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Anti-Hepatitis B Virus Activity of Flavonoids from *Marchantia Convoluta*

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ABSTRACT

Flavonoids from *Marchantia convoluta* (MCF) were studied for their ability to inhibit the proliferation of 2.2.15 cells. All concentration of MCF inhibited the secretion of HBsAg and HBeAg in the cultured medium by 2.2.15 cells. Analysis of morphological changes of MCF-treated cells by phase-contrast microscopy revealed a possible mode of action for MCF: inhibition of proliferation of 2.2.15 cells by inducing apoptosis.

Keywords: *Marchantia convoluta; Flavonoids; Anti-HBV*

Marchantiaceae plants are well-known traditional Chinese medicinal herbs and extensively used to treat tumefaction of skins, protect liver and treat hepatitis. It is used as antipyretic in some regions of China [1-3]. There are several different types Marchantiaceae plants in Guangxi Zhuang Autonomous District such as *Marchantia polymorpha*, *Marchantia convoluta* and *Marchantia paleacea*. These species grow together and it is difficult to distinguish one from the other because of their genetic similarity [4]. *Marchantia convoluta* is found only in China [5].

The major identified constituents in *M. convoluta* are flavonoids, triterpenoids and steroids [1-3, 6-8]. The flavonoids consist mainly of quercetin, luteolin, apigenin and their *O*- and *C*-glycosides [1-3, 7]. The dried leaves are used in China to protect the liver and to treat tumefaction of skin. A high dosage of flavonoids from *Marchantia convoluta* (20 and 40 μg/mL) can significantly reduce the activity of alanine aminotranferease (ALT) and aspartate aminotransferase (AST) in the serum of mice with acute hepatic injury caused by CCl_4. It also increases total protein (TP), alkaline phosphatase (ALP) and inhibits the auricle tympanites of mice caused by dimethylbenzene. Flavonoids from *Marchantia convoluta* strongly inhibit colibacillus, typhoid bacillus, *Staphylococcus aureus*, *Bacillus enteritidis*, hemolytic streptococci type B and *Diplococcus pneumoniae*. They also possess distinct effect of antibiosis, anti-inflammation and induce diuresis in mice [1]. Extracts from *Marchantia convoluta* strongly inhibit tumors in human liver and lung cancer cell lines [2].

Flavonoids are almost universal pigments of plants. It's an important part of the human diet and considered as active principles of many medical plants [9, 10]. Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin.

However, to our knowledge, no cytotoxic effect on human hepatocarcinoma cells has been reported for MCF. In this study, we analyzed the effect of MCF on 2.2.15 cells and found that exposure of 2.2.15 cells to MCF induced cytotoxicity in a dose-dependent manner accompanied with a decreased concentration of HBsAg and HBeAg. It is important to search for more effective agents against HBV, even with an improved therapeutic index.

MATERIALS AND METHODS

Plant Material

The whole plants of *Marchantia convoluta* were collected in Shangling City of Guangxi Zhuang Autonomous District in August 2003. The specimen (No 20041364) was identified by Zhou Zi-jing at Biology Department of Guangxi Chinese Medical University. The leaves were washed with water, air dried for several days and powdered.

Chemicals and Drugs

Methanol (Chromatographic grade, Jiangsu Hanbon Sci. & Tech. Co., Ltd), phosphoric acid (Analytical grade, Hanbon), acetonitrile (Chromatographic grade, Hanbon) and acetic acid (Analytical grade, Hanbon)

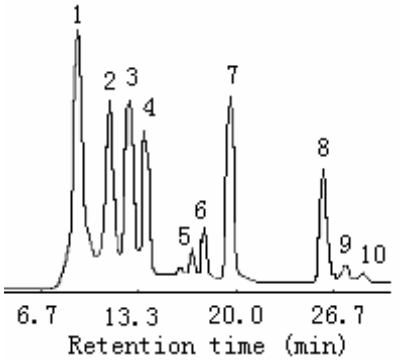

Fig 1. HPLC display of flavonoids peaks in MCF. Peak identifications: 1. Luteolin 7, 4'-di-*O*-glucuronide; 2. Apigenin 7, 4'-di-*O*-glucuronide; 5. Apigenin -7-O-β-D -glucuronide; 6. Quercetin; 7. Luteolin; 8. Apigenin; 3, 4,9 and 10 were not indentified. A Kromasil RP-C_{18} column (250×4.6mm i.d, 5μm) was used. mobile phase: methanol- acetonitrile- acetic acid- phosphoric acid-H_2O (200: 100: 10: 10: 200, V/V); Detecting wavelength: 352 nm; Flow: 0.60 mL/min; Sensitivity: 0.05 AUFS; Quantity of injecting sample: 6.0 μL.

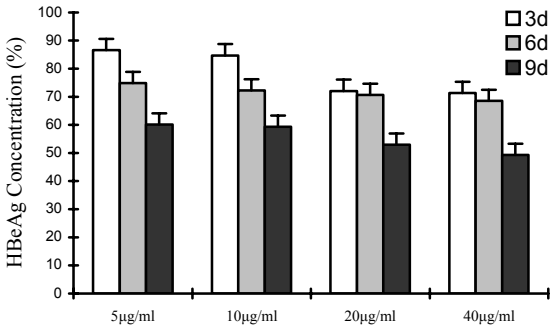

Fig 3. Time and dose-dependent HBeAg release-inhibitory effects of bullatacin on 2.2.15 cells.The HBeAg concentrations, represented as percentage of the control, was determined by ELISA. Values represent the Mean ±SD of 2-5 independent experiments with triplicate wells. (n=6-15) (* $p < 0.05$ vs.control).

were used for the mobile phase preparation. Quercetin, luteolin and apigenin were acquired from Chinese Medicine Checking Institute.

Extraction, Purification and Analysis

The leaf powder of *Marchantia convoluta* (280 g) were extracted with 80% ethanol for one month at room temperature. The extract filtered, vacuum evaporated and was separated on a silica gel column to yield yellow power (5.96 g). The yellow powder was analyzed by HPLC with external standard to identify the main constituents. The content of total flavonoids was determined spectrophotometerically.

HPLC analysis was performed on a Shimadaz LC-2010A LIQUID CHROMATOGRAPH system with a Shimadaz SPD-M10A Diode Array Detector and a Shimadaz Class-vp V6.12 SP4 offline processing system, using a Kromasil RP-C_{18} column (250×4.6 mm i.d, 5μm, Hanbon Science & Technology Co., Ltd) and methanol-acetonitrile-acetic acid-phosphoric acid-H_2O (200:100:10:10:200, V/V) as mobile phase. The mobile phase was filtered through a nylon membrane. Detecting wavelength: 352 nm; Flow rate: 0.60 mL/min; Sensitivity: 0.05 AUFS. The quantity of injected sample was 6.0 μL. The HPLC system was operated at ambient temperature (28±1°C).

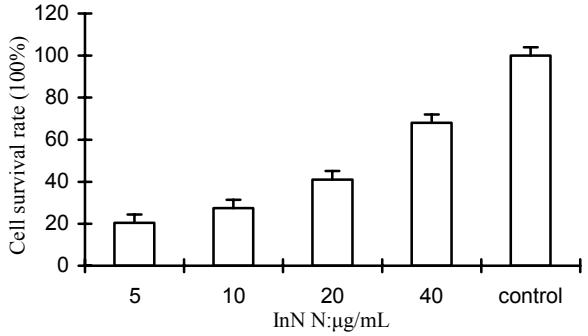

Fig 2. Toxicity of MCF to 2.2.15 cells (N: concentration of MCF).

Cell

The 2.2.15 cells (clone cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) that secrete HBsAg and HBeAg were kindly provided by Chongqing Medical University. The 2.2.15 cells were maintained at 5×10^5 cell/mL in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated FCS, penicillin G (100 IU/mL) and streptomycin (100 pg/mL) and incubated at 37ºC in a humidified atmosphere containing 5% CO_2 and 95% air.

Cytotoxicity Measurement

2.2.15 cells were inoculated at a density of 1×10^5 cell/mL in 96-well tissue culture plates. After 24 h in culture, the cells were treated with various concentrations of MCF (5, 10, 20 and 40 μg/mL) group for a further 24h. A Blank control also set. MTT assays were performed using the cell titer kit^{TM} (Promega) following standard procedure ~~a~~absorbance was measured at 570 nm using a Thermomax (Molecular Devices, San Jose, CA), or a cytoFlour microplate reader (PE Biosystems, Foster City, CA). The data were normalized (A570 nm) and the mean absorbance was plotted against drug concentration. The IC_{50} values were calculated as described above. Determination of Effects of MCF on HbsAg and HbeAg. HBsAg and HBeAg were detected by ELISA (Xinchuang Technology Limited Corporation, Xiamen, China), and the OD value was detected at 450 nm/630 nm in a Microplate Reader. Then suppression rate of HBsAg and HBeAg was calculated: Suppression rate (%) = (OD value of drug well/OD value of negative control well - OD value of control well/OD value of negative control well)/(OD value of control well/OD value of negative control well-2.1).

Analysis of Morphological Changes

After incubating the cultured cells with the indicated concentrations of bullatacin for 24 h, lesions of cell membrane and the compactness of cytoplasmic organ-

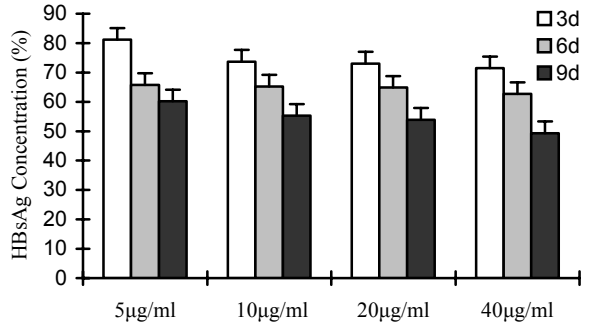

Fig 4. Time and dose-dependent HBsAg release-inhibitory effects of bullatacin on 2.2.15 cells. The HBsAg concentrations, represented as percentage of the control, was determined by ELISA. Values represent the Mean±SD of 2-5 independent experiments with triplicate wells. (n=6-15) ($^{*}p < 0.05$ vs. control).

elles were observed and photographed under an inverted microscope with 200 × magnifications.

Statistical Analysis

The results are expressed as mean±S.E.M. (n=5). Statistical significance was determined by analysis of variance ($p < 0.05$). The analysis was performed using SAS statistical software.

RESULTS

Analysis of MCF

Determination of total flavonoids. The content of total flavonoids was determined through visible spectrophotometer. By studying the factors that affected the determination, the optimal conditions for this experiment were found as follows: $NaNO_2$-$AlCl_3$, color-developing agent; 15 min, color time; 525nm, wavelength. The data of the content and absorbance formed a standard curve, namely Y=-0.0153+0.03003X; the recovery of the samples was 94.61% to 101.59%. The content of total flavonoids in *Marchantia convoluta* is 1.90 %. The content of total flavonoids of the yellow power is 96.35%.

HPLC analysis of MCF. HPLC was used to quantify individual flavonoids by using internal reference. Fig 1 is the HPLC chromatogram of MCF. MCF consists of quercetin, luteolin, apigenin and their *O*-glycosides.

Toxicity Study

The 2.2.15 cell survival rates after 24 h incubation at different concentration of MCF are shown in Fig 2. MCF (40 μg/mL) significantly inhibited the proliferation of 2.2.15 cells early at 24 h. The IC_{50} was 30±1.6 μg/mL. The inhibitory rate of high concentration of MCF (40 μg/mL) was more than 75%.

Inhibitory Effects of MCF on HBsAg and HBeAg

We also wished to determine whether MCF would also influenced the concentration of HBeAg and HBsAg released from 2.2.15 cells. Fig 3 and Fig 4 show the time and dose-dependent inhibitory effects on HBsAg and HBeAg released from 2.2.15 cells by MCF. The average inhibitory rates of MCF 5, 10, 20 and 40 μg/mL for HBsAg were 18.86%, 26.31%, 26.94% and 28.53 %, respectively.

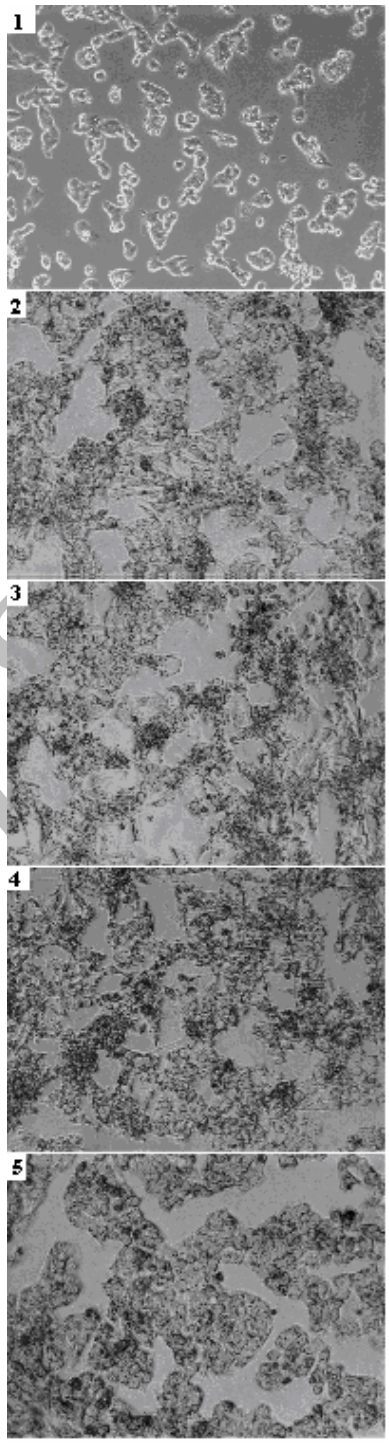

Fig 5. Microscopic appearance of 2.2.15 cells 24 h after incubation with medium alone (1) and medium containing MCF [5 μg/mL, (2); 10 μg/mL, (3); 20 μg/mL, (4); 40 μg/mL, (5)]. Apoptotic cells (arrows in B) are characterized by cellular shrinkage.

Effects of MCF on Morphological Changes in 2.2.15 Cells

It has been reported that several anti-cancer agents cause apoptosis in certain cancer cell lines [11, 12]. To further elucidate whether the toxicity effects of MCF were due to apoptosis, we first observed morphological changes of 2.2.15 cells treated with MCF (40 μg/mL) 24 h.

As shown in Fig 5, when 2.2.15 cells were treated by 40 μg/mL MCF for 24 h, morphological changes similar to morphological characteristics of apoptosis were observed including cellular shrinkage, cytoplasmic blebbing, chromatin margination, and condensation.

DISCUSSION

Measurements of the levels of viral surface antigen and e antigen from the media of cultures treated with MCF revealed that MCF had significant inhibitory effect on HBsAg and HBeAg at low concentrations, compared to the control group ($p < 0.05$). The results show that MCF has the function of inhibiting 2.2.15 cells from generating HBsAg and HbeAg, in addition, it is toxic to the 2.2.15 cells. In our pervious study, flavonoids from *Marchantia convoluta* (20 and 40 μg/mL) can significantly reduce the activity of alanine aminotranferease (ALT) and aspartate aminotransferase (AST) in the serum of mice with acute hepatic injury caused by CCl_4. Therefore it may be of value in developing new chugs. The possible mechanism of action of MCF could be the inhibition of viral DNA polymerase; chain-termination resulting from incorporation into elongated DNA strand, or both and further studies are warranted to elucidate the mechanism of action of this plant extract.

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