

Original Research Article

Apoptosis-inducing effect of 6,7-dimethoxy-4'-hydroxy-8-formylflavon from *Nicotiana tabacum* L leaf in human hepatoma HepG2 cells via activation of mitochondria-mediated apoptotic pathway

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Abstract

Purpose: To study the anti-proliferative and apoptotic influences of 6,7-dimethoxy-4'-hydroxy-8-formylflavon (DHF) from the leaves of *Nicotiana tabacum* L. in human hepatoma HepG2 cells, and the underlying mechanisms.

Methods: The anti-proliferative effect of DHF (10 - 50 µg/mL) on HepG2 cells was assessed by CCK-8 assay. The pro-apoptotic effect of DHF (10, 20 and 30 µg/mL) on HepG2 cells was investigated via flow cytometry, while the mechanisms involved were studied using western blot. Xenograft assay was employed for determination of the *in vivo* effect of DHF (40 mg/kg/day) on HepG2 cell-induced tumor.

Results: The proliferation of HepG2 cells was inhibited by DHF ($IC_{50} = 25.87 \mu\text{g/mL}$) due to apoptosis. In addition, xenograft assay revealed that HepG2 cell-induced tumor growth was significantly suppressed by DHF ($p < 0.05$ or 0.01) without any effects on mice body weights. The expressions of Survivin and Bcl-2 proteins were significantly decreased, while those of Bax, c-caspase-9, and c-caspase-3 proteins were significantly increased by DHF ($p < 0.05$ or 0.01), leading to increase in cytoplasmic levels of Smac and cytochrome c proteins.

Conclusion: The underlying mechanism DHF-mediated apoptotic changes in HepG2 cells *in vitro* and *in vivo* involves induction of the mitochondrial pathway of apoptosis. Thus, DHF is a good drug candidate for the development of an effective therapy for liver cancer.

Keywords: 6,7-Dimethoxy-4'-hydroxy-8-formylflavon, HepG2 cells, Hepatoma, Mitochondria, Apoptosis, Bax, Cytochrome C, Survivin

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INTRODUCTION

The liver performs important functions such as, metabolism, detoxification, deoxidation, glycogen

storage and synthesis of secretory proteins [1], but the capacity of the liver to carry out these functions may be seriously hampered by cancer. Nowadays, with increases in obesity, alcohol

consumption and smoking habits, the incidence of hepatoma has gradually increased [2-5]. Chemotherapy is an important therapy for treating hepatoma [6,7], and successful treatment is often a result of long-term use of chemotherapeutic drug. However, with prolonged use of a chemotherapeutic drug, its anti-hepatoma activity is often reduced or lost altogether, leading to the knotty medical problem of drug resistance. Indeed, drug resistance is a common problem in chemotherapy [8-10], although the problem is often effectively overcome by switching drug. In view of the problem associated with chemotherapy, it is important to develop new and effective anti-hepatoma drugs.

Since many flavonoids exhibit potential anticancer activities [11,12], it is feasible to find new and effective anti-hepatoma agents from flavonoids. A new flavonoid compound, 6,7-Dimethoxy-4'-hydroxy-8-formylflavon (DHF), was isolated from the leaves of *Nicotiana tabacum* L. It has been reported that DHF shows high cytotoxicity against human prostate cancer PC-3 cells and human lung adenocarcinoma A549 cells [13]. The aim of the present study was to investigate the effect of DHF on the proliferation of human hepatoma HepG2 cells, and the likely underlying mechanism.

EXPERIMENTAL

Plant material

The leaves of *Nicotiana tabacum* L. were obtained from Baoshan, Yunnan, China in October 2015 and identified by Wen-Sheng Qiu, a taxonomist. A voucher specimen (no. QDU2015116) was kept at the herbarium of Qingdao University for future reference.

Equipment, reagents and chemicals

Preparative HPLC was carried out on a Venusil MP C₁₈ column with Agilent 1200 preparative liquid chromatograph. Fetal bovine serum (FBS) and RPMI 1640 media were products of Invitrogen (Carlsbad, CA, USA). Extracts kits for total protein, mitochondrial protein and cytoplasmic protein were obtained from BioDee (Beijing, China), Nanjing Biobox Biotechnology Company (Nanjing, China), and Bangyi-Bio (Shanghai, China), respectively. Kit for assay of Enhanced BCA Protein, Cell Counting Kit-8 (CCK-8 kit), and kit for assay of Annexin V-FITC/PI apoptosis were products of Beyotime Biotechnology (Haimen, China). Cell Signaling Technology (Beverly, MA, USA) and Abcam (Cambridge, UK) were the suppliers of primary

antibodies for β -actin, COX IV, Survivin, Mcl-1, Bcl-xl, Bcl-2, Bax, cleaved(c)-caspase-9, c-caspase-3, Smac, cytochrome c as well as Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody. Acetone, methanol, chloroform and silica-gel (200 - 300 mesh) were products of Qingdao Haiyang Chemical Company (Qingdao, China), while dimethyl sulfoxide (DMSO) was supplied by Beyotime Biotech. (Haimen, China). All the solvents used were of analytical grade.

Animals

Five-to-six week old nude mice were obtained from SLRC Laboratory Animal Company (Shanghai, China). The mice were handled in line with provisions of the National Institute of Health Guide concerning the Care and Use of Laboratory Animals [14]. Study was approved by Qingdao University Animal Experimentation Ethics Committee (approval no. QDU2016051).

DHF extraction procedure

The leaves of *Nicotiana tabacum* L. were air-dried and powdered, and the resultant powder (10 kg) were stirred in 10 L of 70 % hydro-methanol. The extraction process was carried out in triplicate with different 10-kg portions of the powder, and the combined filtrate was subjected to low-pressure rotary evaporation to yield 989.5 g of crude methanol extract. This was applied on a 200-300 mesh silica gel column and eluted with systematic gradient of chloroform-methanol (volume ratios of 19:1, 9:1, 8:2, 7:3, 6:4 and 5:5). A total of 6 fractions were obtained. Fraction 2 (53.2 g) was further subjected to separation on silica gel column eluted with acetone-chloroform, followed by purification using high performance liquid chromatography (HPLC, preparative). These purification steps resulted in 40.2 mg of material suspected to be DHF. Then, HPLC, ¹³C-NMR and ¹H-NMR techniques were used to ascertain the purity and identity of the DHF. DHF was dissolved in 0.5 % DMSO to get different concentrations for following assays.

Culturing of HepG2 cells

HepG2 cells from the Shanghai Cell Institute Country Bank (Shanghai, China) were cultured in RPMI-1640 medium containing 10 % FBS and streptomycin/penicillin at 37 °C in a humidified atmosphere of 5 % CO₂.

Cell proliferation assay

The influence of DHF on HepG2 proliferation was determined using CCK-8 assay. The cells at a

density of 2×10^3 cells/well in 96-well plates were exposed to DHF at doses of 10 - 50 $\mu\text{g}/\text{mL}$ for 48 h, after prior incubation for 4 h. The control group received 0.5 % DMSO in place of DHF. At the end of 48-h incubation, CCK-8 solution (10 μL) was added to each well, followed by a further incubation period of 4 h. Thereafter, the absorbance of each well was read at 450 nm in a Bio-Rad Model 680 microplate reader (Hercules, CA, USA). The inhibition brought about by DHF was calculated as in Eq 1 [15].

$$\text{Inhibition (\%)} = \{(Abs_c - Abs_t)/Abs_c\}100 \dots\dots (1)$$

where Abs_c and Abs_t are the absorbance values of control and test samples, respectively.

Cell apoptosis analysis

Flow cytometry was used for determination of cell apoptosis. HepG2 cells were treated for 48 h with DHF at concentrations of 10, 20 and 30 $\mu\text{g}/\text{mL}$, while cells in the control group got 0.5 % DMSO in place of DHF. The cells were placed in staining buffer after harvesting and washing with PBS, and subjected to Annexin V-FITC/PI staining for 20 min in a dark chamber. Finally, the proportion of stained HepG2 cells was analyzed using a flow cytometer (BD Biosciences, MA, USA), and the apoptotic cells were determined by adding the populations of cells that were Annexin V-positive and Annexin V/PI-positive.

Western blot assay

HepG2 cells were exposed to DHF at concentrations of 10, 20 and 30 $\mu\text{g}/\text{mL}$ for 48 h, washed with PBS and harvested (cells in the control group were treated with 0.5 % DMSO in place of DHF). Then, total proteins, cytoplasmic proteins and mitochondrial proteins of the HepG2 cells were extracted separately using corresponding kits. The extracted total proteins were used to investigate the influence of DHF on the expressions of Survivin, Mcl-1, Bcl-xl, Bcl-2, Bax, c-caspase-9 and c-caspase-3. The cytoplasmic proteins and mitochondrial proteins of HepG2 cells were used to study the effects of DHF on the release of Smac and cytochrome c into the cytoplasmic space from the mitochondrial compartment. Enhanced BCA Protein Assay Kit was employed for protein concentration assay.

Equal amounts of proteins (40 μg) were subjected to electrophoretic separation using SDS/PAGE, and subsequently placed on PVDF membrane. The membranes were blocked using non-fat milk (5 %) and then incubated overnight at 4 °C with the appropriate primary antibodies,

and subsequently with HRP-conjugated goat anti-rabbit antibody for 2 h at room temperature. Finally, the proteins were detected by chemiluminescence. Protein loadings were normalized with β -actin and COX IV for total, cytoplasmic proteins, and mitochondrial proteins, respectively.

Xenograft studies

Two groups of nude mice (10 mice/group) were used. The right flank of mice in both groups were subcutaneously injected with HepG2 cells (2×10^6 cells/mouse) in order to induce tumor. At a point when the tumors attained sizes of about 3 mm, mice in the DHF group were given intraperitoneal injection of DHF (40 mg/kg/day), while the control mice received 0.5 % DMSO via the same route. The treatments lasted 20 days. The width (W) and length (L) of tumor were measured at 5-day intervals using Vernier caliper, while mice body weights were determined within the same interval with electronic balance. At the end of the treatment duration, the animals were sacrificed and the tumor tissues were excised and subjected to western blot assay. Tumor volume (TV) was estimated as in Eq 2 [16].

$$TV = (W^2 \times L)/2 \dots\dots\dots (2)$$

Statistical analysis

Data are presented as mean \pm SD, and differences between two groups were assessed using one-way analysis of variance (ANOVA) using SPSS software (version 21.0, SPSS, Inc., Chicago, USA). $P < 0.05$ was taken as indicative of statistically significant difference.

RESULTS

Purity and identification of DHF

The results of HPLC revealed that the purity of target analyte was more than 96 %. It was identified as DHF (Figure 1) through comparison of its NMR data with published data [13].

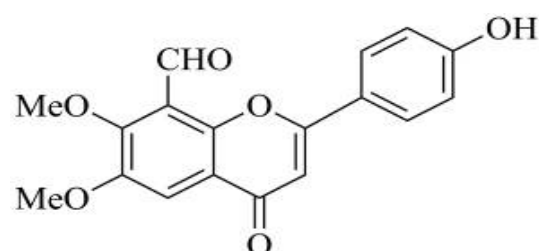


Figure 1: Structure of DHF

DHF inhibits HepG2 cells proliferation

DHF inhibited the proliferation of HepG2 cells in a concentration-dependent manner, with an IC_{50} of 25.87 $\mu\text{g}/\text{mL}$ (Figure 2).

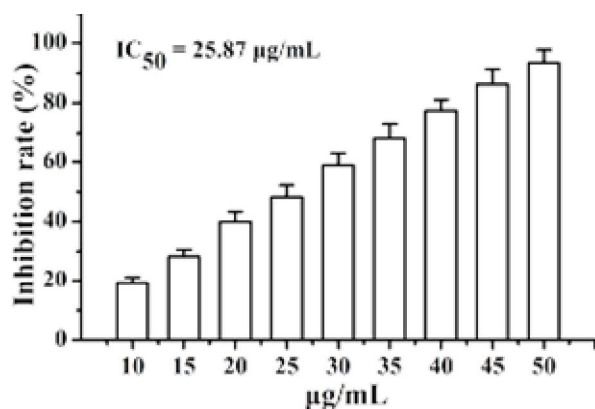


Figure 2: Inhibitory effect of DHF on proliferation of HepG2 cells

DHF induces apoptosis in HepG2 cells

Flow cytometry results revealed that exposure of HepG2 cells to DHF at doses of 10, 20 and 30 $\mu\text{g}/\text{mL}$ led to significant induction of apoptosis, relative to control ($p < 0.01$, Figure 3).

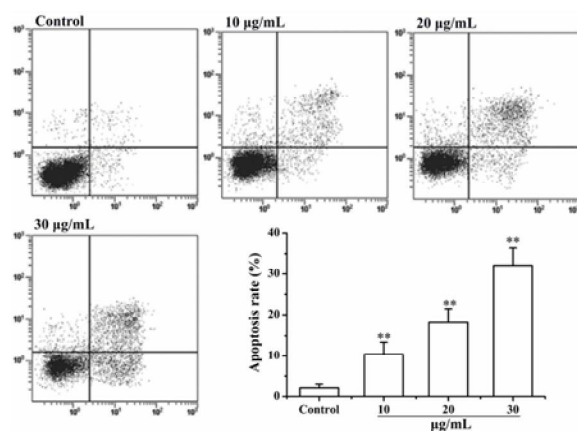


Figure 3: Inductive effect of DHF on apoptosis in HepG2 cells; $**p < 0.01$, versus control

DHF regulates expressions of apoptosis-related proteins in HepG2 cells

Results from western blot (Figure 4) indicated that DHF at doses of 10, 20 and 30 $\mu\text{g}/\text{mL}$ led to significant down-regulation of the expressions of Survivin and Bcl-2 ($p < 0.05$ or 0.01), while there were significant up-regulation of the expressions of Bax, c-caspase-9 and c-caspase-3 ($p < 0.05$ or 0.01) in HepG2 cells, relative to control. However, DHF had no significant effects on Mcl-1 and Bcl-xl expressions.

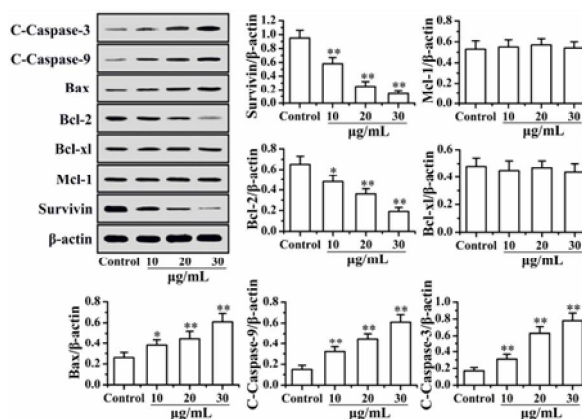


Figure 4: Effect of DHF on the expressions of apoptosis-related proteins in HepG2 cells; $*p < 0.05$, $**p < 0.01$, versus control

DHF promotes release of Smac and cytochrome c proteins from mitochondria to cytoplasm in HepG2 cells

Treatment of HepG2 cells with DHF at doses of 10, 20 and 30 $\mu\text{g}/\text{mL}$ led significant release of Smac and cytochrome c proteins into the cytoplasm ($p < 0.05$ or 0.01), relative to control. These results are shown in Figure 5 A and Figure 5 B.

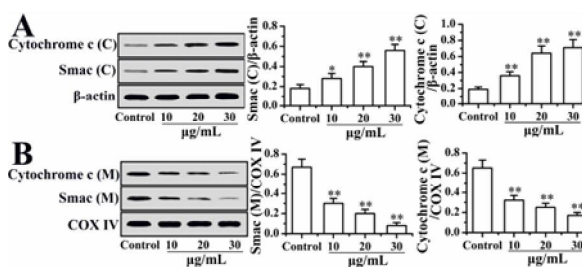


Figure 5: DHF promoted release of mitochondrial Smac and cytochrome c proteins to the cytoplasm in HepG2 cells; (A) Smac (C) and cytochrome c (C) represent Smac and cytochrome c in the cytoplasm, respectively; (B) Smac (M) and cytochrome c (M) represent mitochondrial levels of Smac and cytochrome c, respectively; $*p < 0.05$, $**p < 0.01$, versus control

DHF inhibits xenograft provoked by HepG2 cells

The HepG2 cells xenograft was employed to investigate the effect of DHF on HepG2 cell-induced tumor, and possible mechanisms. The results revealed that DHF exposure brought about significant suppression of the growth of tumor induced by HepG2 cells (Figure 6A), but had no significant influence on body weight (Figure 6B), relative to control. In addition, DHF treatment led to significant increases in the expressions of Bax, c-caspase-3, and c-caspase-9, and significant reductions in the expressions of

Survivin and Bcl-2, relative to control ($p < 0.01$; Figure 7). The releases of Smac and cytochrome c into the cytoplasm increased in a significant manner in HepG2 cell-induced tumor tissue, relative to control ($p < 0.01$; Figure 8A and Figure 8B).

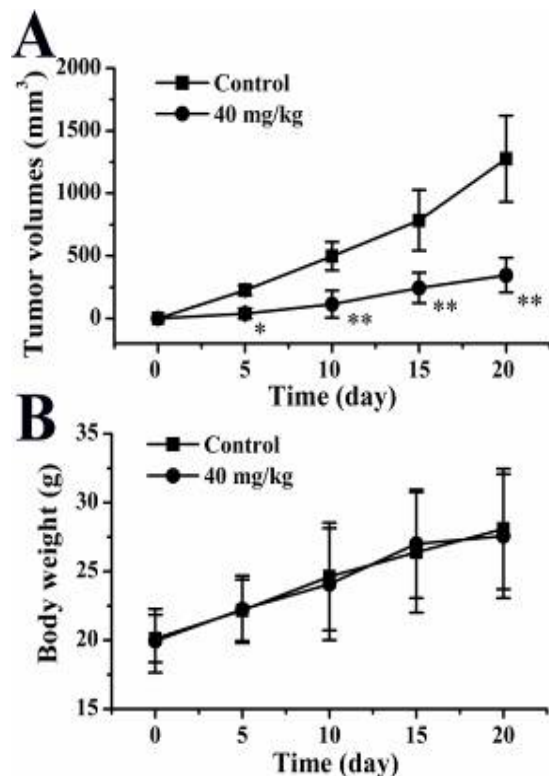


Figure 6: (A) Growth-suppression influence of DHF on HepG2 cell-mediated tumor in nude mice, and (B) its effect on body weight; * $p < 0.05$, ** $p < 0.01$, versus control

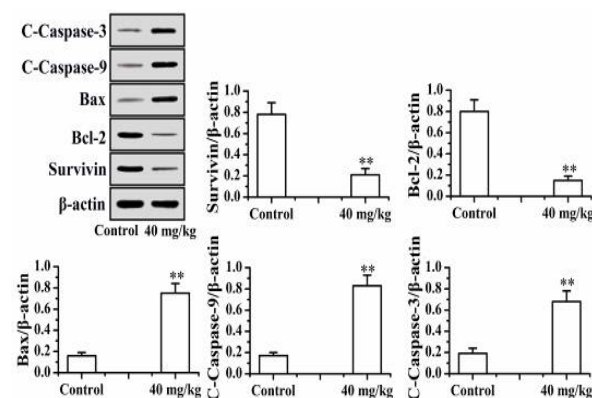


Figure 7: DHF-mediated up-regulation in Bax, c-caspase-9 and c-caspase-3 expressions, and down-regulation of Survivin and Bcl-2 expressions in the tissues of HepG2 cell-induced tumor; ** $p < 0.01$, versus control

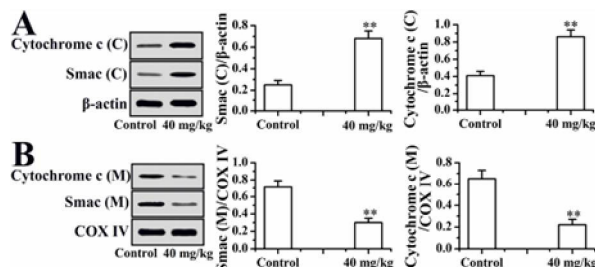


Figure 8: DHF enhanced the cytoplasmic levels of Smac and cytochrome c in the tissues of HepG2 cell-induced tumor; (A) Smac (C) and cytochrome c (C) denote cytoplasmic levels of Smac and cytochrome c, respectively; (B) Smac (M) and cytochrome c (M) represent mitochondrial Smac and cytochrome c, respectively; ** $p < 0.01$, versus control

DISCUSSION

The techniques of flow cytometry and CCK-8 are generally applied in combination for studying the influence of anti-carcinogenic agents on programmed cell death, and cancer cell proliferation and apoptosis [17,18]. In this process, CCK-8 assay is first used to confirm the anti-proliferative effect of the agent under investigation. Subsequently, whether the anti-proliferative property is related to apoptosis is tested through the use of flow cytometry. The results obtained in this study indicate that DHF exerted anti-proliferative influence on HepG2 cells in a process involving apoptosis (Figure 2 and Figure 3).

The mitochondria-mediated apoptotic pathway is a vital process in the apoptosis of cancer cells, and it is under the control of some key apoptosis regulatory proteins such as Survivin, Mcl-1, Bcl-xl, Bcl-2, Bax, c-caspase-9, c-caspase-3, Smac and cytochrome c [19]. When anticancer agents stimulate cancer cell mitochondria, there is increased release of Smac and cytochrome c into the cytoplasm [20]. Studies have demonstrated that that the mitochondrial release of Smac and cytochrome c is blocked by Mcl-1, Bcl-xl and Bcl-2, while Bax has the reverse effect [21-23]. The released cytochrome c promotes the formation of apoptosome by binding Apaf-1 and procaspase-9, the latter of which becomes activated to c-caspase-9 [24]. Furthermore, caspase-3 is activated by c-caspase-9 to obtain the apoptosis-inducing c-caspase-3 [25]. The protein Survivin inhibits the activity of caspase-3 [26], but fortunately, Survivin activity is blocked by Smac [27]. Part of the results obtained in the present investigation reveal that DHF induced apoptosis in HepG2 cells through significant reductions in levels of Survivin and Bcl-2, while increasing those of Bax, c-caspase-9 and c-

caspase-3, and enhancing cytoplasmic concentrations of Smac and cytochrome c.

Xenograft is an accepted model for investigating the effects of anti-carcinogenic agents, as well as their underlying mechanisms [15,16]. The results obtained in the xenograft assay revealed that DHF exerted significant inhibition on the growth of tumor tissue induced by HepG2 cells by reductions in levels of Survivin and Bcl-2, up-regulation of Bax, c-caspase-9 and c-caspase-3 expressions, and increases in cytoplasmic levels of Smac and cytochrome c (Figures 6 - 8).

CONCLUSION

The findings of this investigation demonstrate that DHF suppresses the proliferative capacity of HepG2 cells *in vitro* and *in vivo* via activation of the mitochondrial route of apoptosis. Therefore, DHF is a potential anti-hepatoma agent. More studies on the anti-hepatoma effect of DHF are on-going to further validate these findings.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors 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 them. Qiu-Jie Zhang and Wen-Sheng Qiu contributed equally to this work. Qiu-Jie Zhang and Jun Liang conceived and designed the study while Wen-Sheng Qiu, Hong-Xia Cui, Zhuang Yu, Ru-Yong Yao, Shi-Hai Liu and Zi-Min Liu performed the experiments, collected the data and analyzed same. All authors read and approved the manuscript for publication.

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