Antitumor activity of 2-[(2E)-3,7-dimethyl-2,6-octadienyl]-6-methyl-2,5-cyclohexadiene-1,4-dione isolated from the aerial part of *Atractylodes macrocephala* in hepatocellular carcinoma

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Abstract. 2-[(2E)-3,7-dimethyl-2,6-octadienyl]-6-methyl-1,2,5-cyclohexadiene-1,4-dione (DMD) is a compound isolated from *Atractylodes macrocephala*; however, its antitumor activity has not yet been investigated. Therefore, the present study aimed to investigate the antitumor activity of DMD in the H22 mouse hepatocellular carcinoma (HCC) cell line *in vitro* and *in vivo*. In the present study, the antiproliferative effects of DMD against H22 cells were evaluated using the MTT assay *in vitro*. Furthermore, xenograft nude mice were established to evaluate the antitumor effects of DMD on H22 cells *in vivo*. In addition, apoptosis of H22 cells was determined by flow cytometry with Annexin V-fluorescein isothiocyanate/propidium iodide staining, and western blotting was subsequently performed to examine the expression levels of proteins associated with apoptosis, and c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinases (MAPKs). The results demonstrated that DMD exerts an antitumor effect against H22 cells *in vitro* and *in vivo*, and the underlying mechanism may be associated with mitochondria-mediated apoptosis through upregulation of cytochrome c, cleaved (c)-caspase-3, c-caspase-9, c-caspase-7 and B-cell lymphoma 2 (Bcl-2)-associated X protein, and downregulation of Bcl-2. In addition, the antitumor effects of DMD against H22 cells may be also associated with the MAPK signaling pathway via increased p-JNK and reduced p-ERK1/2 expression. In conclusion, the present study demonstrated the DMD exerts antitumor effects against HCC in mice and provides a scientific basis for the clinical use of DMD for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common forms of liver cancer and is one of the major causes of mortality among patients with chronic liver disease worldwide (1,2). It has been reported that the mean survival rate of HCC is <5% and HCC is the third most common cause of cancer-associated mortality worldwide (3,4). In addition, HCC is one of the most common and malignant cancers, with an increasing incidence rate, particularly in Europe and East Asia (5). At present, chemotherapy with synthetic drugs is commonly used to treat HCC; however, serious side effects are associated with this treatment (6). HCC also exhibits a poor response to treatment modalities and resistance to systemic chemotherapy (7). Therefore, there is an urgent requirement to develop novel anti-HCC agents with improved therapeutic effects.

Traditional Chinese medicine (TCM) has been used for centuries, and it has been reported that plant-derived medicines are safer than synthetic drugs (8). In addition, TCM has been reported to be effective in the treatment of various diseases, particularly those that cannot be treated by modern synthetic drugs (9). The rhizome of *Atractylodes macrocephala*, a recognized herbal medicine in China, has been commonly used to treat conditions including edema, spleen-deficiency, diarrhea and abdominal distention (10). Various other effects of the rhizome of *A. macrocephala* have also been described, including antibacterial, anti-aging and antitumor effects (10). It has previously been reported that 2-[(2E)-3,7-dimethyl-2,6-octadienyl]-6-methyl-2,5-cyclohexadiene-1,4-dione (DMD), the structure of which is presented in Fig. 1, is a compound isolated from the aerial part of *A. macrocephala* (APM), however, its antitumor activities have not yet been investigated (11).

The present study obtained a quantity of DMD from the APM and investigated its antitumor effects against H22 cells *in vitro* and *in vivo*, and explored the potential underlying pharmacological mechanism to provide a scientific basis for the clinical use of DMD in the future.
Materials and methods

Chemicals. Dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RPMI-1640 medium and fetal bovine serum were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cleaved (c)-caspase-3 (cat. no. ab32499), c-caspase-9 (cat. no. ab2324) and c-caspase-7 (cat. no. ab69540) antibodies were purchased from Abcam (Cambridge, MA, USA). Cytochrome c (cat. no. ab13575), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. ab32503), Bcl-2 (cat. no. ab32124), c-Jun N-terminal kinase (JNK; cat. no. ab76125), phosphorylated (p)-JNK (cat. no. ab4821), extracellular signal-regulated kinase (ERK)1/2 (cat. no. ab17942), p-ERK1/2 (cat. no. ab200807), p38 (cat. no. ab31828), p-p38 (cat. no. ab47363) and GAPDH (cat. no. ab8245) antibodies were purchased from Abcam (Cambridge, MA, USA). Bicinchoninic acid (BCA) protein assay reagent was purchased from Beyotime Institute of Biotechnology (Haimen, China). Silica-gel (100-200 mesh) was purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals used in this study were of analytical reagent grade.

Animals. A total 12 BALB/C nude mice (5-6 weeks old; 8-9 g), were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). The animals were maintained under controlled conditions (21±1˚C and 30-70%) with a 12-h light/dark cycle and free access to food and water. All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals (12), and the experiments were performed with the approval of the Animal Experimentation Ethics Committee of The First Hospital of Lanzhou University (Lanzhou, Gansu, China).

Preparation of DMD. The APM was collected from Jiande, China in August 2014. The dried APM was powdered and extracted five times with 75% ethanol by percolation extraction for 3 days each time. The solvent was evaporated under vacuum to obtain the crude extract. Subsequently, the crude extract was suspended in water and partitioned with petroleum ether, ethyl acetate (EtOAc) and n-butanol sequentially. The EtOAc fraction was subjected to repeated column chromatography over silica gel (100-200 mesh) and eluted with petroleum ether-EtOAc (97%:3%, 90%:10%, 80%:20%, 70%:30% and 50%:50%). Combination of similar fractions on the basis of thin layer chromatography (TLC) analysis afforded 5 fractions (I-V). The TLC spots were visualized using a ZF-1 Ultraviolet analyzer (Shanghai King Tech Industry Co., Ltd., Shanghai, China) at 254 nm and 365 nm using the sulfuric acid-alcohol chromogenic agent. As described previously (11), DMD was isolated from fraction IV using further column chromatography over a silica gel (200-300 mesh) and eluted with petroleum ether-EtOAc (50%:50%).

Identification of the compound. The isolated DMD was identified by 1H-nuclear magnetic resonance (NMR) and 13C-NMR. The 1H-NMR and 13C-NMR spectrum data of this compound are as follows: Brown oil; 1H-NMR (CDCl3, 600 MHz, J/Hz) δ: 6.62 (1H, m, H-6), 6.57 (1H, m, H-2), 5.11 (1H, t, J=7.3 Hz, H-2'), 5.03 (1H, t, J=6.8 Hz, H-6'), 3.17 (2H, d, J=7.3 Hz, H-2'), 2.18 (2H, m, H-2'), 2.13 (5H, m, H-2' and H-3'), 1.74 (3H, s, H-9'), 1.67 (3H, s, H-10'), 1.64 (3H, s, H-8'); 13C-NMR (CDCl3, 150M Hz) δ: 189.52 (C-1), 182.54 (C-4), 148.81 (C-2), 143.59 (C-6), 134.96 (C-3'), 132.96 (C-3), 131.12 (C-5), 130.42 (C-7'), 122.43 (C-6'), 113.56 (C-2'), 40.11 (C-4'), 28.01 (C-1'), 25.99 (C-5'), 25.01 (C-9'), 18.03 (C-8'), 16.03 (C-10'), 14.93 (C-7'). According to these spectral data, physicochemical properties and a reported study (13), the compound was identified as DMD.

Cell culture and determination of cell viability. Human HL-60 myeloid leukemia, A549 lung cancer, MCF-7 breast cancer, HCT-8 colon cancer and HeLa cervical carcinoma cell lines, and the H22 mouse HCC cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cell lines were maintained at 37°C in an atmosphere containing 5% CO2/95% air.

Cell viability was determined using the MTT assay according to the previously reported method (14). Cell suspension (100 µl; 5x104 cells/ml) were seeded in 96-well plates and cultured for 24 h at 37°C. Cells were then treated with DMD at various concentrations (10, 20, 40, 60, 80 and 100 µg/ml) and cultured for 24 h at 37°C. The control cells were cultured without DMD treatment for 24 h at 37°C. Subsequently, the MTT was performed to determine the percentage of cell proliferation inhibition (n=4) by detecting the optical density (OD) at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The half maximal inhibitory concentration (IC50) values of DMD on HL-60, A549, MCF-7, HCT-8, HeLa and H22 cells were calculated. In addition, to investigate the dose-dependent and time-dependent effects of DMD, H22 cells were treated with DMD (15, 30 and 60 µg/ml) for 12, 24, 36 and 48 h. The inhibitory rate was calculated according to the following formula: [(ODcontrol-ODtreatment)/ODcontrol] x 100.

Xenograft model in mice. To evaluate the antitumor effects of DMD against H22 cells in vivo, mice were divided into two groups (n=6/group), including control and DMD (40 mg/kg) groups. Nude mice were subcutaneously injected in the back on the right-hand side with H22 cells (0.2 ml; 1x107 cells per mouse). Once the tumors had grown to 2-3 mm in diameter, mice were treated intraperitoneally with DMD (40 mg/kg/day) for 20 days and an equal volume of solvent control (0.5% DMSO). Mice were observed over 20 days, and tumor sizes were measured every 5 days after the tumor inoculation. Tumor diameters were determined using a vernier caliper, and the tumor volumes were calculated according to the following formula (9): Volume = (width2 x length)/2.

Apoptosis assay. Apoptotic cells were detected by flow cytometry on a FACSCalibur cytometer (BD Biosciences). Firstly, 2 ml H22 cells (5x105/ml) were seeded in 6-well
plates for 24 h at 37°C. On the following day, the cells were treated with 15, 30 and 60 µg/ml DMD. After 48 h, cells were trypsinized, washed with PBS and stained using the Annexin V-FITC/PI kit (200 µl Annexin V-FITC and 10 µl PI for every 1x10^5 cells), according to the manufacturer's protocol.

**Western blotting.** Cells were treated with DMD (15, 30 and 60 µg/ml) for 24 h at 37°C. Cells (5x10^6) were then harvested and homogenized with lysis buffer for 10 min and centrifuged at 4°C for 5 min (10,000 x g). Total protein was extracted from cells using the cell lysis buffer for western blotting and IP (Beyotime Institute of Biotechnology; cat. no. P0013), and the protein concentration was determined by one-way analysis of variance followed by a Dunnett’s multiple comparisons post hoc test using SPSS software (SPSS for Windows v19.0; IBM Corp., Armonk, NY, USA). To normalize for protein loading, the expression was expressed relative to GAPDH.

**Statistical analysis.** Data are presented as the mean ± standard deviation and the significance of differences between groups was determined by one-way analysis of variance followed by a Dunnett's multiple comparisons post hoc test using SPSS software (SPSS for Windows v19.0; IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**IC_{50} values of DMD on HL-60, A549, MCF-7, HCT-8, HeLa and H22 cell lines.** The antiproliferative effects of DMD on HL-60, A549, MCF-7, HCT-8, HeLa and H22 cell lines were investigated, and the results are presented in Table I. The results demonstrated that DMD exhibited an antiproliferative effect against the H22 cell line, and the IC_{50} value was 44.29±3.97 µg/ml, which is <50 µg/ml. Furthermore, DMD displayed moderate antiproliferative activities against HL-60 and A549 cell lines (IC_{50} values were 87.43±3.12 µg/ml and 68.19±1.08 µg/ml, respectively). However, no obvious antitumor effects were observed against MCF-7, HCT-8 and HeLa cell lines (IC_{50} >100 µg/ml).

**Inhibitory effects of DMD against H22 cells in vitro and in vivo.** The antitumor activity of DMD against H22 cells in vitro and in vivo was evaluated in the present study. The results demonstrated that DMD possesses significant cytotoxicity against H22 cells, as cell viability was significantly reduced, compared with control cells following treatment with 15 µg/ml (P<0.05), 30 µg/ml (P<0.01) and 60 µg/ml (P<0.01) DMD, and viability was reduced in a concentration-dependent manner (Fig. 2A). In addition, DMD (15, 30 and 60 µg/ml) also exhibited time-dependent cytotoxic effects against H22 cells (Fig. 2B). Furthermore, the antitumor activity of DMD against H22 cells in vivo was further evaluated in a mouse xenograft model. As demonstrated in Fig. 2C, DMD (40 mg/kg) significantly inhibited tumor growth, compared with the control group (P<0.01).

**Proapoptotic effects of DMD on H22 cells.** The aforementioned results demonstrated that DMD exhibits notable antitumor activity against H22 HCC cells. To determine whether the antitumor activity of DMD resulted from induction of apoptosis, apoptotic H22 cells were detected by staining with Annexin V-FITC/PI followed by flow cytometry. As presented in Fig. 3, the percentage of apoptotic cells increased gradually when treated with increasing concentrations of DMD (15, 30 and 60 µg/ml), and the percentage was significantly increased, compared with control cells at all doses (P<0.01). These results indicated that DMD may induce H22 cell death by inducing apoptosis.

**Exposure of H22 cells to DMD results in upregulation of cytochrome c, c-caspase-3, c-caspase-9, c-caspase-7 and Bax, and downregulation of Bcl-2.** To investigate the potential mechanism by which DMD induces apoptosis of H22 cells, the protein expression levels of cytochrome c, c-caspase-3, c-caspase-9, c-caspase-7, Bax and Bcl-2 in H22 cells were detected. As demonstrated in Figs. 4-6, the expression levels of cytochrome c (P<0.01), c-caspase-3 (P<0.01), c-caspase-9 (P<0.01) and Bax (P<0.01) were significantly upregulated following treatment with DMD (15, 30 and 60 µg/ml) in a concentration-dependent manner. In addition, c-caspase-7 expression was upregulated in response to DMD, in a concentration-dependent manner. C-caspase-7 was significantly upregulated following treatment with 30 µg/ml (P<0.05) and 60 µg/ml DMD (P<0.01), compared with the control group; however, no significant difference was observed in c-caspase-7.
Apoptosis, which is a form of programmed cell death, is considered to be a major apoptotic pathway, and is a physiological cell suicide process that is regulated by the Bcl-2 family proteins (14). Apoptosis is a process that can be induced by a variety of factors, including DNA damage, oxidative stress, and the release of cytochrome c from mitochondria (15). The Bcl-2 family proteins play a crucial role in regulating the balance between cell survival and cell death. They form a complex that controls the mitochondrial membrane potential and the release of pro-apoptotic factors, such as cytochrome c (16).

In the present study, we investigated the antitumor activity of DMD isolated from A. macrocephala. DMD significantly inhibited the proliferation of H22 HCC cells in vitro (Fig. 2A). The IC50 values of DMD on various cancer cell lines are presented in Table I. DMD also induced apoptosis in H22 cells in vivo (Fig. 2B). The effects of DMD on p38, p-p38, JNK, p-JNK, ERK1/2, and p-ERK1/2 protein expression were examined (Fig. 3). DMD significantly upregulated the expression levels of p-JNK (P<0.01) and reduced the expression levels of p-p38, p-p38, p-JNK, and p-ERK1/2 in a concentration-dependent manner, compared with the control group (P<0.01). These results indicated that DMD-induced apoptosis of H22 cells may be associated with the mitochondria-mediated apoptotic pathway.

### Discussion

The present study systematically investigated the antitumor activity and mechanism underlying the effects of DMD isolated from the aerial part of APM on a HCC cell line in vitro and in vivo. The results indicated that DMD exhibited an antitumor effect against the H22 HCC cell line in vitro and in vivo, which may be associated with mitochondria-mediated intrinsic apoptosis and the MAPK pathway.

It has previously been reported that uncontrolled cell proliferation and insufficient apoptosis may be regarded as the leading causes behind the development of cancer (15). Apoptosis, which is a form of programmed cell death, is a physiological cell suicide process that is regulated by several proteins (16). In addition, it is considered to be an ideal target for cancer therapy (17). Mitochondria-mediated apoptosis is considered to be a major apoptotic pathway, and this pathway relies on the Bcl-2 family proteins to control the cell death process.

### Table I. IC50 values of DMD on various cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (µg/ml)</th>
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<tbody>
<tr>
<td>HL-60</td>
<td>87.43±3.12</td>
</tr>
<tr>
<td>A549</td>
<td>68.19±1.08</td>
</tr>
<tr>
<td>MCF-7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HCT-8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HeLa</td>
<td>&gt;100</td>
</tr>
<tr>
<td>H22</td>
<td>44.29±3.97</td>
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</tbody>
</table>

Cells were treated with DMD for 24 h and cell viability was determined by MTT assay. Data are presented as the mean±standard deviation. DMD, 2-((2-methyl-2,5-cyclohexadiene-1,4-dione; IC50, half maximal inhibitory concentration.

### Figure 2. Antitumor effects of DMD against H22 cells in vitro and in vivo.

(A) Inhibitory effects of DMD on the proliferation of H22 cells. Cells were treated with DMD at three concentrations (15, 30 and 60 µg/ml) for 24 h and cell viability was determined by MTT assay. (B) Cells were treated with DMD (15, 30 and 60 µg/ml) for 12, 24, 36 and 48 h, and cell viability was determined by MTT assay. (C) Tumor growth curves of xenograft mice injected with H22 cells and treated with DMD (40 mg/kg/day, i.p.) for 20 days or solvent control. Data are presented as the mean±standard deviation (n=4). *P<0.05 and **P<0.01 vs. the control group. DMD, 2-([2E]-3,7-dimethyl-2,6-octadienyl]-6-methyl-2,5-cyclohexadiene-1,4-dione.

### Effects of DMD on p38, p-p38, JNK, p-JNK, ERK1/2 and p-ERK1/2 protein expression.

To investigate other potential mechanisms of DMD action on H22 cells, the expression levels of proteins in the mitogen-activated protein kinase (MAPK) pathway were examined, including p38, JNK, ERK1/2, p-p38, p-JNK and p-ERK1/2. As presented in Fig. 7, the effects of DMD on p38, p-p38, JNK and ERK1/2 were not significant (P>0.05). However, DMD (15, 30 and 60 µg/ml) significantly upregulated the expression levels of p-JNK (P<0.01) and reduced the expression of p-ERK1/2 (P>0.01) dose-dependently, compared with the control group. Therefore, DMD-mediated apoptosis of H22 cells may also be associated with the MAPK apoptotic pathway.

### Figure 3. Effects of DMD on p38, p-p38, JNK, p-JNK, ERK1/2 and p-ERK1/2 protein expression.

The results indicated that DMD exerted an antitumor effect against the H22 HCC cell line in vitro and in vivo, which may be associated with mitochondria-mediated intrinsic apoptosis and the MAPK pathway.

The present study systematically investigated the antitumor activity and mechanism underlying the effects of DMD isolated from the aerial part of APM on a HCC cell line in vivo and in vitro. The results indicated that DMD exhibited an antitumor effect against the H22 HCC cell line in vitro and in vivo, which may be associated with mitochondria-mediated intrinsic apoptosis and the MAPK pathway.

It has previously been reported that uncontrolled cell proliferation and insufficient apoptosis may be regarded as the leading causes behind the development of cancer (15). Apoptosis, which is a form of programmed cell death, is a physiological cell suicide process that is regulated by several proteins (16). In addition, it is considered to be an ideal target for cancer therapy (17). Mitochondria-mediated apoptosis is considered to be a major apoptotic pathway, and this pathway relies on the Bcl-2 family proteins to control the cell death process.

Bcl-2 family proteins have important roles in mitochondria-mediated apoptosis, and are considered to be the initial regulatory step in the induction of mitochondrial apoptosis (17). Bcl-2 and Bax are recognized as apoptosis-associated proteins.
Bcl-2 directly binds and suppresses the proapoptotic proteins of the Bcl-2 family; however, Bax directly causes cytochrome c to be released into the cytoplasm or inhibits antiapoptotic Bcl-2 proteins (18). To explore the potential mechanism underlying DMD-induced apoptosis of the H22 cell line, the present study determined the expression of various proteins associated with the mitochondria-mediated apoptotic pathway, including cytochrome c, c-caspase-3, c-caspase-9, c-caspase-7, Bax and Bcl-2. The results indicated that DMD upregulated the expression of cytochrome c, c-caspase-3, c-caspase-9, c-caspase-7 and Bax, and downregulated the expression of Bcl-2, compared with control cells. Therefore, the results of the present study indicated that DMD may induce mitochondria-mediated apoptosis in the H22 mouse HCC cell line.

MAPKs are serine/threonine kinases that function in the regulation of a wide range of cellular processes, including proliferation, differentiation and apoptosis (9). MAPKs consist of three families: JNK, ERK and p38 MAPK (25,26). ERK is involved in functions that include cell proliferation and the prevention of apoptosis, and exhibits a cytoprotective role in the MAPK apoptosis pathway (27). Conversely, the JNK and p38 MAPK cascades are associated with proapoptotic effects (28).
Figure 6. Effects of DMD on the protein expression levels of Bax and Bcl-2. Total protein was extracted and detected by western blot analysis using antibodies against Bax and Bcl-2; GAPDH was used as an internal control. Data are presented as the mean ± standard deviation (n=4). **P<0.01 vs. the control group. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; DMD, 2-[(2E)-3,7-dimethyl-2,6-octadienyl]-6-methyl-2,5-cyclohexadiene-1,4-dione.

Figure 7. Effects of DMD on the protein expression levels of (A) p-p38, (B) p38, (C) p-JNK, (D) JNK, (E) p-ERK1/2, (F) ERK1/2 and (G) GAPDH. Total protein was extracted and detected by western blot analysis using antibodies against ERK1/2, p-ERK1/2, JNK, p-JNK, p38 and p-p38; GAPDH was used as an internal control. Data are presented as the mean ± standard deviation (n=4). **P<0.01 vs. the control group. DMD, 2-[(2E)-3,7-dimethyl-2,6-octadienyl]-6-methyl-2,5-cyclohexadiene-1,4-dione; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated-.
To investigate whether DMD-induced apoptosis of H22 cells was associated with the MAPK pathway, the present study detected the expression levels of JNK, ERK1/2 and p38 proteins, and the level of phosphorylation of these proteins. The results indicated that DMD significantly upregulated the expression levels of p-JNK and downregulated the expression levels of p-ERK1/2 dose-dependently, whereas DMD exhibited no obvious effect on JNK, ERK1/2, p38 and p-p38 protein expression, compared with control cells. These results indicated that DMD-mediated apoptosis may be associated with the MAPK pathway.

In conclusion, the results of the present study demonstrated that DMD isolated from APM exhibited a notable antitumor effect against H22 cells, and the potential underlying mechanism may be associated with mitochondria-mediated apoptosis via increased expression of cytochrome c, c-caspase-3, c-caspase-9, c-caspase-7 and Bax, and reduced expression of Bcl-2. In addition, the antitumor effects of DMD against H22 cells may also involve the MAPK pathway, as the results demonstrated increased p-JNK expression and reduced p-ERK1/2 expression, which serve proapoptotic and cytoprotective roles, respectively.

References