Food and Chemical Toxicology 50 (2012) 3166–3173

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

A flavonoid component from *Docynia delavayi* (Franch.) Schneid represses transplanted H22 hepatoma growth and exhibits low toxic effect on tumor-bearing mice

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ARTICLE INFO

Article history:
Received 20 January 2012
Accepted 23 May 2012
Available online 8 June 2012

Keywords:
Chrysin
H22 transplanted tumor
Anti-tumor activity
Hepatic cancer
Toxic effect
Angiogenesis

ABSTRACT

The fruit of *Docynia delavayi* (Franch.) Schneid is a kind of popular food in southwestern areas of China. Additionally, its rhizome has been long used as a folk medicine in the treatment of liver cancer by local people. Chrysin is a kind of flavonoid which induces cancer cell death *in vitro*. However, its anti-tumor activity *in vivo* and toxicological effects on the tumor-bearing animals still remain poorly understood. In this study, we obtained four flavonoids from this herb. Among them, chrysin showed the strongest cytotoxic effect on an array of cultured tumor cells. Further investigations revealed that it significantly repressed transplanted H22 ascitic hepatic tumor cell growth *in vivo*. Moreover, this compound displayed little toxic effects. Additionally, we demonstrated that in transplanted tumor tissues, chrysin not only activated caspase-3 and induced apoptosis, but also inhibited the production of vascular endothelial growth factor (VEGF) and suppressed angiogenesis. These data showed that chrysin exhibited prominent anti-tumor activities and low toxic effects *in vivo*.

1. Introduction

Hepatocellular carcinoma is a serious disease that threats global public health. Every year, more than 700,000 new cases of liver cancer are diagnosed worldwide, with most of them detected in developing countries (Rehman et al., 2009). The five-year survival rate after diagnosis of this disease is very low and it is the third leading cause of cancer-related deaths. Although several chemically synthesized medicines are currently available for treating this disease, these drugs are quite expensive and frequently result in severe side effects. Therefore, identification of novel drugs with better effectiveness and lower toxicity is necessary. Recently, natural products have received more and more attentions as a potential origin of new therapeutic anti-tumor drugs (Harhaji et al., 2008; Rashid et al., 2011; Yin et al., 2007).

Docynia delavayi (Franch.) Schneid (D. delavayi) is an evergreen plant commonly grown in some areas such as Diqing Tibetan Autonomous Prefecture (shangri-la) and Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China. It is named "Duo-yi" by Dai people, one of the local ethnic minorities in Southwestern China. The rhizome of this plant has been used for the treatment of a variety of diseases including liver cancer. Besides as s a folk medicine, its fruit is also eatable (Lin et al., 2003). However, the chemical constituents responsible for its anti-tumor effect are not well defined.

Flavonoids constitute a group of polyphenolic compounds that occur ubiquitously in plants, which exist at a high level in food and can be easily ingested by human. They have been shown to have an array of pharmaceutical effects, such as antioxidant, antiviral, and anti-cancer activities (Kandaswami et al., 2005; Middleton et al., 2000). Chrysin (5,7-dihydroxy-2-phenyl-4H-chromen-4-one) shares a common structure of flavones with two additional hydroxyl groups. A series of biological activities of chrysin have been reported, including *in vitro* anti-tumor activity (Cho et al., 2004; Critchfield et al., 1996; Khoo et al., 2010; Woodman and Chan, 2004). A recent study revealed that chrysin dramatically sensitizes human HepG2 hepatomas to tumor necrosis factor (TNF) alpha-induced apoptosis (Li et al., 2010). Another investigation showed that 8-bromo-7-methoxychrysin, a derivative of chrysin, is able to effectively induce

Abbreviations: ALT, alanine aminotransferace; AST, aspartate aminotransferace; BUN, blood urea nitrogen; CDDP, cisplatin; CRE, creatinine; *D. delavayi*, *Docynia delavayi* (Franch.) Schneid; H22, H22 ascitic hepatic cancer cells; Vero, African green monkey kidney epithelial cells; Huh7, human hepatic carcinoma cell; EC1, esophageal cancer cells; Accm, adenoid cystic carcinoma cells; A549, alveolar basal epithelial carcinoma cells; HGB, hemoglobins; MTT, methyl thiazolyl tetrazoliym; PLT, platelets; RBC, red blood cells; UA, uric acid; VEGF, vascular endothelial growth factor; WBC, white blood cells.

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http://dx.doi.org/10.1016/j.fct.2012.05.039

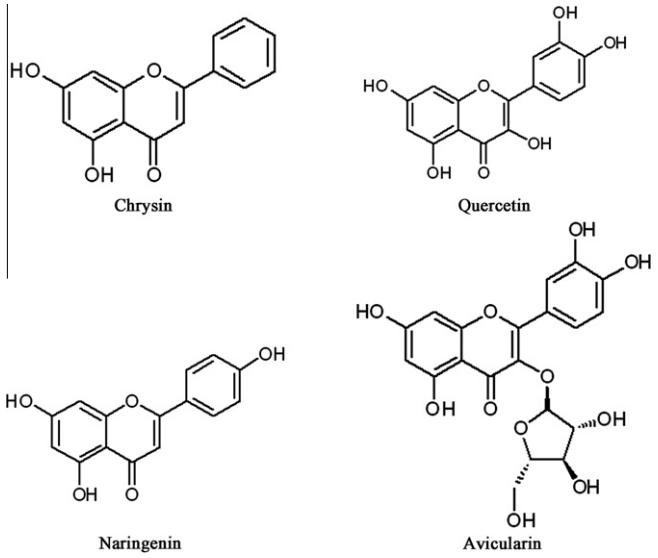

Fig. 1. Chemical structures of four flavonoids purified from *D. delavayi*.

apoptosis in cultured liver cancer cells (Yang et al., 2010). These findings imply a possibility that chrysin can be used for treating hepatocyte carcinoma clinically. However, relatively little is known about the effect of chrysin against hepatocyte carcinoma *in vivo*. Additionally, there are very few studies about the toxicological effect of long-term administration of chrysin to the animals, which is also an important aspect for the clinical application of this compound, except for its cytotoxic effect to the tumor cells.

In this study, we have purified and identified four different flavonoids from this plant, namely chrysin, quercetin, naringenin and avicularin (Fig. 1). We determined their *in vitro* cytotoxic effects on an array of cancer cells originated from different tissues. The *in vitro* investigations demonstrated that chrysin was the strongest cell death inducer, and Huh7 hepatic carcinoma cells were more sensitive to chrysin treatment than all the other tumor cells we tested. Our further investigations were designed to evaluate the *in vivo* therapeutic activity of chrysin in H22 ascitic hepatocyte carcinoma transplant solid tumor model and its toxicology effects on tumor-bearing mice.

2. Material and methods

2.1. Phytochemical investigations

The raw materials of the fresh rhizome of *D. delavayi* were collected in June 2008 in Yunnan Province, China. The plant material was identified by Prof. Dingrong Wan, College of Pharmaceutical Sciences, South-Central University for Nationalities, China. A voucher sample is deposited at the Herbarium situated in College of Pharmaceutical Sciences. The air-dried powder of the raw plant material (5 kg, 40-mesh) was extracted with 95% ethanol (1:10 w/v) three times, each time 2 h. These solutions were combined, filtered, centrifuged and concentrated under reduced pressure to obtain dried crude total extract. Content of flavonoids in the total extract was determined and expressed as rutin equivalents (RE) using the methods as described previously (Sun et al., 2011). The crude extracts contained 209 ± 8.8 μg/mg (RE) total flavonoids.

To separate and purify individual compounds, the total extract was suspended in methanol: water (9:1) and extracted successively with ethyl acetate and butyl alcohol. The ethyl acetate extract was then subjected to extensive chromatography on silica gel column and eluted with cyclohexane–ethyl acetate or ethyl acetate-acetone gradient solvent system. Four flavonoids were obtained at last and the purity of these compounds was determined by high-performance liquid chromatography (HPLC) as described previously (Liu et al., 2007). The chemical structures of these compounds were identified as chrysin, quercetin, naringenin and avicularin by spectrum methods (UV, IR, MS, ^{1}H NMR and ^{13}C NMR).

2.2. Cells and animals

Vero untransformed African green monkey kidney epithelial cells, human Huh7 hepatic carcinoma cell, EC1 esophageal cancer cells, Accm adenoid cystic carcinoma cells, and A549 alveolar basal epithelial carcinoma cells were cultured in RPMI 1640 medium plus 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37 °C incubator supplied with 5% CO_2 in a humidified atmosphere. Murine ascitic H22 hepatoma cells were maintained by weekly transplantation of the tumor cells into the peritoneal cavity of Kunming mice.

Male and female Kunming mice (18–22 g) were obtained from the Experimental Animal Center, Institute of Health and Epidemic Prevention (Wuhan, China). The animals were housed in standard specific pathogen free (SPF) environmental conditions. All the animal experimental procedures are approved by the Animal Care and Use Committee of South-Central University for Nationalities (Wuhan, China).

2.3. Cell viability assay and IC_{50} value calculation

Cells were seeded on 24-well plates and allowed to adhere overnight. After treating them by different concentrations of chemicals, the medium was replaced by 250 μL of medium containing MTT (methyl thiazolyl tetrazoliym, 0.5 mg/mL) and followed by incubating the cells at standard culture condition for 3 h. Then, the mediums were discarded and 500 μL of DMSO was added to each well and mixed thoroughly. Cell viabilities were indicated by the optical density (OD) values of the solutions at 570-nm wavelength.

IC_{50} value of a compound is a concentration at which this compound is able to inhibit cell growth by 50%. It was calculated according to the values of cell viability corresponding to the concentration of a chemical using Origin Software.

2.4. H22 transplanted tumor model establishment and drug administration

The transplanted tumor model was established as previously described (Rashid et al., 2011). Briefly, 1×10^6 of H22 cells were injected into the mice subcutaneously. The mice were randomly divided into six groups with 10 mice for each group. Drug administration began 24 h later. The groups for chrysin administration received different dosages (15, 30, 60 mg/kg), respectively. The positive control group was treated with cisplatin (CDDP, 5 mg/kg) and the vehicle control group received 0.9% normal saline. All the drugs were administrated by intraperitoneal injection for 10 days. Then, all the mice were sacrificed and the whole bodies, the segregated tumor, liver, thymus and spleen of the mice were weighed immediately. The tumor inhibitory ratio was calculated by the following formula:

$$\text{Tumor inhibitory rate } (\%) = (W_{\text{Control}} - W_{\text{Treated}})/W_{\text{Control}}$$

W_{Treated} and W_{Control} were the average tumor weight of the treated and control mice, respectively.

2.5. Blood physiochemical assays

The blood samples were acquired from those experimental mice before scarified under diethyl ether anesthesia, and collected in heparinized tubes and EDTA tubes. White blood cells (WBC), Red blood cells (RBC), Hemoglobins (HGB) and Platelets (PLT) were recorded by automatic blood cell counting apparatus (Mindray, Shenzhen, China). Blood serum were separated by centrifugation at 3000*g* for 10 min, physiochemical indexes, including aspartate aminotransferace (AST), alanine aminotransferace (ALT), blood urea nitrogen (BUN), uric acid (UA) and creatinine (CRE) were recorded by automatic biochemical analyzer (Sysmex, Japan) using reagents purchased from Sysmex Incorporation.

2.6. Hochest 33258 staining

Hochest 33258 staining was performed as previously described (Deng et al., 2006a,b) with some modifications. Briefly, at the end of the experiments, the mice were euthanized and the transplanted tumor were dissected out and fixed in 10% neutral buffered formalin solution. We got 10 tumors from each group, and randomly chose three ones out of them. Then, these samples were cut into 10-μm-thick sections and stained by Hoechst 33258 (0.5 μg/mL). After washed by PBS three times, they were observed and photographed under a fluorescent microscope (Olympus, BX-60, Japan).

To quantify the fragmented and condensed staining which indicated apoptotic nucleus in the slides, we randomly chose five regions from the pictures of each tumor. These pictures were blinded and counted by two people, and their mean values were used in the statistical analysis. To avoid interobserver difference, a datum is valid only if the discrepancy between these two observers is <10 percents.

2.7. Organ index calculation

At the end of the study, the mice were euthanized and relevant organs were dissected out and weighted. The organ indexes were calculated according to the formulas described below (Chen et al., 2011).

$$\text{Organ index} = \text{mean organ weight(in milligrams)}/\text{mean body weight(in grams)}.$$