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Original Research Article

Essential oil from *Chenopodium ambrosioides* L. induces mitochondrial-mediated pathway and endoplasmic reticulum stress-related apoptosis in human liver cancer SMMC-7721 cells

Qian Rui-Hua[1], Zhu Xiao-Huan[1], Hao Jun-Mei[1], Li Hai-Ying[1], Song Bo-Han[1], Ren Qiu-Rong[1], Ma Dan-Wei[1], Wang Ya-Nan[1]*, Chen Zhao-Bin[2,3]

[1]College of Life Science, Sichuan Normal University, Chengdu 610101, [2]Department of Public Health Laboratory Sciences, West China School of Public Health, Sichuan University, Chengdu 610041, [3]Shenzhen Nanshan Center for Disease Control and Prevention, Shenzhen 518054, PR China

For correspondence: **Email: 273218760@qq.com; **Tel:** +86 (28) 84480655*

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Abstract

Purpose: *To evaluate the cytotoxic effect of essential oil derived from Chenopodium ambrosioides L. in Sichuan Province on human liver cancer SMMC-7721 cells, as well as its possible molecular mechanisms.*
Methods: *Cytotoxicity was characterized by MTT assay and transmission electron microscopy (TEM) of SMMC-7721 cells ultrastructure. The apoptotic effect of the essential oil was evaluated by changes in mitochondrial membrane potential and Western blot assay.*
Results: *MTT assay data indicate that the essential oil was cytotoxic to SMMC-7721 cells, while TEN revealed that there were vacuoles and nucleus fragmentation in the SMMC-7721 cell cytosol, cell swelling, and a large amount of leakage. Mitochondrial membrane potential assay and Western Blot data indicate that the essential oil induced cell apoptosis.*
Conclusion: *The essential oil of Chenopodium ambrosioides L. in Sichuan Province seems to induce apoptosis of human liver cancer SMMC-7721 cells via the mitochondrial-mediated pathway and endoplasmic reticulum stress. Thus, this plant requires further investigation as a potential source of an anti-liver cancer drug.*

Keywords: *Chenopodium ambrosioides Essential oil; Anti-tumor activity, Liver cancer Apoptosis, SMMC-7721 cells*

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INTRODUCTION

Traditional medicinal plants are natural sources of bioactive compounds, which can be used as food additives, and are used as ingredients in functional foods and nutritional products in health promotion [1]. *Chenopodium ambrosioides* L. is an annual or perennial fragrant herb belonging to the *Chenopodiaceae* [2]. Its common names include fairy grass, red zeeland, and smelly

Chenopodium [3]. It is native to tropical America and is now widely distributed across temperate, subtropical, and tropical countries [3]. The extracts of roots, leaves and anthotaxis of *C. ambrosioides* have been used by the local people as dietary flavorings and as Chinese medicines for hundreds of years [4].

C. ambrosioides has anti-rheumatic, insecticidal, and analgesic effects [5]. It is commonly used in the treatment of skin rheumatism, skin eczema, dysmenorrhea, amenorrhea, snake bites, and other diseases [5]. Currently, *C. ambrosioides* is known for its anti-molluscocidal, anti-fungal, anti-infection, anti-oxidation, and anti-tumor effects [3]. At present, it has been reported that whether the *C. ambrosioides* essential oil and flavanoids are cytotoxic to SMMC-7721 cells [6] and MCF-7 cells and ultimately induce apoptosis in such cells [7]. However, the mechanism for this cytotoxicity remains unclear.

Apoptosis usually occurs in multicellular organisms. The process is split into the internal pathway and the external pathway [8]. The internal pathway is also known as the mitochondrial apoptotic pathway, and many studies have shown that when this pathway is activated, cell survival depends largely on the functional status of the mitochondria [9]. The external pathway is in charge of surveying the conditions of the extracellular and intracellular environments to indicate whether the cell should survive or die [10,11]. The function of the endoplasmic reticulum (ER) is to fold and embellish proteins during the synthesis of proteins [12].

ER pressure is the outcome of various internal and external pressures, and it causes apoptosis when all attempts to adapt to stress fail [13]. Previous studies have shown that two main components of the essential oil under study, 1-isopropyl-4-methylbenzene and a-terpinene, are not as good as essential oil in their anticancer activity [14]. Therefore, this study, further explores the molecular mechanism of apoptosis induced by the essential oil of *C. ambrosioides* in SMMC-7721 cells.

EXPERIMENTAL

Materials

C. ambrosioides plants were gathered from the street of Jingju Temple, Chengdu, Sichuan in September 2016 and confirmed as *C. ambrosioides* by Dr. Ma Danwei, College of Life Science, Sichuan Normal University, China. It is now stored in the Cell Biology Laboratory of the College of Life Sciences, Sichuan Normal University.

Preparation of extract of *C. ambrosioides* essential oil

The gathered plants were shade-dried in a cool place. The *C. ambrosioides* essential oil was obtained by steam distillation [15], then dried (anhydrous sodium sulfate) and filtered (0.22 μm membrane). Finally, the extracted oil was stored in a brown agentia bottle sealed at -20 ºC.

Cell culture

SMMC-7721 cells were provided by the State Key Laboratory of Sichuan University and cultured in RPMI-1640 medium supplemented with fetal bovine serum and antibiotics. Cells were grown and kept at the 37 ℃ incubator aerated with 5 % CO_2.

Evaluation of cytotoxicity

SMMC-7721 cells (1 × 10^5 cells /mL, 100 μL/hole) were inoculated in a 96-orifice plate for 20 h in moist air at 37 ℃ and 5 % CO_2. Then, the cells were attached to the tablet and treated with *C. ambrosioides* essential oil using 5 concentrations (6.25, 12.5, 25, 50, and 100 μg/mL). 1 % dimethyl sulphoxide (DMSO) treated cells as the negative control group, and 80 μg/mL fluorouracil treated cells as the positive control group. After treatment at 24, 48, and 72 h with essential oil, 20 μL MTT (5 mg/mL) was added to the culture solution and incubated for 4 h. Then, the culture-medium was removed and 150 μL DMSO was added to each pore. After 10 min of oscillation, absorbance (A) was measured at 490 nm. All experiments were conducted in triplicate. Cell viability (V) was calculated as in Eq 1.

V (%) – (Ae/Ad)100 …….. (1)

where Ae and Ad are the absorbance of essential oil- and DMSO-treated cells, respectively.

Assessment of apoptosis via AO/EB staining

Single-layer SMMC-7721 cells were incubated in the presence and absence of the essential oil derived from *C. ambrosioides* at various concentrations. After 24 h, the cell suspension was mixed with the AO/EB solution (Both AO and EB are 100 μg/mL). When viewed with a fluorescence microscope, living cells with AO/EB staining were green, necrotic cells were red,

early apoptotic cells were green, and late apoptotic cells were red.

Examination of cell ultrastructure by transmission electron microscopy (TEM)

SMMC-7721 cells (1.5 × 10^5 cells /mL) were inoculated in six-orifice plates. After 20 h of incubation, the cells were transferred into in 4 mL fresh RPMI-1640 culture-medium containing *C. ambrosioides* essential oil of 100 µg/mL. The negative control group cells contained 1 % DMSO. The cells were incubated at 37 ℃, in 5 % CO_2 for 24 h.

The treated cells were pre-fixed in electron microscopy fixative at 4℃ for 2 h, washed with PBS for 20 min, treated with 1 % osmium tetroxide at 4 ℃ for 1 h, dewatered with a gradient set of ethanol and buried in epon. The ultra-thin slices were double dyed with saturated acetic acid solution and lead acetate, and Tecnai transmission electron microscope (Most Technology Development Co. Ltd, Beijing) was used to observe the cells using 200 kV [16].

Assessment of mitochondrial membrane potential

SMMC-7721 cells (3 × 10^5/mL) were grown in six-orifice plates, then with increasing concentration of essential oil (12.5, 50, 100 µg/mL) and processed, as above. The negative control had 1 % DMSO, while the mitochondrial electron transport chain inhibitor carbonylcyanide-p-chlorophenyl hydrazone (CCCP) served as the positive control. After incubation at 37 ℃ in 5 % CO_2 for 24 h, the treated cells were washed with PBS and stained with 1 mg/mL tetraethylbenzi-midazolylcarbocyanine iodide (JC-1, Biyun days) dye was used for half an hour at 37 ℃ in the darkness [17]. The reduction of cell mitochondrial depolarization was detected, as shown by the transformation from red fluorescence to green fluorescence, and imaged under inverted fluorescent microscope [18].

Western blot assay

The essential oil derived from *C. ambrosioides* (12.5, 50, 100 µg/mL) or carrier-treated cells were lysed on ice in the presence of a complete protease inhibitor mixture in RIPA buffer and centrifuged at 12,000 rpm for 10 min. Bicinchoninic acid (BCA) protein assay kit (KGP903 Kaiji, Biological Company, Nanjing) was used to determine protein concentration. The same amount of protein specimens (100 µg) were electrophoresed on SDS-polyacrylamide gel, then blotted onto the PVDF membrane (Hybond company, USA).

PVDF membrane was incubated for 1 h in 5 % skimmed milk powder at 4 ℃, overnight with antibodies to: Cytochrome c (dilution 1:2000, ab133504), Bax (dilution 1:2000, ab32503), Bcl2 (dilution1:200, ab32124), Caspase 3 (dilution1:200, ab44976), Caspase 9 (dilution1:200, ab69514), Caspase 12 (dilution 1:500, ab62484), β-actin (dilution1:5000), and rabbit polyclonal anti-actin (all the antibodies were purchased from Abcam Abbott (Shanghai) Trading Co., Ltd., USA). After washing with PBST, the PVDF membranes were incubated in anti-rabbit IgG (H + L) of goat with biotin (dilution1:5000, ab6721) and biotinylated anti-mouse IgG (H + L) in goats (dilution1:5000, ab6789) secondary antibodies (Abcam Abbott Trading Co., Ltd., USA) for 2-3 h at room temperature.

Finally, after three 10 min of washing, immunoreactive signals were detected using an ECL detection system. These membranes were then exposed at various time points to ensure the best density. The relative protein levels were checked using β-actin as an internal standard. The experiments were repeated 3 times.

Statistical analysis

SPSS 17.0 (SPSS Inc, USA) was used for data analyses. Significant difference was analyzed by least significant difference (*LSD*) test, and $p < 0.05$ was deemed statistically significant. Correlation was analyzed via bivariate method. All data are presented as mean ± standard deviation (SD). Data were plotted using Microsoft Excel 2003 (Microsoft, USA).

RESULTS

Cytotoxic activity against SMMC-7721 cancer cells

Figure 1 shows the cytotoxic effects of *C. ambrosioides* essential oil on SMMC-7721 cell line. Time- and concentration-dependent inhibition were observed at 24, 48 and 72 h with IC_{50} 26.28, 12.50 and 7.49 µg/mL respectively. During the 24 h period of treatment, the cell viability percentage values at 6.25 and 12.5 µg/mL concentrations of the essential oil reduced cell viability to 94 and 72 %, respectively compared with the control. They were further reduced to 43, 32 and 16 % at concentrations of of 25, 50, and 100 µg/mL, respectively.

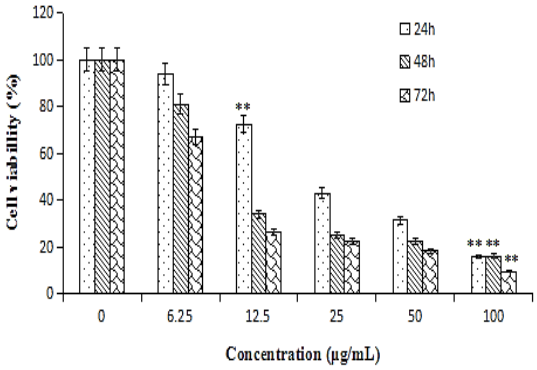

Figure 1: Cell viability between the SMMC-7721 cancer cell and the sample concentration was evaluated by MTT assay to evaluate the cytotoxic potential of essential oil compared with fluorouracil; the data are expressed as mean ± SEM (n = 3); *p < 0.05, **p < 0.001 compared to control group (1 % DMSO)

Effect of *C. ambrosioides* essential oil on apoptosis of SMMC-7721 cells

Figure 2 shows the apoptosis of SMMC-7721 cells caused by 24 h of *C. ambrosioides* essential oil treated at concentrations of 12.5, 50, and 100 µg/mL. With increase of essential oil concentration, the number of late apoptotic cells increased. This indicates that the essential oil can lead to apoptosis of SMMC-7721 cells.

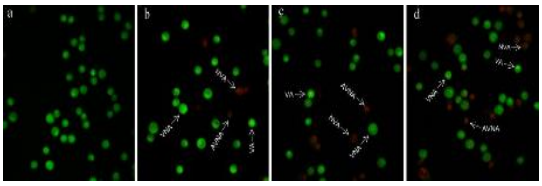

Figure 2: Morphological features of *C. ambrosioides* essential oil-treated SMMC-7721 cells via AO/EB double staining. 1 % DMSO (a), 12.5 µg/mL (b), 50 µg/mL (c), and 100 µg/mL (d) essential oil VNA indicates viable non-apoptotic cells; VA indicates the early apoptotic cells; NVA indicates late apoptotic cells; NVNA indicates necrotic cells

Effect of *C. ambrosioides* essential oil on ultrastructure of SMMC-7721 cells

The electron microscopy results showed that chromatin were distributed along the nuclear membrane in the SMMC-7721 cells treated with 100 µg/mL essential oil for 24 h contrasted with that of untreated control cells (Figure 3a). They also showed swollen mitochondria that had become spherical-shaped, a ruptured mitochondria ridge, a faded matrix, and vacuoles of various sizes in the cytoplasm (Figure 3b).

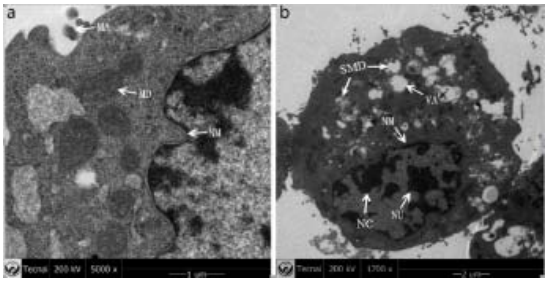

Figure 3: Effect of *C. ambrosioides* essential oil on ultrastructure of SMMC-7721 cells. After 24 h of treatment with 1 % DMSO (a) and 100 µg/mL essential oil (b), the ultrastructure of SMMC-7721 cells was observed by transmission electron microscopy. CM indicates cell membrane; MA indicates microvilli; NM indicates nuclear membrane; NU indicates nucleoli; MD indicates mitochondria; VA indicates empty bubble; SMD indicates swelling mitochondria; NC indicates nuclear chromatin

Effect of essential oil on mitochondrial membrane potential in SMMC-7721 cells

From the supplementary photo taken with inverted fluorescence microscope (Figure 4), it is evident that the decrease of the mitochondrial depolarization in the cells exposed to essential oil and the transition from red to green fluorescence, is different in intensity in a concentration- dependent manner.

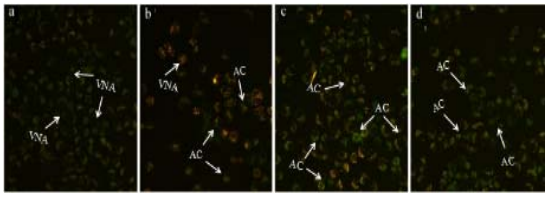

Figure 4: Effect of *C. ambrosioides* essential oil on mitochondrial membrane potential in SMMC-7721 cells. 1 % DMSO (a), 12.5 µg/mL essential oil (b), 50 µg/mL essential oil (c), 100 µg/mL essential oil (d) were treated for 24 h, to induce apoptosis in the SMMC-7721 cells. VNA indicates viable non-apoptotic cells; AC indicates apoptotic cells

Effect of the essential oil on expression of apoptosis-associated proteins

Mitochondria mediated the role of mitochondrial pathway in essential oil induced apoptosis by studying the release of mitochondrial Cytochrome C into the cytoplasm, the consequent increase of Caspase-9 and Caspase-3 activity, and the mitochondrial apoptotic balance of protein Bax and antiapoptotic protein Bcl-2. As shown in Figure 5, along with the increase of essential oil, the activities of Caspase-9, Caspase-3, and Caspase-12 gradually increased as well. The

western imprinting analysis also revealed that Cytochrome C released by mitochondria from SMMC-7721 cells increased after exposure to essential oil. This is because Cytochrome C activities Caspase-9 thereby triggering a cascade of apoptotic cells, activation of Caspase-3 which promotes apoptosis [8]. We also examined the activities of Bax and Bcl-2 by western blot analysis. This western blot analysis revealed that after essential oil administration the activity of proapoptotic Bax was raised, while the activity of Bcl-2 protein reduced.

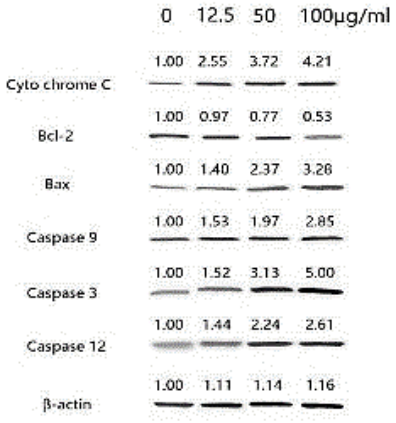

Figure 5: Western blot data for cytochrome C, Bcl-2, Bax, Caspase 9, Caspase 3, and Caspase-12 in SMMC-7721 cells treated with different concentration of *C. ambrosioides* essential oil for 24 h; β-actin was used as an internal control

DISCUSSION

Natural plant products serve a vital function in the treatment of many diseases, and they are continually being studied to identify novel therapeutic agents [17]. Liver cancer is among the most common pernicious tumors in China. Liver cancer cells are not sensitive to chemotherapy and are prone to drug resistance, and because conventional chemotherapy drugs damage normal cells, the treatment of hepatocellular carcinoma is difficult [18,19]. Apoptosis is controlled by genetic material, and cells follow their own pathway to voluntarily undergo the death process [20].

Many studies have shown that the activation of apoptotic pathway by natural products in cancer cells can serve as a main protective mechanism to the occurrence development and progression of cancer [21,22]. In this study, the results of AO/EB staining showed that the nuclei of SMMC-7721 cells exposed to *C. ambrosioides* essential oil exhibited nuclear condensation and fragmented chromatin typical of apoptosis in a dose-dependent manner. The change of MMP is a significant characteristic of apoptosis [23]. JC-1 staining demonstrated that the essential oil resulted in the decrease of MMP in SMMC-7721 cells. Under transmission electron microscopy, swollen mitochondria appeared in SMMC-7721 cells treated with the essential oil. These outcomes indicate that essential oil-induced apoptosis originated from to the mitochondria (Figure 6).

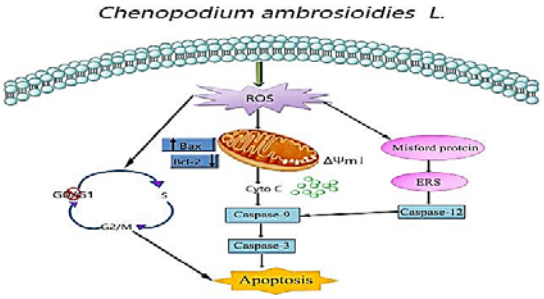

Figure 6: The mechanism of *Chenopodium ambrosioides* L. essential oil inducing apoptosis in an SMMC-7721 cell

Previous research had shown that *C. ambrosioides* essential oil induced apoptosis was caspase-dependent using the caspase inhibitor Z-VAD-FMK in SMMC-7721 cells, and that the essential oil also caused G_0/G_1 phase arrest, inducing SMMC-7721 cell death [6]. The essential oil and its main components, α-terpinene and 1-isopropyl-4-methylbenzene, can induce ROS production, interfere with antioxidant activity, and cause oxidative damage [23]. In this study, the activities of Cytochrome C, Caspase-9, Caspase-3, and proapoptotic Bax increased and that of Bcl-2 decreased in SMMC-7721 cells treated with the essential oil. Thus, the essential oil induces ROS generation, which promotes MMP loss, and this leads to Bcl-2 and the expression of Bax levels to change, and then releases Cytochrome C, which activates Caspase-9 and Caspase-3 to induce SMMC-7721 cell apoptosis (Figure 6).

Endoplasmic reticulum stress is a physiological response that is part of a cell's resistance to adverse stimuli, but prolonged endoplasmic reticulum stress will activate Caspase-12 and then activate Caspase-9 and Caspase-3 cascade reactions, inducing apoptosis [24]. In this study, as the concentration of essential oil increased, the expression level of Caspase-12 protein increased in SMMC-7721 cells. This suggests that apoptosis induced by the essential oil in SMMC-7721 cells could also be through the endoplasmic reticulum stress pathway (Figure 6). The specific mechanism requires further study.

CONCLUSION

The findings of this work show that the cytotoxic activity of *Chenopodium ambrosioides* L. essential oil against human hepatocellular carcinoma SMMC-7721 cell line is pronounced and that it induces apoptosis via mitochondrial-mediated and endoplasmic reticulum stress pathways. Thus, the essential is capable of being developed into a drug for the treatment of liver cancer.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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