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苦瓜對 HepG2 及 HepG2.2.15 肝癌細胞的抗病毒及抗腫瘤作用

臧世芳[1]、葉怡琳[1]、全中和[2]、徐雪瑩[1]

[1]慈濟大學生命科學系所
[2]行政院農業委員會花蓮區農業改良場

摘　要

爲了由天然資源中尋找具抗病毒及抗腫瘤活性的有效成分，過去已經有相當多的植物及食品組成進行評估。苦瓜（學名 *Momordica charantia*）因熟知其具有降血醣、抗醣尿、抗病毒及抗腫瘤之功能，而被廣泛應用於日常保健食材及傳統醫學上。爲了釐清不同品系苦瓜所具有的多項生物功能及作用情形，本研究乃以肝癌細胞株 HepG2 及感染 B 型肝炎病毒（HBV）的 HepG2.2.15 肝癌細胞株爲研究對象，同時進行苦瓜抗病毒及抗腫瘤的活性評估。苦瓜處理的細胞是以 MTT 個別進行細胞毒性的比較分析，在所有以乙醇或二氯甲烷分別萃取苦瓜種子、果實、莖和葉等不同部位所得到的粗萃取成分中，只有乙醇萃取的果實成分，對 HepG2 及 HepG2.2.15 細胞具有較高的生長抑制作用。其中有一部份苦瓜萃取物同時對 HepG2 及 HepG2.2.15 細胞生長的抑制作用與處理的時間和苦瓜的劑量相關。研究結果顯示 HM483 及 HM17 兩個品系苦瓜的乙醇萃取物，在處理劑量高於 0.4mg/ml 的情況下，對兩株肝癌細胞就都具有明顯的生長抑制效果。本研究結果顯示，各項不同的苦瓜萃取成分，對 HepG2 及 HepG2.2.15 兩株細胞的作用並沒有明顯的差異，而因 HepG2.2.15 細胞中 HBV 的存在，也間接顯示 HBV 基因體的表現可能與 HepG2 肝癌細胞對苦瓜萃取成分的反應無關。

關鍵詞：抗病毒、抗腫瘤、苦瓜、HepG2 肝癌細胞、B 型肝炎病毒

INTRODUCTION

Hepadnavirus is a small hepatotropic DNA-containing virus that replicates through an RNA intermediate (). Huamn hepatitis B virus (HBV), a member of the hepadnavirus family, is responsible for both acute and chronic liver infection. Chronic HBV infection, which causes liver cirrhosis and hepatocellular carcinoma (HCC), is a major global public health problem (Hino et al., 2002). Over 350 millions people are chronically infected with HBV and about 1 million people die of HBV related diseases each year, although an effective vaccine has been available for 20 years, there is still no effective treatment for HBV infected individuals (Ocama et al., 2005). The clinical treatments such as interferon-α (INF-α), nucleoside analog and gene therapy strategy are of limited efficacy because of low rates of seroconversion and the rapid development of drug-resistant mutants (Shindo et al., 1999; Chiou et al., 2001; Liaw, 2002; Wolters et al., 2002). There is an urgent need for more effective antiviral therapies or alternative approaches that can completely reduce or inhibit HBV replication.

Herbal medications are currently being promoted for clinical use in preventing virus infection or cancer therapy. Many of these claims are based on anecdotes in traditional Chinese medicine. Nevertheless, it is conceivable that certain herbs or natural products could have potent pharmacological properties. *Momordica charantia*, a cucurbitaceae fruit widely consumed as a vegetable in Asia, has been reported to possess several bioactivities including antidiabetic activity, antimutagenicity, antiviral activity and cytotoxicity (Grover & Yadav, 2004). It is one of the plants that has been frequently used as medicine. MC is commonly known as bitter gourd or bitter melon in English and cultivated throughout the world for use as vegetable as well as medicine. Some of its common uses in most countries are for treatment of diabetes (Day et al., 1990; Grover et al., 2001). It was also reported to have prominent anti-virus activities on human immunodeficiency virus (HIV) and herpes simplex virus (HSV) by the MC-derived ribosome inactivating proteins, MAP30 and GAP31 (Lee-Huang et al., 1995; Bourinbaiar & Lee-Huang, 1996). Previous studies have shown that crude MC extract and its various purified fractions from some

species have anticancer activity against several malignancies such as lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumor, prostatic cancer, squamous carcinoma of tongue and larynx, human bladder carcinomas and Hodgkin's disease (Battelli et al., 1996; Sun et al., 2001). In addition to the reported anti-tumor activity of MAP30 and GAP31, momordin, cucurbitacin and two glycoproteins, α and β momorcharins, from MC seeds have also been elucidated to inhibit guanylate cyclase of pathogenesis and replication of leukemia and other cancers (Takemoto et al., 1982; Ng et al., 1992; Rybak et al., 1994; Arazi et al., 2002). HBV is highly associated with the development of hepatocellular carcinoma. However, the bioactivities of MC on HBV and the HBV related hepatocellular carcinoma remain to be elucidated. To clarify the anti-tumor effect of MC on hepatocellular carcinoma, HBV-infected cells will simultaneously aid to reveal the possible anti-virus effect on the cells and the HBV regulatory mechanisms respond to MC. The aim of this study intends to primarily evaluate the effect of MC extracted by different parts or processes in human hepatocellular carcinoma HepG2 cells and that HBV-infected HepG2.2.15 cells.

MATERIALS AND METHODS

Preparation of MC Extracts

Fruits, stems, leaves and seeds of MC were certified and kindly donated by the District Agricultural Research and Extension Station, Council of Agriculture (Hualien, Taiwan). Different parts of the fresh M. charantia were chipped and extracted with CH_2Cl_2 and ethanol under room temperature, respectively. The CH_2Cl_2 and ethanol extracts were separately concentrated under reduced pressure to give a brownish residue. The extracts were consequently diluted with DMSO and PBS to the appropriate concentration before using for various treatments.

Cell Lines and Culture

HepG2.2.15 cells (human hepatoma cell line HepG2 stably transfected with HBV genome, kindly provided by Prof. W.L. Shih) and HepG2 cells

(human hepatocellular carcinoma, obtained from American Type Culture Collection) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 100 ml/l fetal bovine serum (FBS), 1.5g/l sodium bicarbonate, 1.0 mmol/l sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37℃ in a humidified incubator with 5% CO_2. A final concentration of 200μg/ml G418 was contained in the medium for the maintenance of HepG2.2.15 cells. Before the treatment, the cell count was adjusted to 1×10^6 /ml and cell viability to higher than 90% by trypan blue exclusion test. All cultures were free of mycoplasma.

MC Extracts Treatment and Cell Viability Assay

For MC extract treatments, 1×10^4 HepG2 and HepG2.2.15 cells were seeded onto a 96-well plate and allowed to grow for one day before treatment with different concentrations of MC extracts. After drug treatment, the cell viability was measured based on the reduction of MTT in mitochondria. A microculture tetrazolium colorimetric assay was performed to determine the cytotoxicity and cell proliferation of MC by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, Sigma Chemical Co., St Louis, MO). After treating with MC for 0, 24 and 48 h, the media were removed and cells were washed with phosphate-buffered saline (PBS). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was then added to each well to a final concentration of 0.5 mg/ml and cells were incubated at 37℃ for 4 h. The reaction was terminated by removing the supernatant gently and the purple formazan crystals were dissolved by adding 100 μl of DMSO, following by thorough mixing for 30 min. Plates were read at 570 nm using the microplate reader (μQuant, Kcjunior, Bio-Tek Instruments, Inc. U.S.A.) according to the manufacturer's instruction. The means and standard deviations were calculated from eight replicates. Relative cellular growth was calculated by a ratio of average absorbance or counts of various treatments to that of the control ones.

Nuclear Staining with DAPI and PI

Cellular changes after treatments were evaluated by staining with PI and DAPI. HepG2 and HepG2.2.15 cells were treated with the extracts of MC for 24h before staining. PI stained cells were fixed with 80% ethanol for 30 min and incubated with a 5 μM Propidium iodide solution for 30 min in the dark. DAPI stained cells were fixed with 4% paraformaldehyde for 10 min and incubated with a 0.4 μg/ml DAPI solution for 10 min in the dark. The nuclear morphology of the cells was examined by fluorescence microscopy (Axiovert 200, Zeiss, Germany).

Statistical Analysis

For all the treatments, data were expressed as mean±SE of six independent experiments. Statistical analysis of variance followed by Student's t-test was used to assess the differences between control and cells treated with the extraction of MC.

RESULT

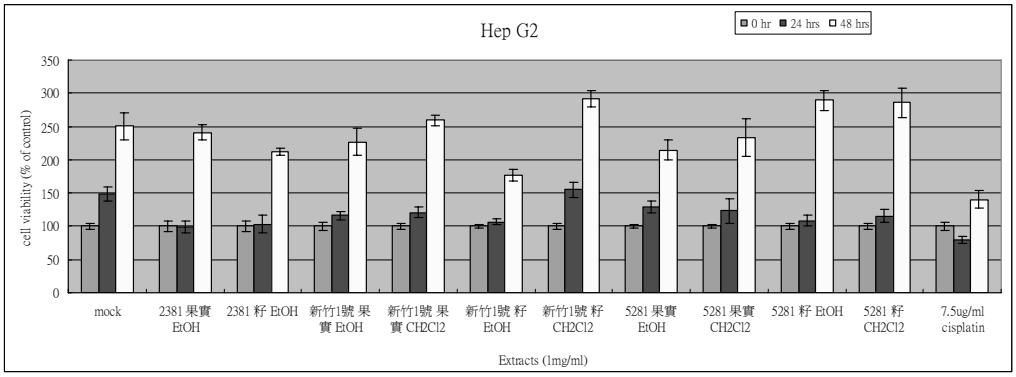
Inhibition of Cell Growth by MC

As shown in Figure 1, the growth of HepG2 and HepG2.2.15 cells were not inhibited significantly by MC after treating at a concentration of 1 or 2 mg/ml. The growth inhibition was comparatively evaluated with the control cells and the cells treated by cisplatin which is an effective chemotherapeutic agent. From the cell viability, fruit of 2381 and 5281 extracted by ethanol showed a higher inhibitory effect on cell growth at the treated concentration of 2mg/ml. In general, at the same treatment condition, the growth inhibition of MC on HepG2.2.15 cells was slightly higher than that on HepG2 cells. Among all the different species of MC, ethanol extract of HM483 and HM17 showed significant cell growth inhibition on HepG2 and HepG2.2.15 in a dose- and time-dependent manner. As shown in Figure 2, the effect of MC on HepG2 cells was slightly higher than that on HepG2.2.15 cells. The effective dose used for inhibiting the cell growth was lower than other MC extracts. In addition, the MC from different cultivated area showed significant different growth

inhibition on both cells (data not shown).

Morphological Observations

After treating with HM483 and HM17, both the cells declined significantly with an increased dose. The nuclear condensation could be observed at 24 h after treatments. The difference of cellular changes between HepG2 and HepG2.2.15 was not significant.


Hep G2
0 hr
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48 hrs
cell viability (% of control)
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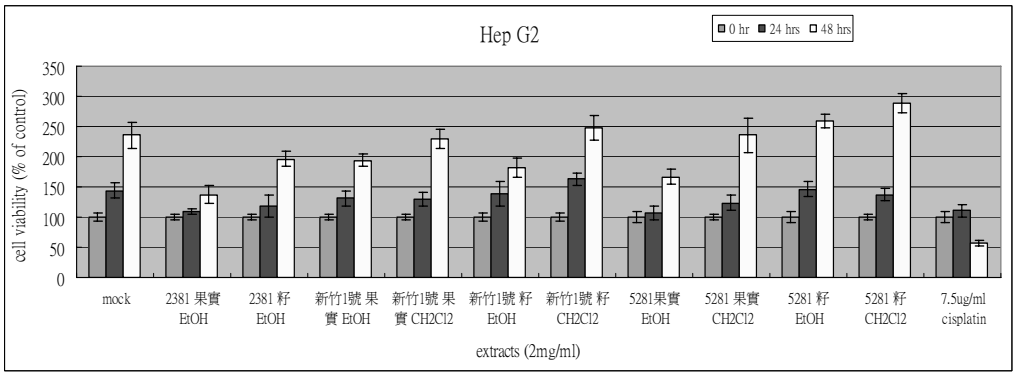
7.5ug/ml cisplatin
Extracts (1mg/ml)


Hep G2
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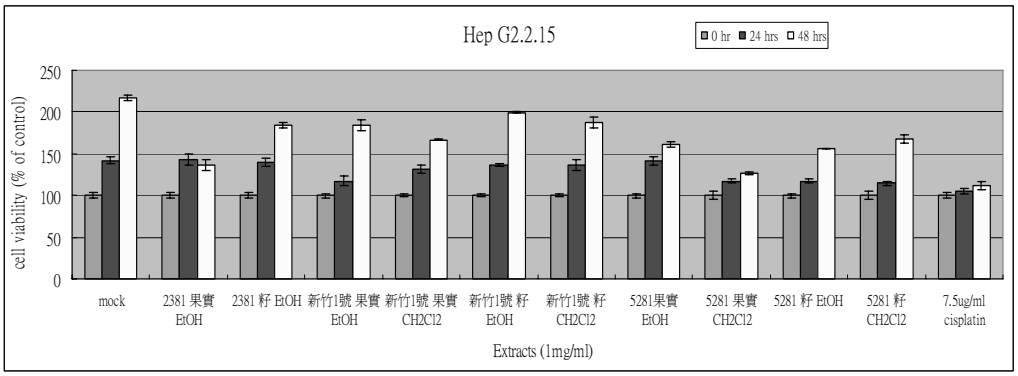
7.5ug/ml cisplatin
extracts (2mg/ml)


Hep G2.2.15
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cell viability (% of control)
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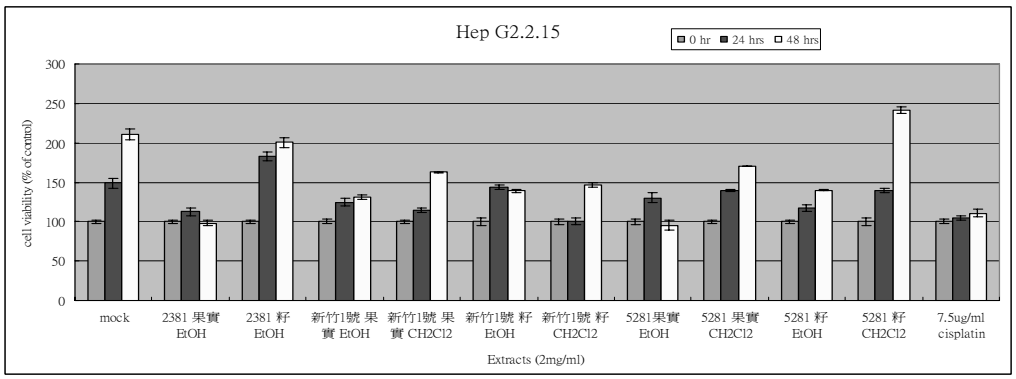
7.5ug/ml cisplatin
Extracts (1mg/ml)


Hep G2.2.15
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cell viability (% of control)
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5281 籽 CH2Cl2
7.5ug/ml cisplatin
Extracts (2mg/ml)

Figure 1.Effect of growth inhibition on HepG2 and HepG2.2.15 cells by various MC extracts. HepG2 and HepG2.2.15 cells were treated with the indicated concentrations of MC extracts for two days. MTT assays were processed as described in Materials and Methods. Data represent the cell growth inhibition rate to untreated controls (MOCK).

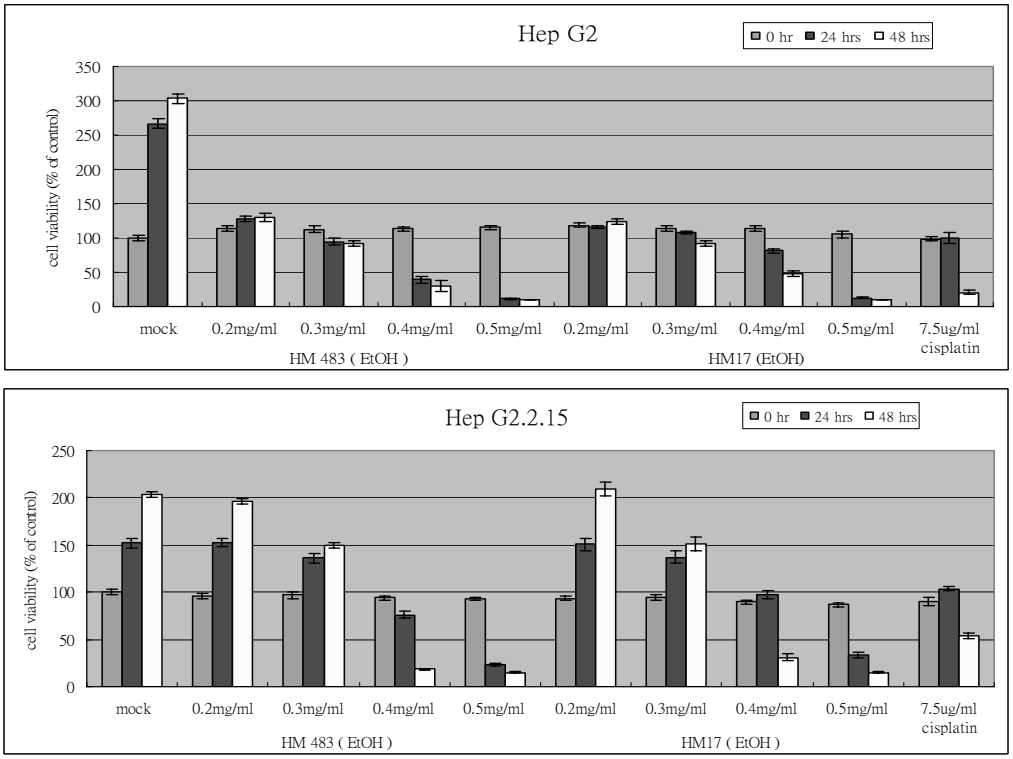

Figure 2.Effect of growth inhibition on HepG2 and HepG2.2.15 cells by ethanol extracted HM483 and HM17. HepG2 and Hep2.2.15 were treated with a gradient concentration from 0.2 to 0.5mg/ml.

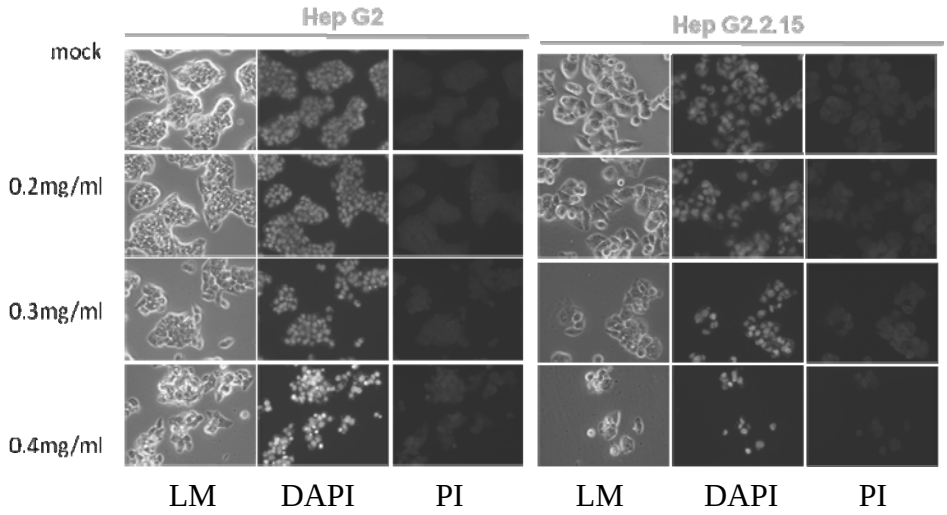

Figure 3.DAPI/PI double staining of HepG2 and HepG2.2.15 cells by treating with the ethanol extracted HM483.

DISCUSSION

Numerous attempts have been made to identify treatment for HBV infections because of the consequent diseases (Colacino, 1996; Huang et al., 2006). Among them, several components from natural products and many small molecules of herbal origin have been shown to have some anti-HBV activities in vitro. These results partly explain the beneficial effects of complementary or alternative strategy in the treatment of HBV-infected patients. According to previous report using by MAP30, a plant protein obtained from Momordica charantia, the production of HBV and expression of HBsAg and HBeAg could be inhibited by MAP30 (Fan et al., 2007). Our result from the cytotoxic analysis of MC on both HepG2 and HepG2.2.15 cells could just show a significant anti-tumor activity by an appropriate MC extract, but it didn't demonstrate any information concerning the anti-virus activity on HBV.

MAP30 and another anti-HIV protein from *Gelonium multiflorum*, GAP31, have been reported to possess dual ability to act on both DNA and RNA substrates. MAP has a novel DNA topological inactivation ability to convert viral DNA into topologically inactive forms and interrupting DNA function. The ability of MAP30 to interrupt essential topological

interconversions of viral DNA and ribosomal function of rRNA in viral-infected cells may provide novel mechanisms for its antiviral actions. MAP30 also has potent anti-tumor activity against tumor cells (Rybak et al., 1994). In addition to MAP30, extracts of MC contain many other components which are not well characterized and may contain undefined biological activities. The importance of MC in regulating cell viability is mediated by several components.

This study on dosage-cell growth relationships showed that several species of MC with appropriate extraction could inhibit the growth of HepG2 and HepG2.2.15 cells. The differences of growth inhibitory effect on HepG2 and HepG2.2.15 by specific MC extracts are not significant. With the anti-HBV data of the HepG2.2.15 cells that didn't be shown in this study, the effective MC extracts for anti-virus and anti-tumor bioactivities are different. It indicated that these bioactivities of MC extract on HepG2 cells with or without HBV were mediated by different components.

Our results in this study indicated that MC is a potent anticancer drug on both hepatocellular carcinoma cells with or without HBV infection. However, the anti-HBV activity of MC extracts on HBV-infected hepatocellular carcinoma cells and the relationship between anti-virus and anti-tumor activity in HepG2.2.15 cells remain to be investigated.

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The Anti-virus and Anti-tumor Effect of *Momordica charantia* on HepG2 and HepG2.2.15 Cells.

Shih-Fang Tsang[1], Yi-Ling Yeh[1], Jong-Ho Chyuan[2], Hsue-Yin Hsu[1]

Institute and Department of Life Science, Tzu-Chi University[1].
Hualien District Agricultural Research and Extension Station[2].

ABSTRACT

To search for virus and cancer chemopreventive agents from natural resources, many phytochemicals and food additives have been screened. Bitter gourd (Momordica charantia, MC), widely known for its hypoglycemic, antiviral, antidiabetic and antitumor activities, has been used throughout the world as both food and traditional medicine. To clarify the multifunctional activities of MC with different species, the in vitro anti-virus and anti-tumor activities of crude extracts from MC were evaluated in this study by hepatocarcinoma cell lines, HepG2 cells and Hepatitis B virus (HBV)-infected HepG2 cells, HepG2.2.15 cells. MTT analysis was used to compare the cytotoxicity of cells treated with extracts of MC. Among the ethanol or CH2Cl2 extracts from MC seed, fruit, stem and leaves tested, only the ethanol extracts from the fruit inhibited the growth of HepG2 or HepG2,2,15 cells. Some extracts inhibited cell growth in both HepG2 and HepG2.2.15 cells in a time- and dose-dependent manner. Ethanol extracts from fruits of HM483 and HM17 showed significant growth inhibition on both cell lines at concentrations higher than 0.4mg/ml. There was no difference between HepG2 and HepG2.2.15 cells to all the MC extracts tested. It showed the presence of the HBV genome in the HepG2.2.15 cells did not change the response of HepG2 cells to MC extracts.

Key words：Anti-virus, Anti-tumor, *Momordica charantia*, HepG2, HBV.