traditional anti-tumor chemo drugs lack the ability to eliminate CSCs, resulting in survival of CSCs which later

give rise to recurrent tumors often at metastatic sites.^{42,43} New agents inhibiting CSCs are promising to comprehensively inhibit tumor growth, metastasis and recurrence, and to conquer drug resistance. In this study, we demonstrated that 2 medicinal plant extracts, Pao and Rau, significantly inhibited ovarian CSCs *in vitro*. Previously, we have reported that Pao and Rau induced apoptosis in ovarian cancer cells and potentiated the effects of carboplatin against ovarian cancer cells.^{33,34} Data here showed that Pao and Rau inhibited ovarian CSCs at lower concentrations than those needed to achieve inhibition in the bulk of ovarian cancer cells. These data are consistent with our studies in pancreatic cancer,^{35,36} and suggest that Pao and Rau may preferentially target the CSCs population in the bulk of tumor. The effects of Pao and Rau inhibiting CSCs are worth investigating *in vivo*. Whereas Pao and Rau extracts are widely available dietary supplements to the public for uses including anti-cancer, there are to date very few research studies done on the anti-cancer effects of Pao and Rau. Data reported here confirm that Pao and Rau inhibited CSCs not only in one type of cancer. As cancer is a disease of many types and has high heterogeneity, these effects are now validated in 2 different cellular models. This may imply that the effects and

mechanism of Pao and Rau can be extended to other types of cancers.

The mechanisms for CSCs formation are not fully understood. Several cellular signaling pathways have been demonstrated to be important in the formation and maintenance of CSCs, including the canonical Wnt/ β -catenin signaling pathway, Notch-signaling pathway and Hedgehog signaling pathway.⁴⁴ The canonical Wnt/ β -catenin signaling pathway regulates the degradation/nuclear translocation of β -catenin, which acts as a transcription factor and leads to the cancer cell “gain of

self-renewal ability.”⁴⁵⁻⁴⁸ Our data here clearly showed that both Pao and Rau reduced the nuclear β -catenin levels at 24 and 48 hours.

Plant preparations often are complex mixtures of natural compounds. There is the possibility that Pao or Rau affect multiple molecular targets and pathways which lead to CSC inhibition. We examined 10 other genes and transcription factors that are important in cancer stem cell initiation and maintenance. Data did not show significant changes of these genes with Pao or Rau treatment. While the in-depth mechanism(s) by which Pao and Rau induce CSC inhibition

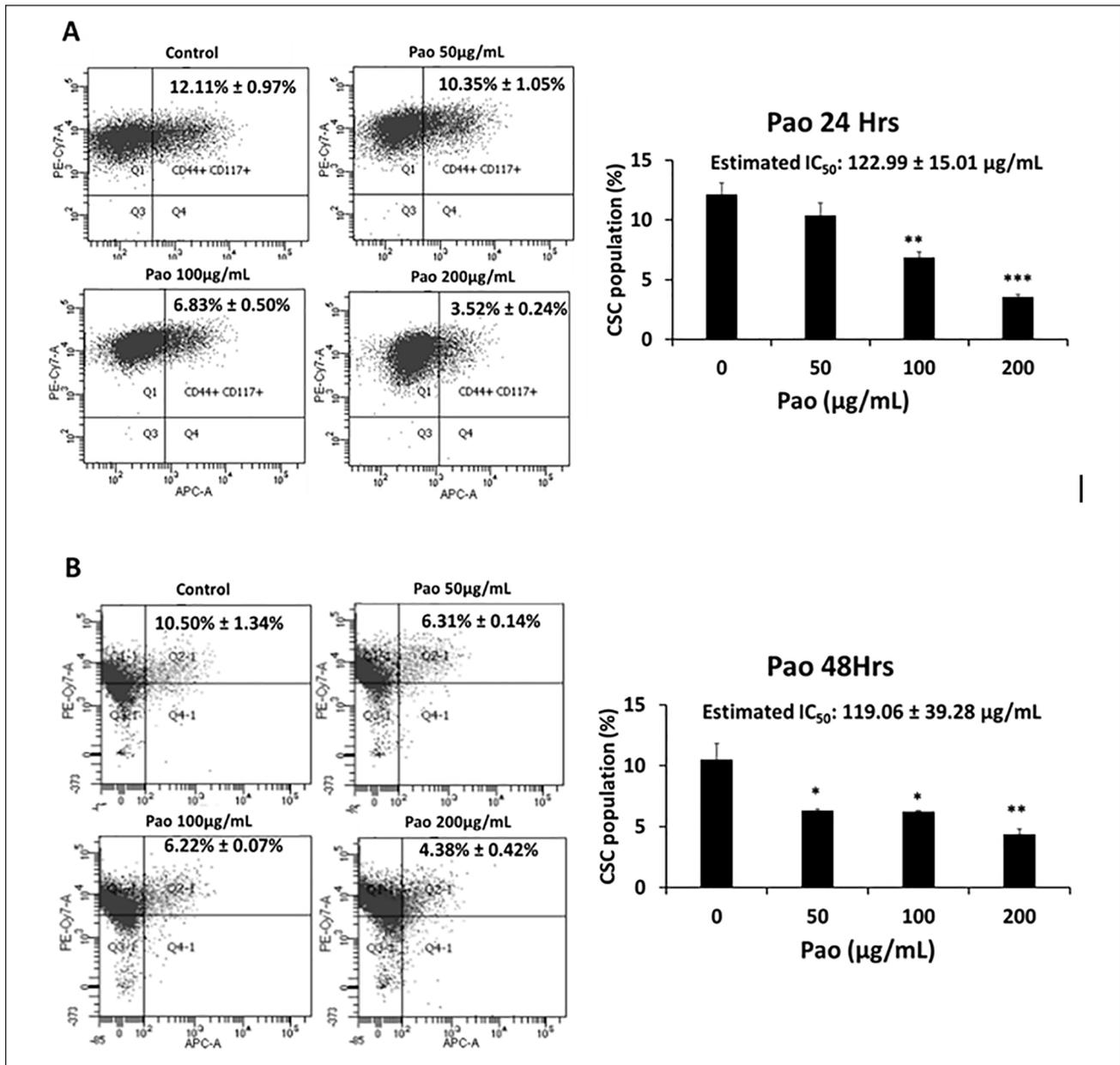


Figure 3. (continued)

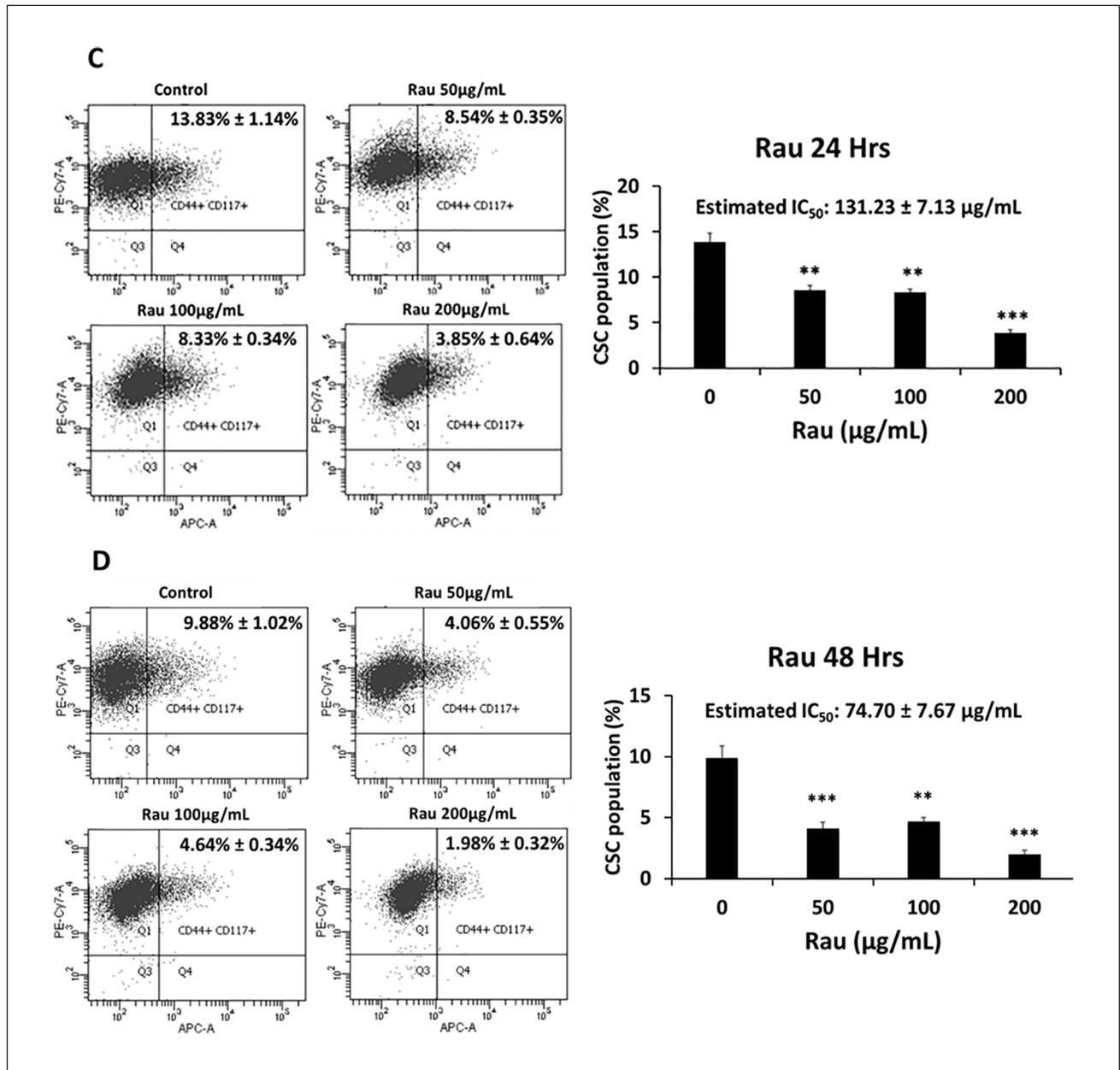


Figure 3. Pao and Rau reduced ovarian CSC population. SKOV3 cells were treated with Pao or Rau for 24hours (A and C), and 48hours (B and D) at the indicated concentrations. Cells were then stained with fluorescent conjugated antibodies for CD44 and CD117, followed by flow cytometry analysis. The left panels represent the distribution of CD44 (PE-cy7) and CD117 (APC) positive cells by flow cytometry. The right panels show the percentages of CD44+CD117+ cells (Mean ± SD of 3 experiments). The data were normalized to cell death. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared to untreated control by *t*-tests.

need to be further investigated, it is likely that Pao and Rau share the mechanism of inhibiting ovarian cancer CSCs through inhibition of the Wnt/ β -catenin pathway. This mechanism of action needs to be further validated in appropriate animal models.

Taken together, data here showed that both Pao and Rau possess the activities of inhibiting ovarian cancer stem cells

in vitro. Pao and Rau are consumed as dietary supplements by the general population in the USA, indicating their potential low toxicity. Given the driving role of CSCs in tumor metastasis, drug resistance, and tumor recurrence, the benefits of Pao and Rau in ovarian cancer treatment are worth investigation *in vivo* and clinically, especially in combination with current chemotherapies.

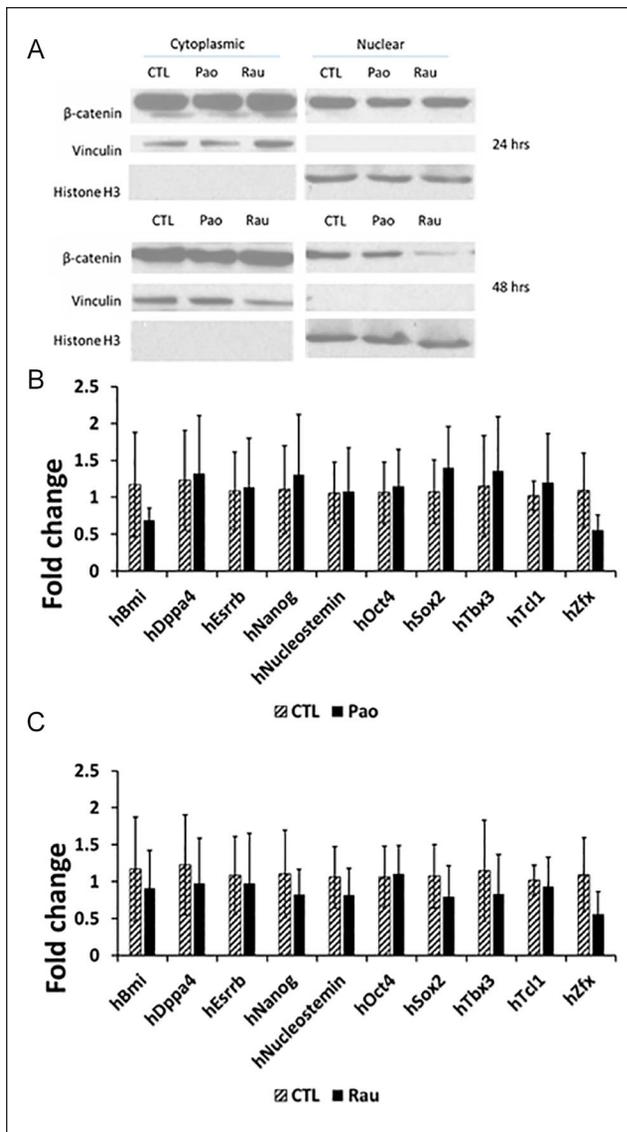


Figure 4. Pao and Rau decreased nuclear β -catenin levels in ovarian cancer cells. SKOV3 cells were treated with Pao or Rau at 100 μ g/mL for 24 and 48 hours. (A) The expression of β -catenin was detected by western blots in cytoplasmic and nuclear fractions. Vinculin was a loading control for cytoplasmic proteins, and histone H3 was a loading control for the nuclear fractions. (B and C) The expression of 10 CSC-related genes were examined by RT-qPCR at 24 hours of treatment by Pao (B) or Rau (C). Error bars show SD with 12 repeats.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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