ORIGINAL ARTICLE

Anticancer Effects of Crude Extract from *Melia toosendan* Sieb. et Zucc on Hepatocellular Carcinoma *In Vitro* and *In Vivo*

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**ABSTRACT** Objective: To investigate the anti-cancer effects of crude extract from *Melia toosendan* Sieb. et Zucc and its possible molecular mechanisms *in vitro* and *in vivo*. Methods: Transonic alcohol-chloroform extraction method was used to extract toosendanin from the bark of *Melia toosendan* Sieb. et Zucc, and the content of toosendanin in the crude extract was measured by high performance liquid chromatography (HPLC). Anti-cancer effects of crude extract from *Melia toosendan* Sieb. et Zucc were investigated in *in vivo* and *in vitro* studies. In the *in vitro* experiment, human hepatocellular carcinoma cell lines SMMC-7721 and Hep3B were co-incubated with toosendanin crude extract of different concentrations, respectively. In the *in vivo* experiment, BALB/c mice were subcutaneously inoculated with mouse hepatocellular carcinoma H22 cells and treated with crude extract.

Results: HPLC revealed the content of toosendanin was about 15%. Crude extract from *Melia toosendan* Sieb. et Zucc inhibited cancer cells growth in a dose- and time-dependent manner. The 50% inhibitory concentration (*IC*$_{50}$, 72 h) was 0.6 mg/L for SMMC-7721 cells and 0.8 mg/L for Hep3B cells. Both high-dose [0.69 mg/(kg·d)] and low-dose [0.138 mg/(kg·d)] crude extract could markedly suppress cancer growth, and the inhibition rate was greater than 50%. Hematoxylin and eosin staining showed necrotic area in cancers and transmission electron microscopy displayed necrotic and apoptotic cancer cells with apoptotic bodies. Immunohistochemistry showed that the expression of Bax and Fas increased and the expression of Bcl-2 reduced. Conclusion: Toosendanin extract has potent anti-cancer effects via suppressing proliferation and inducing apoptosis of cancer cells *in vivo* and *in vitro*. The mechanism of apoptosis involves in mitochondrial pathway and death receptor pathway.

KEYWORDS crude extract, *Melia toosendan* Sieb. et Zucc, anti-cancer activity, SMMC-7721 cell, Hep3B cell, murine hepatocellular carcinoma

*Melia toosendan* Sieb. et Zucc. (also named *Melia azedarach*. L. or *Azadirachta indica* A. Juss) originally grew in China and India, and was later transferred to all over the world. *Melia toosendan* Sieb. et Zucc. is a traditional Chinese medicine with insecticidal, analgesic and anti-inflammatory characteristics. With a history of 2,000 years, the roots, barks, trunks, fruits, seeds and leaves of melia plants have been used in the treatment of gastrointestinal parasitic diseases and the prevention and control of agricultural pest.\(^{(1)}\)

Toosendanin (TSN, C$_{30}$H$_{38}$O$_{11}$, FW 574), a triterpenoid derivative as shown in Figure 1, isolated from the barks of *Melia toosendan* or chinaberry, is colorless and acicular crystal. Traditionally, toosendanin can be extracted with organic solvent or water. Recently, supercritical carbon dioxide extraction, microwave-assisted organic solvent extraction and ultrasound-assisted organic solvent extraction have been developed based on traditional

\[\text{Figure 1. Chemical Structure of Toosendanin}\]
methods. It is confirmed that each method has its own advantages and ultrasound-assisted organic solvent extraction is relatively superior to other methods.\(^2\,^3\) The present study modified the ultrasound-assisted organic solvent extraction and toosendanin was crudely extracted by ethanol under the assistance of ultrasound, and then extracted in chloroform.

Toosendanin has been used as a botanical insecticide without public harm and residual toxicity in the fruit and vegetable production. In addition, toosendanin has been applied in the clinical practice as an anthelmintic vermifuge.\(^4\,^6\) Previous study showed that toosendanin selectively blocked acetylcholine (ACh) released from nerve terminals and had antitoxin role in vitro and in vivo.\(^1\,^7\,^8\) Previous study also showed that toosendanin could confer anti-proliferative and pro-apoptotic effects on human prostate adenocarcinoma cell line (PC3), hepatoma cell line (BEL7404), glioblastoma cell line (U251), neuroblastoma cell line (SHSY5Y), promyelocytic leukemia cell line (HL-60), histiocytic lymphoma cell line (U937) and rat pheochromocytoma cell line (PC12) in vitro experiments.\(^9\,^11\) Our previous study indicated that toosendanin could induce apoptosis of hepatocellular carcinoma cells mediated by mitochondria-dependent pathway in vivo and in vitro.\(^12\) Recently, liver injury was reported when high concentration of toosendanin was used in rats and rhesus monkey models.\(^13\,^15\) But the effects of toosendanin crude extract on hepatocellular carcinoma cell remains largely unknown.

### METHODS

#### Drugs and Reagents

Toosendanin with purity over 98% was purchased from the Zhuokang Biotech, Shanghai, China. Toosendanin was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C before use. The designed concentration was achieved through diluting with cell culture medium (the final concentration of DMSO in medium was less than 0.02%). Cyclophosphamide (CTX) with purity of 99% was purchased from Hualian Pharmaceutical Co., Ltd. (China). RPMI-1640 medium was purchased from GIBCO (USA). MEM medium was purchased from Hyclone (USA). Fetal bovine serum (FBS) was purchased from GIBCO (USA). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma (USA). Other reagents of analytical purity were purchased from Sigma (USA).

#### Animals and Cell Lines

The clean grade BALB/c mice weighing 18–22 g with a male/female ratio of 1:1 were supplied by Animal Center of Chongqing Medical University, Chongqing, China [license No: SYXX(Yu) 20020007]. All animal experiments were approved by the Research Ethics Committee of Chongqing Medical University. The animals were housed five per plastic cage with wood chip bedding in an animal room with a 12 h light and 12 h dark cycle at room temperature (24 ± 2 °C) and allowed free access to standard laboratory diet (purchased from the Laboratory Animal Center of Chongqing Medical University, Chongqing, China). Mouse hepatocellular carcinoma cell line (H22), human hepatocellular carcinoma cell lines (SMMC-7721 and Hep3B) were purchased from China Center for Type Culture Collection. SMMC-7721 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Hep3B cells were cultured in MEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

#### Preparation and Identification of Toosendanin Crude Extract

The bark of *Melia toosendan* was collected from the Shapingba District of Chongqing in August 2007, and identified by Chongqing Institute of Traditional Chinese Medicine, China. Clean and dry tree barks of *M. toosendan* (1 kg) were cut into small pieces and smashed into powder about 40 mesh in the grinder. The powder was dried at 56 °C and mixed with 60% ethanol at a solid-liquid ratio of 1:9. Ultrasonic treatment (200 W) was performed for 30 min at 40 °C in water bath. After reflux for 2 h, the solution was filtered with cotton gauze. The filtrate was then collected and the incubation procedure was repeated three times with the residue mixed with 60% ethanol. All the filtrates were pooled together and concentrated with a rota-evaporator under reduced pressure at 70–80 °C. After the concentration procedure, the remaining syrup was then mixed with distilled water and the chloroform extraction fraction was separated using a separatory funnel for three times. After dried by anhydrous sodium sulfate for 2 h, the brown oily residue was dissolved with anhydrous ethanol, then decolorized by active carbon for 30 min at 60 °C. The residues were washed with ethanol. After the filter and concentration procedures, the remaining fraction was then mixed with methanol and chloroform. Heat and stir it when petroleum ether...