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Original Article

Screening flavonoids and their synthetic analogs to target liver cancer stem-like cells

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Abstract: *Objective*: Hepatocellular carcinoma (HCC) is one of the most malignant tumors having the highest death rate in the world. In this research, we aimed to screen effective compounds that targeted liver cancer stem-like cells (LCSLCs) from 9 flavonoids and synthetic analogues. *Methods:* Magnetic activated cell sorting and stem cell conditioned suspension culture were used to separate LCSLCs from Huh7 hepatoma cell lines. MTT assay and tumor sphere formation ability were used to detect inhibitory effects of flavonoids and synthetic analogues on parental cells and LCSLCs. *Results:* 4 flavonoids and synthetic analogues (curcumin, casticin, luteolin and 8-bromo-7-methoxy chrysin (BrMC)) resulted in greater survival and self-renewal inhibitory effects on LCSLCs than that of parental cells. Among these compounds, curcumin and casticin had the highest inhibitory capability on LCSLCs. *Conclusion:* By determination, 4 flavonoids and synthetic analogues including curcumin, luteolin, casticin and 8-bromo-7-methoxy chrysin (BrMC) affected the viability and self-renewal of LCSLCs.

Keywords: Hepatocellular carcinoma, liver cancer stem like cell, flavonoid, self-renewal, therapeutic action

Introduction

Hepatocellular carcinoma (HCC) is one of the most common clinical malignant tumors affecting human health. Clinically, about 7.8 million patients die due to liver cancer all over the world annually according to the report of World Health Organization in 2015. HCC is often diagnosed at a late stage and medical treatments including chemotherapy, chemoembolization, ablation, and proton beam therapy have poor outcomes. There is an urgent need for new therapies for this aggressive disease. HCC progression has been thought to be driven by a small subset of cells, namely liver cancer stem cells (LCSC) [1]. They are tumor-initiating cells that retain stem cell-like properties. They have high capability for self-renewal, production of heterogeneous progeny, resistance to chemotherapy, and limitless proliferation, enable liver cancer resistance to radiotherapy and chemotherapy [2, 3].

Although liver cancer has a wide range of treatments, most therapies eventually fail with a short survival period and a high recurrence and mortality rate. Therefore, specifically killing the liver cancer stem-like cells (LCSLCs) play key roles in individual programs of liver cancer clinical treatment. At present, there are several methods for separating and identifying LCSLCs including serum-free suspension tumor spheroid formation [1], CD133 immunomagnetic bead sorting method [4], and the side population by flow cytometry [5]. Previous studies have shown the cells cultured in 6-well low adherence plates with serum-free stem cell conditional medium which containing DMEM/F12 plus 20 ng/mL EGF, 10 ng/mL bFGF, 1×B27 and 0.4 μg/mL insulin have cancer stem cell specific characteristics [6, 7].

Flavonoids are the most common group of polyphenolic compounds in the human diet. Widely distributed in food items such as parsley, onions, blueberries, and other berries, black tea, green tea, and oolong tea, bananas, all citrus fruits, Ginkgo biloba, red wine, seabuckthorns, and dark chocolate. Research show flavonoids have a wide range of biological and pharmacological activities in anti-allergic [8], anti-inflammatory [8, 9], antioxidant [9], anti-microbial [10-13], anti-cancer [9, 14], and anti-diarrheal activities [15]. Previous studies

including genistein [16], quercetin [17], resveratrol [18], curcumin [19], chrysin [20, 21], apigenin [22], luteolin [23], casticin [24], and 8-bromo-7-methoxy chrysin [25] have shown flavonoids and synthetic analogues can inhibit tumor growth. While recently some research revealed quercetin [26], resveratrol [27], curcumin [28], chrysin [29], casticin [30], and 8-bromo-7-methoxy chrysin [6, 31] inhibit the growth of tumor stem cells. In this study, we detected the inhibitory effects of 9 types of flavonoids contrast drugs in liver cancer cells and liver cancer stem-like cells (LCSLCs) by MTT assay and sphere formation ability assay. We found these drugs affect the number and the size of tumor spheroids differently, which provides experimental data for clinic hepatoma research.

Materials and methods

Cell culture

The Huh7 cells were obtained from the Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM cell culture medium supplemented with 10% FBS, 10 U/mL penicillin and medium 100 μg/mL streptomycin at 37°C and 5% CO_2 in the humid incubator. When the cells reached 80% confluence, adherent cells were digested with 0.25% trypsin and passaged for further drug treatments. For liver cancer stem cell culture, the derived $CD133^+$ cells were cultured in low adherence plates with serum-free stem cell conditional medium that containing DMEM/F12 (Gibco Invitrogen) plus 20 ng/mL EGF (Peprotech, NJ, USA), 10 ng/mL bFGF (Peprotech, NJ, USA), 1×B27 (Invitrogen, CA, USA) and 0.4 μg/mL insulin (PeproTech). The study was approved by the Ethics Committee of Hunan Normal University.

Drugs and reagents

Dulbecco's minimum essential medium (DMEM), serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium and Trypsin were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Biowest (Loire Valley, French). Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazo-2-yl)-2,5-diphenyltetrazolium (MTT), genistein, quercetin, resveratrol, curcumin, chrysin, apigenin, luteolin, and casticin were purchased from Sigma (St. Louis, MO, USA). 8-bromo-7-methoxy chrysin (BrMC) were synthesized by our laboratory, College of Medicine, Hunan Normal University. Trypan blue, insulin, penicillin and streptomycin were purchased from Dingguo Changsheng Biotech Co.Ltd (Beijing, China). All drugs were dissolved in DMSO, and stored at -20°C.

Immunomagnetic separation of $CD133^+$ hepatoma cells

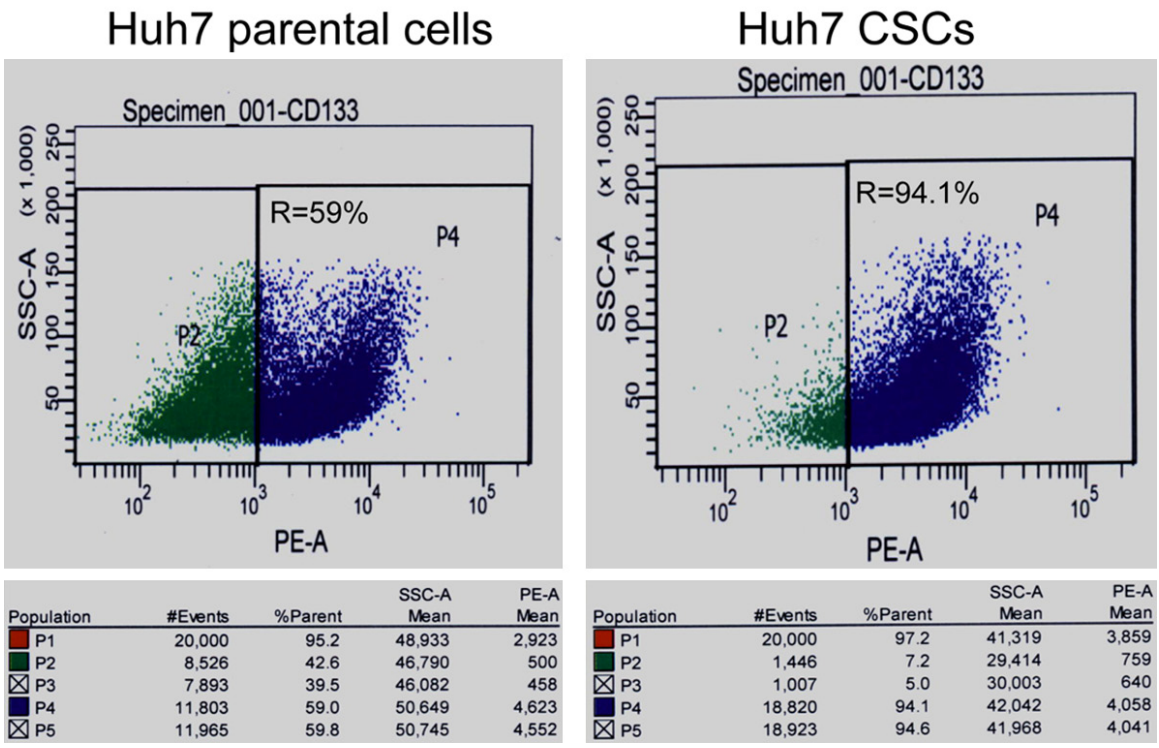
Cells were suspended with PBE incubation solution (0.5% bovine serum albumin, 0.08% EDTA in PBS, pH 7.2) to a final concentration of 1×10^8 cells in 0.5 mL, incubated with anti-CD133 antibody (final concentration 20 μg/mL) at 4°C for 30 min, then incubated with antibody-coated superfine magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at 10°C for 15 minutes and suspended in 20 times the total volume of PBE solution. The separation column was installed into a magnetic field and pretreated with 0.5 mL PBE which was naturally eluted due to gravity. The incubated cell suspension was added to the separation column and naturally eluted. The column was rinsed twice and then separated from the magnetic field and subsequently inserted into a new tube, followed by administration of 1-2 mL PBE along the needle core to remove the $CD133^+$ cells. By this way, negative cells were collected and two types of cells were rinsed with medium.

Flow cytometry (FCM)

The freshly isolated cells from each fraction were prepared at a concentration of 10^5 cells/ml using William's E medium (containing 20% FBS) and blocked for 15-30 minutes at room temperature. These cells were then washed twice with PBS and re-suspended in 990 μL PBS. Subsequently, 10 μL of antibodies, including CD133 (PE-conjugated, Biolegend, USA) and isotype control IgG2b (PE-conjugated, Biolegend, USA), were added to each cell suspension. After 30 minutes of incubation at 4°C in the dark, the cells were washed twice with PBS, fixed in 0.1% formaldehyde and analyzed by the FACS CaliburTM system (BD Immunocytometry Systems, San Jose, CA).

MTT assay

The Huh7 cells were incubated in 96 well plates. The LCSLCs of Huh7 cells were seeded in ultra-



Population	#Events	%Parent	SSC-A Mean	PE-A Mean
P1	20,000	95.2	48,933	2,923
P2	8,526	42.6	46,790	500
P3	7,893	39.5	46,082	458
P4	11,803	59.0	50,649	4,623
P5	11,965	59.8	50,745	4,552

Population	#Events	%Parent	SSC-A Mean	PE-A Mean
P1	20,000	97.2	41,319	3,859
P2	1,446	7.2	29,414	759
P3	1,007	5.0	30,003	640
P4	18,820	94.1	42,042	4,058
P5	18,923	94.6	41,968	4,041

Figure 1. FACS results shows that the percentage of $CD133^+$ population is much higher than that of Huh7 parental cells.

low adhesion 96 well plate with conditional serum-free stem cell media, the cell density is 5×10^3 cells for each well. After 24 hours, the cells were treated with 9 different flavonoids and synthetic analogues (genistein, quercetin, resveratrol, curcumin, chrysin, apigenin, luteolin, casticin and BrMC). Each of the flavonoids and synthetic analogues was tested at different concentrations (10 μM, 20 μM, 40 μM). After 48 hours of treatment, 10 μL MTT was added to each well and incubated for another 4 hours, then DMSO was added to each well. The cells were measured at 490 nm wavelength by microplate reader (Biotek, SYNERGY HTX, Vermont, and USA). The relative inhibition rate (IR) for cell proliferation was calculated as following formula: (1 - average absorbance of experimental group/mean absorbance of control group) ×100%. The experiment was repeated three times.

Sphere-formation assay

The LCSLCs of Huh7 cells were seeded in ultra-low adhesion 6 well plate with conditioned serum-free stem cell media at a density of 1.5×10^5 cells for each well. After 24 hours, the cells were incubated with various flavonoids and synthetic analogues for another 6 days. Then the cells were monitored under microscope (Olympus, CX41, Japan) at day 4 and day 6.

Equal numbers of LCSLCs were seeded in 96-well ultra-low attachment plates at a density of 500 cells/well and/or 1,000 cells/well in 100 μL of conditional stem cell media. Four or more replicate wells were plated for each sample. Sphere formation in each well was monitored on day 7 after seeding.

For stem cell culture, specific stem cell culture medium containing flavonoids and synthetic analogues were added every 2 days to compensate for evaporation and provide fresh growth factors.

Statistical analysis

To test for differences in cell viability before and after flavonoid treatment and tumor ball formation in the treated and control groups

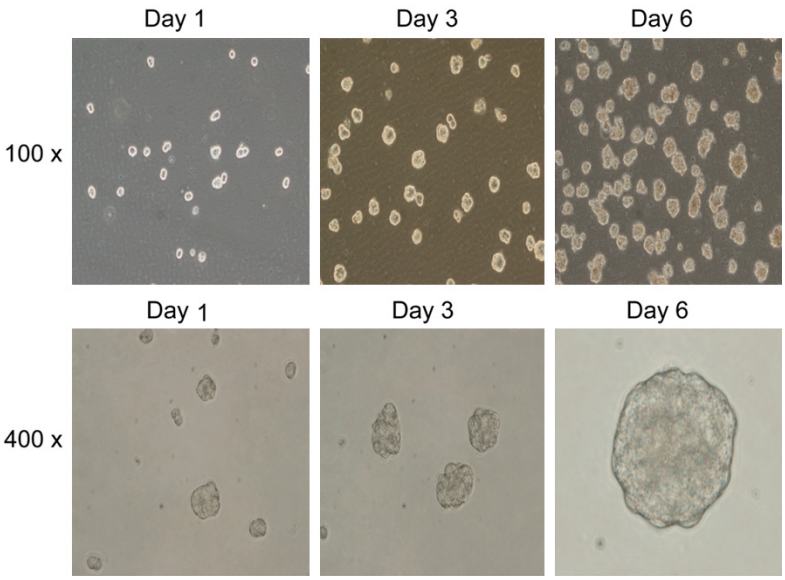


Figure 2. Culture of Huh7 derived stem-like cells (LCSLCs). The LCSLCs were suspended grown in stem cell conditioned medium. Above: tumor spheroid ball images under microscope at day 1, day 3, and day 6 (magnification, ×100). Below: tumor spheroid ball images at day 1, day 3, and day 6 (magnification, ×400).

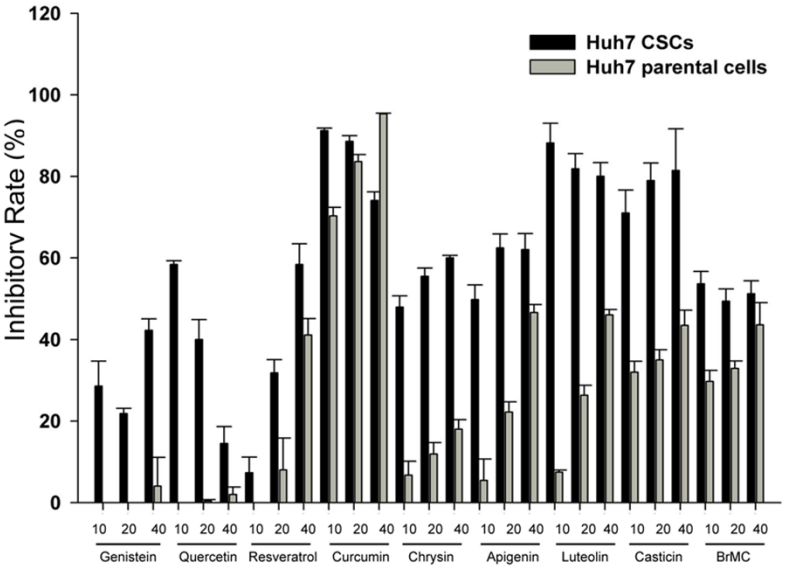


Figure 3. Screen of active drugs to Huh7 LCSLCs and parental cells. MTT assay showed the inhibition rate (%) of 9 types of active drugs to Huh7 LCSLCs and parental cells, the inhibition rates were normalized to untreated control cells. *, P < 0.01 compare to NC; ***, P < 0.05 compare to NC.

before and after treatment, Dunnett's two-tailed t test was done and the mean difference was considered significant at the P < 0.05 level. The relationship was statistically analyzed using Pearson's correlation test based on their H-scores and was considered significant at P < 0.05. All statistical analysis was done with SPSS version 15 (SPSS).

Results

Derivation and culture of LCSLCs

Human hepatoma carcinoma cell line Huh7 was cultured as normal condition and the cells adhered to the culture plates. In order to isolate LCSLCs, Huh7 cells were enzyme and suspended into single-cells. Then, the separated cells were immunostaining with an anti-CD133 antibody (Miltenyi Biotec Inc.) and checked by FACS analysis. FACS results showed that the percentage of $CD133^+$ population was about 94.1% (**Figure 1**), which was much higher than that of Huh7 parental cells (59%), indicating that our immunomagnetic separation system was effective. On the other hand, a major characteristic of cancer stem cells (CSCs) was their capacity to form three-dimensional colonies or spheres. We cultured $CD133^+$ cells in low adherence plates with conditional stem cell medium, in which those cells aggregated into tumor spheroids. The morphological change of tumor spheroids over time is shown in **Figure 2**. Obviously, the size and the cell number of the tumor spheroids increased during culture.

Comparison of cell survival inhibitory effects of the parental cells and LCSLCs by flavonoids and synthetic analogues

In order to analyze the inhibitory rate of flavonoids and synthetic analogues to LCSLCs and that of parental cells, we treated cells with 9

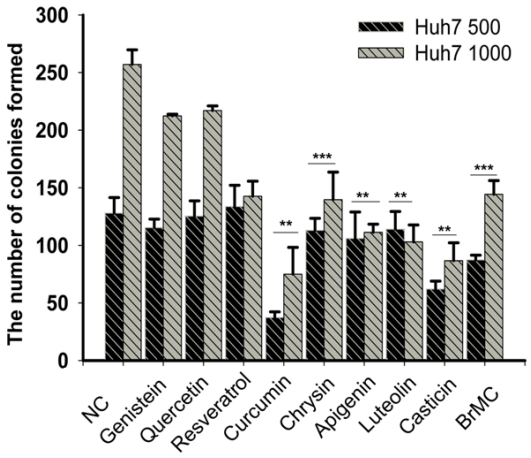


Figure 4. Screening active componds by sphere formation ability. All tested-agents affected the number of tumor spheroids balls, specifically curcumin luteolin, casticin and BrMC, compared with the control group (NC). **, $P < 0.01$ compare to NC; ***, $P < 0.05$ compare to NC.

types of drugs at different concentrations (10 μM, 20 μM and 40 μM), and the inhibitory rate of each tested agents were evaluated by MTT assay. As shown in **Figure 3**, 7 test-compounds had high inhibitory rates, which were resveratrol, curcumin, chrysin, apigenin, luteolin, casticin and BrMC. They dose dependently decreased the cell viability. Among these active compounds, the curcumin had the highest inhibition efficiency. If we compare the groups of Huh7 LCSLCs and that of parental cells, we found these flavonoids and synthetic analogues had higher inhibitory effect on LCSLCs than on parental cells.

Impact of flavonoids and synthetic analogues on the number of tumor spheroids

In order to detect the impact of flavonoids and synthetic analogues on tumor spheroid formation capability, the same number of LCSLCs were seeded in ultra-low adhesion 96 well plate with conditioned serum-free stem cell media containing active compounds, and the cell density was 500 cells/well and/or 1000 cells/well. After 7 days of treatment, the number of tumor spheroids in the control and experimental group were counted. The results showed the number of tumor spheroids decreased in all of the experimental groups, particularly in the group that treated by curcumin, chrysin, apigenin, luteolin, casticin and BrMC (**Figure 4**).

Inhibition of the growth of tumor spheroids of LCSLCs by flavonoids and synthetic analogues

The same number of LCSLCs were seeded in ultra-low adhesion 6-well plate with conditional serum-free stem cell media, and treated with the same concentration of drugs (10 μM). As shown in **Figure 5**, 4 tested-agents inhibited the growth of tumor stem like cells in the experimental group compared that in the negative control, they are curcumin, luteolin, casticin and BrMC, respectively.

Discussion

HCC is one of the most common clinical malignant tumors threating human life. Clinically treatments with surgery, chemotherapy, radiotherapy, and biological therapy are presently used followed by poor effectiveness. The research on cancer stem cells has opened up new avenues to accelerate the development of novel diagnostic and treatment strategies. Most cancer research experts focused on isolation and targeted killing of LCSLCs by using cancer stem cell biomarkers including CD133 (prominin-1), CD90, CD44, CD13, EpCAM, and OV6. CD133 is generally regarded as one of the most important molecular markers for stem cells, such as colon cancer [32-36], glioblastoma multiforme (GBM) cell line [37, 38], HCC [39-43], pancreatic cancer [44], gastric cancer [45-48], lung cancer [49-51], etc. Other groups isolated $CD133^+$ cells from liver cancer cell line by magnetic activated cell sorting system and stem cell conditioned suspension culture, $CD133^+$ populations were highly tumorigenic and characteristic of CSC [6, 31]. In our research, we sorted the $CD133^+$ cells by magnetic system, the population of $CD133^+$ is 59% in Huh7 parental cells, which is relative high in liver cancer cells, but consistent with other's report [52]. LCSLCs could be cultured in serum free stem cell conditioned suspension culture in ultra-low plates, which obtain the characteristic of LCSLCs [6, 7]. Recently, another team found that flavonoids inhibit growth of LCSLCs. Lin et al. found that ursolic acid chalcone treatment caused a decrease in self-renewal capability and increase in sensitivity to doxorubicin and vincristine drugs in $CD133^+$ LCLSCs [53].

In our study, the results of MTT and tumor spheroid formation assay revealed that 9 of flavonoids and synthetic analogues had higher

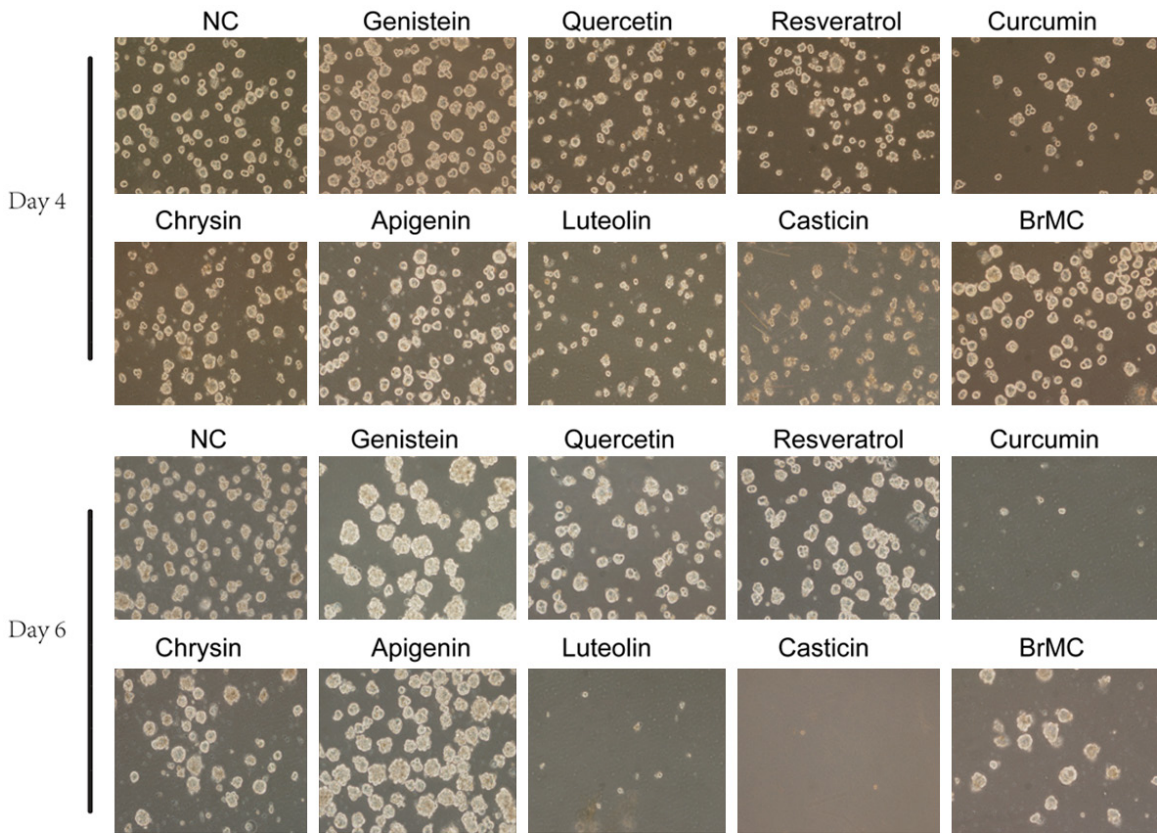


Figure 5. Screening of active compound drugs to LCSLCs. Equal amount of $CD133^+$ cells were seeded in 6-well ultra-low attachment plates containing 9 types of different flavonoids and synthetic analogues flavonoid drugs at a working the tested concentration of 10 μM respectively. After 4 or 6 day of culture, the cells were taken photos under microscope. (Magnification, ×100).

survival inhibitory rates on LCSLCs (the sorted stem cell) than on parental cells. These compounds were genistein, guercetin, resveratol, curcumin, chrysin, apigenin, luteolin, casticin, and BrMC (P all < 0.05). Six of 9 flavonoids and synthetic analogues were able to affect the number of tumor spheroids, they were curcumin, chrysin, apigenin, luteolin, casticin and 8-bromo-7-methoxy chrysin (P < 0.05). While 4 drugs were able to affect the size of tumor spheroids, these were curcumin, luteolin, casticin and BrMC (P < 0.05). Among these compounds, curcumin and casticin had the highest inhibitory activity.

In conclusion, we present supportive evidence for the inhibitory effects of flavonoids and synthetic analogues on LCSLCs, which may help us with screening for effective novel agents targeted to liver cancer and further research on therapeutics for human hepatocellular carcinoma.

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Disclosure of conflict of interest

None.

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