Supporting Information to:

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Escin, a Natural Mixture of Triterpene Saponins, Exhibits
Antitumor Activity Against Hepatocellular Carcinoma

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Fig. 1S Comparison of mice tumors for the four groups. (I): the tumor control, physiological saline of the same volume and times of escin solution was given by peritoneal injection. (II): peritoneal injection was done with cyclophosphamide (CTX) 30 mg/kg, once on d 2. (III) and (IV): peritoneal injection was done with escin, 2.8 mg·kg$^{-1}·$d$^{-1}$ and 1.4 mg·kg$^{-1}·$d$^{-1}$, respectively, for 7 days.

Fig. 2S Cells were analyzed by flow cytometry after PI staining then the relative percentage of cells in different cell cycle phases are reported.
Fig. 3S Apoptosis induced by escin by annexin V and PI staining assay. Cell number is plotted. Cells staining positive for Annexin V are apoptotic. The 5, 10, 15, 20 μg/mL escin could induce 6.1%, 11.7%, 23.5%, 39.2% apoptosis. The percentage of apoptosis in control is 3.2%.

Concrete Operation of Methods

SRB assay
Briefly, exponentially growing cells were harvested and seeded in 96-well plates with the final volume of 100 μL containing 5 × 10³ cells per well. After 24 h incubation, cells were treated with various concentrations of escin for 24 h. The cultures were then fixed at 4 °C for 1 h by addition of ice-cold 50% trichloroacetic acid to give a final concentration of 10%. Fixed cells were rinsed five times with deionized water and stained with 0.4% SRB dissolved in 0.1% acetic acid for 10 min. The wells were washed five times with 0.1% acetic acid and left to dry in room temperature overnight. The absorbed SRB was dissolved in 150 μL unbuffered 1% Tris base (pH 10.5). The optical density (OD) of each well was measured using a microplate reader (BIO-TEK) at 570 nm with reference at 630 nm.

Pulse field gel electrophoresis
For pulse field gel electrophoresis (PAGE), DNA was prepared from agarose plugs (1×10⁶ cells) [15] digested twice with proteinase K (1 mg/mL; 50 °C; 12 h) in NDS buffer (0.5 M EDTA, 10 mg/mL lauroyl sarcosine), washed in TBE 3 0.5, followed by electrophoresis in a Bio-RadCHEF-DR II (Richmond, Calif.) equipment (1% agarose; TBE 3 0.5; 200 V; 24 h; pulse wave 60 s; 120° angle).

Cell cycle analysis by flow cytometry
Cells (2×10⁵ cells/well) were plated in 6-well tissue culture dishes and treated with escin at the concentrations indicated for 48 h, then trypsinized, collected, washed in cold PBS. Cells were subsequently fixed in 70% ethanol, washed, treated with 50 μg/mL RNase A at 37 °C, and incubated with 50 μg/mL PI at 4 °C for 30 min, then analyzed with a FACSCaliber flow cytometer.
Assessment of apoptosis by flow cytometry

Briefly, after 48 h incubation with escin, cells were harvested, stained with 5 μL annexin V-Fluorescein isothiocyanate (FITC) and 5 μL PI to each sample and incubated for 15 min in the dark at room temperature. Cells were washed twice in PBS, and then stained cells were analyzed by flow cytometer to be viable (annexin V and PI negative), early apoptotic (annexin V positive and PI negative), or late apoptotic (annexin V and PI positive). The degree of apoptosis was quantified as a percentage of the annexin V positive cells.

Preparation of whole cell extracts and subcellular fractionation

Cells were washed and scraped into ice-cold PBS and collected by centrifugation, then resuspended in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM dithiothreitol and 10 μL/mL protease inhibitor cocktail). The extracts were centrifuged and the clear supernatants were stored in aliquots at -70°C for further analysis. Subcellular fractionation was performed using mitochondria isolation kit obtained from Sigma (Sigma Chemicals, St Louis, MO) and different compartmental proteins were used to study the translocation of AIF and cytochrome c.

Immunoblotting

The aliquots of lysates (20 μg of protein) were boiled with sample buffer for 5 min, and resolved by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween 20) containing 3% nonfat dried milk overnight at 4 °C. The blots were incubated with various primary antibodies, followed by incubation for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase in TBST. Actin and histone expression was used as loading control.