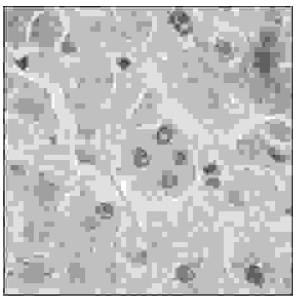
ORIGINAL **Prof-1058**

HEPATITIS B VIRUS



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ABSTRACT... jianbo_xiai@yahoo.com.cn. Dried leaves of Marchantia convoluta are largely used to protect livers, and to treat tumefaction of skins in China. Flavonoids from Marchantia Convoluta (MCF) was one of the most potentially effective anti-inflammatory. MCF was studied here for its ability to inhibit the proliferation of 2,2,15 cells (clone cells derived from HepG2 cells that were transected with a plasmid containing HBV, DNA). All concentrations (5,10,20 and 40 µg/ml) of MCF inhibit hepatitis B surface antigen (HbsAg) and hepatitis B E antigen (HbeAg) in the cultured medium released from 2.2.15 cells. Analysis of morphological changes of MCF-treated phase- contrast microscope revealed a possible model of action for MCF to inhibit Proliferation of 2.2.15 cells by inducing apoptosis.

Key words: Marchantia Convoluta; Flavonoids; Anti- HBV

INTRODUCTION

Marchantia plants are well- known traditional Chinese medicinal herbs and extensively used to treat tumefaction of skins, protect liver and treat hepatitis and used as antipyretic in country side[1-3]. There is a large number of Marchantiaceae plants in Guangxi Zhuang Autonomous, District such as Marchantia Polymorpha, M. convoluta and M. paleacea. These species grow together and it is difficult to distinguish one from the others because of their genetic similarity. M. convoluta is found only in China[4] and is quite rare.

The major identified constituents in M. convoluta are flavonoids, triterpenoids and steroids[1-3,5-8]. The flavonoids consist mainly of quercetin, luteolin, apigenin and their 0- and C-glycosides[1-3,6]. The dried leaves are used in China to protect the liver and to treat tumefaction of skin. A high dosage of flavonoids from M. convoluta (20 and 40 ug/ml) can significantly reduce the activity of alanine amino tranferease (ALT) and aspartate amino transferase (AST) in the serum of mice with acute hepatic injury caused by CCl_4 and increase the contents of total protein (TP) and alkaline phosphates (ALP), as well as inhibit the auricle tympanites of mice caused by dimethylbenzene. Flavonoids from M. convoluta strongly inhibit colibacillus, typhoid bacillus, Staphylococcus aureus, Bacillus enteritidis, hemolytic streptococci type B and Diplococcus pneumoniae and possess distinct effect of antibiosis, anti-inflamation and diuresis in mice[1]. Extracts from M. Convolus strongly inhibit tumors in human liver and lung cancer cell lines[2].

In this study the effect of MCF on anti-HBV was investigated.

Flavonoids are almost universal pigments of plants. It is an important part of the human diet and considered as active principles of many medical plants. Fla vonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Over 4000 different flavonoids have been described and they are categorized into flavonols, flavones, flavonones, anthocyanidins and isoflavonoids and many are ample in plant. More than 20 percent of plants have flavonoids constituents. The flavonoids have attracted considerable attention in recent years because of their special structures and wide range of biological activity. Several activities have been attributed to them, i.e, radical scavengers[9-11], antioxidant[12-13], enzyme inhibitor[14-16], antimicrobial activity[17], antiulcerogenic activity[18], and antimalarial activity[19].

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. That is important to search for more effective agents against HBV, even with an improved therapeutic index.

MATERIAL AND METHODS

Plant material

The whole plants of Marchantia covoluta were collected in Shangling City of Guangxi Zhou Zi-jng at Biology Department of Guangxi Medical University. The leaves, after being washed with water and dried in the shade for several days, were 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) were used for the mobile phase prepration. Quercetion, luteolin and apigenin were acquired from Chinese Medicine Checking Institute.

Leaves, purification and analysis

The leaves powder of Marchantia convoluta (280g) were extracted with 80% ethanol for one month at room temperature. The suspension, after filtration of the solvents were removed under vacuum to give a residue, which was separated on silica gel to yield yellow powder (5.96g). The yellow powder was analyzed by HPLC with external standard to identify the main constituents. The content of total flavonoids was determined through visible spectrophotometer[20].

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 (250x4.6mm 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 filtrated through a nylon membrane. Detecting wavelehgth:352 nm; Flow rate: 0.60 mL/min; Sensitivity: 0.05 AUFS. The quantity of injection sample was 6.0 μL. The HPLC system was operated at ambient

temperature(28±1 °C).

Cell

The 2.2.15 cells (clone cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) that secrete hepatitis B virus were kindly provided by Chongqing Medical University. The 2.2.15 cells were maintained at 5 x 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 37°C in a humidified atmosphere containing 5% CO_2 and 95% Air.

Cytotoxicity measurement

2.2.15 cells were inoculated at a density of 1 x 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. Blank control also set. Then MTT assays were performed using the cell titer kit™ (Promega) following the standard procedure Absorbance was measured at 570 nm using a Thermomax (Molecular Devices, San Jose, CA), or a cyto Flour micro plate reader (PE Bio systems, Foster City, CA). The data were normalized (A570 nm) and the mean absorbance was plotted against drug concentration. The $1C_{50}$ values were calculated as described above.

Analysis of MCF against HBV replication in cultures of 2.2.15 cells

The protocol for assaying anti-HBV activity in culture of 2.2.15 cells is briefly summarized as follows. 2.2.15 cells were inoculated at a density of 1 x10^5 cell/ml in 96-well tissues culture plates and grown to confluence. After 24 h in culture, the cells were treated with various concentrations of MCF (5, 10, 20 and 40µg/ml) for 9 days with changes of medium every 3 d. Blank control also set. On the 9^{th} day, the culture medium was harvested. Culture medium was collected and stored for analysis of extracellular (virion) HBV DNA after 0, 3, 6 and 9 days of treatment. Treated cells were lysed for 24 h following day 9 of treatment for the analysis of extracellular HBV genomic forms.

An aliquot of the culture medium (5µl) was used for estimation of HBV surface antigen (HbsAg) and HBV e antigen (HBeAg). The remaining medium was processed to obtain virus by a polyethylene glycol precipitation method. The HBV DNA recovered from the secreted particles was subjected to Southern blot analysis. Inhibition of HBV DNA was determined by comparison of the HBV DNA from positive control and no treatment control. Quantitative and qualitative manner for extracellular HBV DNA and the relative of HBV replication were performed by Vuego Scan (Brisa-620ST) density scanning with the Discovery Series Volume One Software.

Determination of effects of MCF on HBsAg and HBeAg

The HBV-producting 2.2.15 cell cultures were derived from HepG2 cells were transected with a plasmid vector containing G418-resistence sequences and 2 head-to-tail dimmers of the HBV genome (Sells et al, 1987). The cells were found to produce elevated level HBsAg and HBeAg, secrete infection virions into the culture medium and contain chromosomally integrated HBV DNA sequence, as well as relaxed circular, covalently closed incomplete episomal copies of the genome.

The different concentration of MCF (5, 10, and 40µg/ml) were added to the hapatocyte cultures on day 1 and maintained in culture with medium changed every 3 d until day 9. Cells were harvested at day 9.

Analysis of morphological changes

After incubating the cultural cells with the indicate concentrations of bullatactin for 24h, lesions of cell membrane and the compactness of cytoplasmic organelles were observed and photographed under an inverted microscope with 200 X magnification.

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 flavonoid in Marchantia convoluta is 1.90%. The content of total flavonoids of the yellow powder is 96.35%.

3.1.2 HPLC analysis of MCF

HPLC was used to quantify individual flavonoid by using internal reference. Fig.1 is the HPLC spectrum of MCF. MCF consist of quercetin, luteolin, apigenin and their O-glycosides.

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 identified. A Kromasil RP-C_{18} column (250x4.6mm i.d, 5μm) was used.

Fig-1.

Toxicity study

MCF (40 μg/ml) significantly inhibited the proliferation of 2.2.15 cells early at 24h. The IC_{50} was 30 ± 1.6 μg/ml. The viable cell number, determined by the trypan blue day exclusion, showed almost the same results (data not shown).

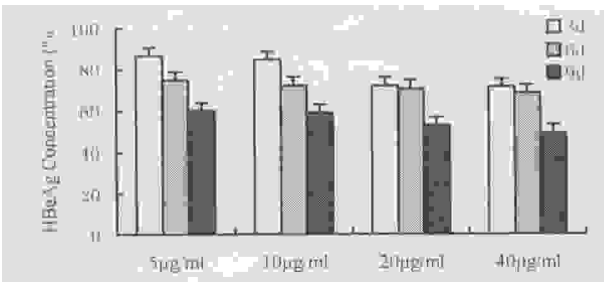

Fig-2. Time and dose-dependent HbeAg release inhibitory effects of bullatacin on 2.2.15 cells. The HbeAg concentrations, represented as percentage of the control, was determined by MTT. Values represent the Mean ±SD of 2-5 independent experiments with triplicate wells. (n=6-15) (*P<0.05 vs. control).

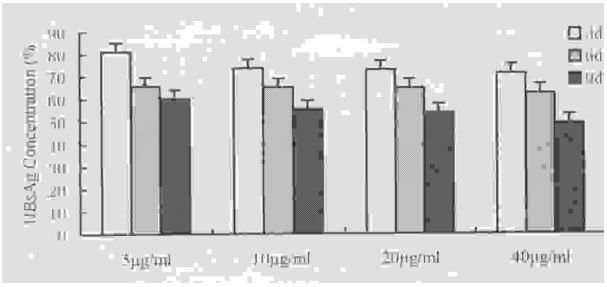

Fig-3. Time and dose-dependent HbsAg release-inhibitory effects of bullatacin on 2.2.15 cells. The HbsAg concentrations, represented as percentage of the control, was determined by MTT. Values represent the Mean ± SD of 2-5 independent experiments with triplicate wells. (N=6-15) (*P<0.05 vs. control)

Inhibitory effects of MCF on HBsAg and HBeAg

We also wished to determined whether MCF would also influence the concentration of HBeAg and HBsAg released from 2.2.15 cells. As can be seen in Fig. 2 and

Fig. 3, MCF showed time and dose dependent inhibitory effects on HBsAg and HbeAg released from 2.2.15 cells. The average inhibitory rate of MCF 5, 10, 20 and 40 µg/ml for HBsAg were 18.86%, 26.31%, 26.94% and 28.53% respectively for 3d.

Apoptotic cells (arrows in B) are characterized by cellular shrinkage. Phase contrast, x200.

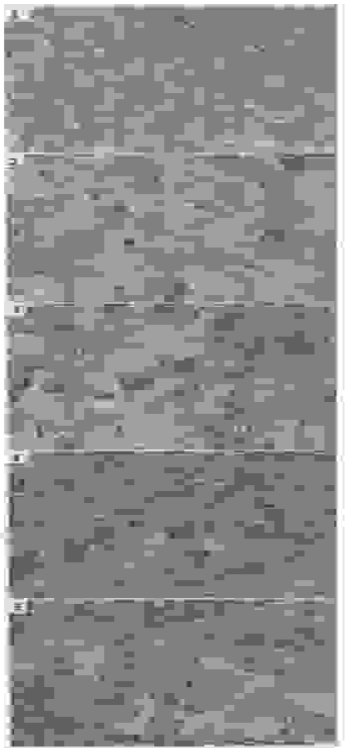

Fig-4. Microscopic appearance of 2.2.15 cells 24h after incubation with medium alone (4-1) and medium containing MCF (5 µg/ml, 4-2; 10 µg/ml, fig. 4-4; 40 µg/ml, 4-5).

Effects of MCF on morphological changes in 2.2.15 cells

It has reported that several anti-cancer agents cause apoptosis in certain cancer cell lines. To further elucidate whether the cytotoxicity effects of MCF were due to apoptosis, we first observed morphological changes of 2.2.15 cells treated with MCF (40 µg/ml) 24h. As shown in Fig. 4, when 2.2.15 cells were treated by 40 µg/ml MCF for 24h, morphological changes similar to morphological characteristics of apoptosis were observed including cellular shrinkage, cytoplasmic blabbing, chromatin margination, and condensation.

DISCUSSION

The flavonoids are a heterogeneous groups of phenol compounds approx. 4000, ubiquitous in the plant world. They are the pigments responsible for the autumnal explosion of plants color, and for the shades of yellow, orange and red in flowering plants. Flavonoids are also important factors for plant growth, development and immunity. The vasoprotective, anti-inflammatory, anti-allergic, anti-microbial, antihepatotoxic, anti-osteoporotic and anti-neoplastic action of flavonoids were well documented. Cytotoxic, mutagenic and or carcinogenic effects have also been reported.

Phenolic acids and flavonoids are widespread in nature, occurring in all plant families, and are found in considerable quantities in fruits, vegetables, grains, cola, tea, coffee, cocoa, beer and red wine[21-22]. In the United States, the daily dietary intake of flavonoids is estimated to be in the range of 500 to 1,000 mg, and even several grams in supplementing diets with flavonoids or flavonoid-containing herbal preparations such as Ginkgo biloba, Pycnogenol 227, or grape seed extract[21]. The bio-activities of the dietary phenolic acids and flavonoids are reported to be anti oxidative, anti-inflammatory, and anti carcinogenic. Therefore, high consumption of the dietary phenolic acids and flanovoids may have profit for prevention of oxidative damage, cardiovascular diseases, and cancer[21-22].

Despite the availability of an efficient vaccine, chronic Hepatitis B virus (HBV) infection remains a major pubic health problem worldwide. Indeed, according to the world Health Organization, more than 400 million people are chronic carries of the virus, and more than one billion have been in contact with HBV[23]. Chronic carries are exposed to the complications of the disease which include the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[24].

In clinical practice, treatment relies mainly on the use of IFN alpha, or nucleoside analogs[25], such as Lamivudine or adefovir dipivoxil. However, results of meta-analysis of IFN clinical trials showed that only a minority of patients are long-term responders (approximately 20%)[26].

Lamivudine as one of DDNs has been used widely in clinic, and has a rapid potent, anti-HBV effect, but there is a rebound of HBV DNA after treatment, and drug resistance and viral mutants may appear after a long-term treatment with Lamivudine. Its antiviral effects is also limited by the numerous side effects of this treatment. On the other hand, nucleoside analogs are well tolerated and exhibit an early and potent antiviral effect limited by the selection of resistant mutants during long-term therapy[27-28].

Therefore, antiviral therapy of chronic hepatitis B remains a clinical challenge. Effective antiviral therapy against HBV infection has not been fully developed, and studies have been hampered by he extremely narrow host range and limited access to experimental culture systems. The 2.2.15 cells (clone cells derived from HepG2 cells that were transfected with a plasmid containing HBV DNA) that secrete hepatitis B virions, make it possible to examine the effectiveness of potential anti HBV-associated drugs.

Plants are rich in antioxidant substance that protect cells from oxidative stress caused by chlorophyll photosynthesis and cellular respiration. Attention is currently being focused on widely distributed plant flavonoids with antioxidizing activity. Our team has been studying the effects of plant extracts and purified flavonoids on the activity of anti-HBV. Efforts are being directed toward purifying the compounds, determining their action mechanism and evaluating their ability to modulate the activity of native and acquired immunity cells.

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