Ya'an Tibetan Tea Exerts Anti-tumor and Chemotherapy Sensitizing Effect against Human HepG2 Hepatocellular Carcinoma Cells

Lang He
Chengdu Medical College
Yu Yang
Chengdu Medical College
Siwei Deng
Chengdu Medical College
Guoqi Yu
Zhejiang Engineering Research Center of Fat-soluble Vitamin
Yuping Gong
Chengdu Medical College
Pei Yuan
Chengdu Medical College
Shujuan Cao
Chengdu Medical College
Haiyang Zhu
Chengdu Medical College
Junli Hao
Chengdu Medical College
Dan Wang (wangdan@cmc.edu.cn)
Chengdu Medical College

Research Article

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Abstract

Chinese dark tea is post-fermented tea possessing various biological. Ya'an Tibetan tea, which is unique to China, is one of those dark tea. The purpose of this study was to observe the antiproliferative activity and chemotherapy sensitizing effect of Ya'an Tibetan tea water extract against human hepatocellular carcinoma cell line HepG2, so as to preliminarily explore the molecular mechanism of its anti-tumor effect. HepG2 cells were treated with different concentrations of Tibetan tea water extract and Tea-paclitaxel combination. The cytotoxicity was measured. The apoptotic features were detected by Hoechst 33258 fluorescence staining, flow cytometry and caspase activity assay kit of Caspase-3/8/9. Crystal violet staining method was adopted to observe the colony formation capacity of different treatment groups. The results revealed that Ya'an Tibetan tea water extract inhibited the proliferation of HepG2 cells in a time-and dose-dependent manner. Meanwhile, it also enhanced the cytotoxic effect of paclitaxel on HepG2 cells, while had no cytotoxic effect on human liver cells LO2. HepG2 cells exhibited obvious morphological changes of apoptosis after treatments. The enzyme activity of Caspase-3/8/9 in HepG2 cells were increased. Furthermore, compared with treatment alone, Tibetan tea in combination with paclitaxel significantly induced the cell apoptosis ($P < 0.05$) and inhibited the colony formation capacity of HepG2 cells ($P < 0.05$). This study provides an experimental basis for the further development and clinical application of Ya'an Tibetan. However, its anti-cancer potential needs further study.

Introduction

Hepatocellular carcinoma (HCC) ranks as the third leading cause of cancer-related deaths worldwide. HCC patients had several treatment options, but the five-year survival is poor compared with other malignancies and the rate of tumor recurrence after surgery is very high (70–80% of cases)[1]. The development of more effective novel agents is thus urgently needed.

Tea is one of the most consumed beverages in the world. Habituating with tea in daily habit has been linked to the reduced risk of various diseases, such as cancer, disease of cardiovascular system and obesity. Pure polyphenol from tea may be used as therapeutic agent along with conventional therapy for better cancer treatment[14, 19]. Although green tea is most studied for its health benefits, emerging data is suggesting that other kind of tea may also possess similar health promoting attributes and anti-cancer effects[4, 5]. Chinese dark tea is post-fermented. The special post-fermentation makes its active ingredients different from those of other kinds of tea. Its biological activity includes oxidation resistance, hypolipidemic effects[7], antihyperglycemic[17] and immune enhancement[9].

Ya'an Tibetan tea is a sort of Chinese dark tea which was initially produced in Southwestern China and then carried via the mountains to Tibet[20]. In order to further explore the medicinal properties of Ya'an Tibetan Tea, we looked for the potential anti-cancer property of Ya'an Tibetan Tea on human hepatocellular carcinoma cell line. The results suggest that constituents of Ya'an Tibetan Tea may affect hepatocellular carcinoma cell on a cellular level. Combined treatment with Ya'an Tibetan Tea and

paclitaxel significantly enhanced the cytotoxic effect. Further studies are important to elucidate the precise mechanism of inhibition of hepatocellular carcinoma by Ya'an Tibetan Tea.

Materials And Methods

1.1. Materials

Ya’an Tibetan tea was obtained from Ya’an Friendship Tea Co., Ltd.; Paclitaxel injection (Taiji Group Sichuan Taiji Pharmaceutical Co., Ltd.); human hepatocellular carcinoma cell line HepG2 and human liver cell line LO2 were purchased from the Cell Culture Bank of the Chinese Academy of Sciences; Dulbecco's modified Eagle's medium (Gibco, USA); Fetal bovine serum (Sijiqing Company Ltd. Hangzhou, China); Trypsin 0.25% solution, Penicillin-Streptomycin solution (HyClone, USA); Cell Counting Kit-8 (MCE, China); Apoptosis cell Hoechst 33258 detection Kit (Solarbio Life sciences, Beijing, China); Caspase 3, Caspase 8 and Caspase 9 enzyme activity detection kits, Bradford protein concentration determination kit (Beyotime Institute of Biotechnology, Shanghai, China); AnnexinV-FITC Apoptosis detection kit (KeyGEN Biotechnology Co., Ltd., Jiangsu, China); paraformaldehyde (Biosharp Beijing, China**);** Crystal violet (Cologne Chemicals Co., LTD, Chengdu, China).

1.2 Methods

1.2.1 The extraction of Ya’an Tibetan tea

Pulverized Ya'an Tibetan tea was decocted 3 times in distilled water at 100 ℃ for 3-5min. After 5 minutes’ centrifugation at 5000rpm, the supernatant was collected and then concentrated. 50ml concentrated solution was divided into three 90mm glass plates which were placed in -40°C pre-cooled for 12h, freeze-dried at -75°C for 24h. Finally, lyophilized aqueous extract of Tibetan tea (LATT, extraction yield 27%) was prepared. LATT was stored at 4 ℃ for further exploration.

1.2.2 Cell culture and treatment

HepG2 cells and LO2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) Fetal bovine serum (FBS) and 1% (0.01 g/ml) Penicillin-Streptomycin solution at 37°C in a humidified incubator under 5% CO_2 atmosphere. Cells were passaged when the density was observed to be 80%-90%. HepG2 cells or LO2 cells in logarithmic growth phase were cultured in medium with different concentrations of Tibetan tea water extract (400 μg/mL, 600 μg/mL, 800 μg/mL, 1000 μg/mL), paclitaxel 0.8 μg/mL, Tibetan tea water extract(600 μg/mL) and paclitaxel(0.8 μg/mL) combination .

1.2.3 Cell Viability Assay

HepG2 cells or LO2 cells were seeded at a density of 3,000 cells/well in 96-well microplates and cultured in 100 μL of DMEM supplemented with 10% FBS at 37°C with 5% CO_2 for 12h, then treated with different concentrations of Tibetan tea water extract as well as paclitaxel for 24h or 48h. Cell proliferation was measured by using the Cell Counting Kit-8 assay (CCK-8). The absorbance was taken at 450 nm. The

results were calculated as follows: inhibition rate = [(mean control absorbance - mean experimental absorbance) / (mean control absorbance - control blank absorbance)] ×100%.

1.2.4 Apoptosis assay

Hoechst 33258 fluorescence staining HepG2 cells were seeded into 6-well plates at a density of 5×10^6 cells/well and cultured at 37°C with 5% CO_2 for 12h. Tibetan tea water extract treated and non-treated cells were fixed with Hoechst fixative for 10 min at room temperature and were washed three times with PBS. Then the cells were incubated in dark place with Hoechst 33258 solution for 5 min at room temperature and washed twice with PBS. Morphological changes in nuclei were observed by using fluorescence microscope.

Caspase-3/8/9 Activity Assay The enzyme activities of Caspase-3, Caspase-8 and Caspase-9 were measured by a colorimetric assay using the corresponding Caspase Activity Assay Kit. Briefly, Cells of each group were collected. Total proteins were extracted with provided lysis buffer. Proteins were quantitated by Bradford method. Finally, Caspase-3/8/9 enzyme activities were detected according to the protocol recommended by the manufacturer.

Flow cytometry analysis HepG2 cells (4.5×10^4 cells/mL) were seed into 12-well plates and treated for 24h. Treatment groups and control are as described above. Induction of apoptosis were examined using Annexin V-FITC Apoptosis Detection kit according to the manufacture's protocol. The apoptosis cells were detected by NovoCyte Quanteon Flow Cytometer and the data was analyzed with NovoExpress software.

1.2.5 Crystal violet staining

HepG2 cells were seed into 12-well plates at a density of 700 cells/well in 2 ml medium. Treatment groups are Tibetan tea 300 μg/mL, paclitaxel 0.1 μg/mL and combination. The medium was replaced every 3 days. As cultured for 7 days and cell colonies were observed, the cells were washed three times with PBS, fixed with 4% paraformaldehyde at room temperature for 20 min. After stained with 0.5% crystal violet for 20 min, the plates were washed with RO water until the cell clones were purple, but the background was clean and transparent in the wells. After drying in the air, the cell colonies were counted.

1.2.6 Statistical analysis

All experiments were repeated independently three times. Data were analyzed with Microsoft Excel 2016 and reported as means ± standard deviation. Student's t-test was performed to compare means between two groups, and one-way analysis of variance was used for multiple groups. $P < 0.05$ was considered as statistically significant.

Results

3.1 Morphological observation of HepG2

As shown in Figure1, after treatment with various concentrations of Ya'an Tibetan tea water extracts for 24 h, compared with the control group, HepG2 cells became round, shrunken and brighter, and gradually detached from the bottom of culture plates. In addition, as the concentration of Tibetan tea water extract increasing, the growth density gradually decreased obviously. Therefore, the morphological observation preliminarily shows that Tibetan tea water extract can inhibit proliferation of HepG2 cells in a dose-dependent manner.

3.2 Cytotoxic effect of Ya'an Tibetan tea water extract on HepG2 cells and normal human liver cells LO2

The results of CCK-8 method, shown in Table 1 and Figure 2, demonstrated that Tibetan tea water extract significantly inhibited the proliferation of HepG2 cells in does- and time-dependence manners ($P < 0.05$). Meanwhile, as presented in Table 2 and Figure 2, in the LO2 hepatocytes, the very same Tibetan tea water extract had no cytotoxic effect on normal human liver cells LO2 ($P > 0.05$).

Table 1 Inhibitory rate of HepG2 cells of different concentrations of Tibetan tea water extract

	Control	Tibetan tea water extract (μg/mL)			
		400	600	800	1000
24 h	1.31±0.05	1.16±0.02^a	0.79±0.17^a	0.33±0.04^c	0.2±0.01^d
48 h	1.58±0.07	1.17±0.11^a	0.85±0.2^a	0.35±0.04^c	0.2±0.01^c

Values are expressed as mean ± SEM (n=3) and different letters within a column indicate significant difference between samples ($P < 0.05$); Compared with control cells in the corresponding period, $^aP < 0.05$ $^bP < 0.001$

$^cP < 0.0001$ $^dP < 0.00001$

Table 2 Inhibitory rate of LO2 cells of different concentrations of Tibetan tea water extract

	Control	Tibetan tea water extract (μg/mL)			
		400	600	800	1000
24 h	0.38±0.08	0.35±0.01	0.37±0.01	0.43±0	0.51±0
48 h	0.66±0.21	0.43±0.03	0.45±0.03	0.46±0.01	0.49±0.01

Values are expressed as mean ± SEM (n=3); Compared with control cells in the corresponding period, $P > 0.05$

3.3 Ya'an Tibetan tea water extract induces apoptosis in HepG2 cells

After treated with 600 μg/mL Tibetan tea water extract for 24 h, Hoechst 33258 fluorescence staining was used to monitor the apoptotic cells. Morphological changes of nuclei were observed by fluorescence

microscope at a magnification of 200×, Figure 3. The characteristic chromatin agglutination, nuclear fragmentation and apoptotic bodies were clearly shown in Tibetan tea water extract-treated cells, but cells without Tibetan tea water extract treatment displayed health characteristics with a large round nucleus and normal chromatin patterns. The results suggested that the proliferation inhibitory effect of Tibetan tea water extract may due in part to induction of apoptosis.

To assess the apoptosis population induced by Tibetan tea water extract, treated HepG2 cells and control were harvested and stained with Annexin V-FITC and PI for apoptosis analysis by flow cytometry. The percentage amount of viable(Q4-3), dead(Q4-1), early(Q4-4) and late(Q4-2) apoptotic HepG2 cells after 24h of exposure is shown in Figure 4A. Compared with control, the treatment with Tibetan tea water extract was able to increase the early apoptotic rate and total apoptotic rate of apoptosis in HepG2 cells ($P < 0.05$), Figure 4B. These results further demonstrated that Tibetan tea water extract induced apoptosis significantly.

Notes: (A) HepG2 cells distribution at several phases. (B) The early and total apoptosis rate of HepG2 cells. As compared to untreated group (control) apoptosis, $*P < 0.05$ indicates significant differences; Cell death were assessed using Annexin V-FITC apoptosis marker and PI dye. Viable cells were considered Annexin V-FITC −/PI −; Early apoptotic cells were considered Annexin V-FITC +/PI −; Late apoptotic/dead cells were identified as Annexin V-FITC +/PI +; Total apoptosis rate was the sum of the early and late apoptosis rates.

In order to explore the pathway that Tibetan tea water extract induces apoptosis of hepatocellular carcinoma cells as well as to investigate the molecular mechanism of the anti-hepatic Carcinoma effect, the enzyme activity of Caspase-3/8/9 were measured. Figure 5 shows that a significant increase in the enzyme activity of caspase-3, caspase-8 and caspase-9 in Tibetan tea water extract-treated HepG2 cells compared with control cells ($P < 0.05$).

3.4 Chemosensitization induced by Ya'an Tibetan tea water extract

To further study whether Tibetan tea water extract could increase the chemosensitivity of HepG2 cells, the effects of paclitaxel combined with Tibetan tea on the proliferation and apoptosis of human hepatocellular carcinoma cells were investigated. CCK-8 method was used again to detect the cytotoxicity of combination group. Notably, compared to monotherapy, combining Tibetan tea water extract and paclitaxel improved the growth inhibition rate of HepG2 cells (Figure. 6, $P < 0.05$).

Moreover, colony-formation assays were carried out to test the effect of combination on the clonogenicity of HepG2 cells. The hepatoma carcinoma cells treated with combination showed remarkably reduced colonies in comparison to that of monotherapy group, Figure 7.

Thirdly, to determine whether apoptosis was involved in the cytotoxic effects, stained with Annexin V-FITC/PI, a significant increase in the apoptotic populations of HepG2 cells were induced by combining Tibetan tea water extract and paclitaxel, Figure 8. The apoptotic rates of HepG2 cells were 0.59% in the

control group, 10.6% in the paclitaxel-treated group, 11.3% in the Tibetan tea -treated group, while 23.7% in the paclitaxel- Tibetan tea group. Compared to the monotherapy group, the apoptosis rate of HepG2 cells treated with combination was significantly increased ($P < 0.05$).

Discussion

Cancer is one of the leading causes of morbidity after cardiovascular diseases. According to the GLOBOCAN 2020 by the International Agency for Research on Cancer (IARC), there were 19.3 million new cancer cases and a total of 10.0 million cancer deaths globally in the year 2020[13]. Natural product-derived compounds such as paclitaxel are one of the major sources of medicine especially for cancer. Several chemotherapeutic drugs are using for the treatment of different types of cancer. Most of them block cell replication, therefore tumor growth. However, most of these drugs cause undesirable and potentially toxic effects for both malignant and normal cells because of their nonselective mode. Thus, more selective and less toxic molecules can help to treat cancer with better success and without side effects.

Ya'an Tibetan tea has been reported about its antioxidant and cytoprotective effects, it also can effectively lower blood pressure, remove blood lipids, and reduce the generation of atherosclerosis[16]. Tea polyphenols have been widely explored and the most attention focuses on catechins from green tea. The inhibitory activities of tea catechins against carcinogenesis and cancer cell growth have been demonstrated in cancer of liver, esophagus, stomach, small intestine, and colon[11, 18]. Tibetan tea also has been reported that it comprises at least five phenolic components, including gallic acid, and four catechins[16].

In this study, we investigated the anti-tumor effect of Ya'an Tibetan tea in the human hepatocellular carcinoma cell HepG2. Our results in vitro showed that compared to the control, the extract of Ya'an Tibetan tea inhibited the proliferation of HepG2 cells, which was in a dose- and time-dependent manner. Meanwhile, the extract was also tested for the toxicity on normal human cell lines (LO2) and was found to be nontoxic.

Most of chemotherapeutic agents used for the treatment of cancer cause apoptosis in cells. Apoptosis is a program cell death which has morphological characteristics including nuclear chromatin condensation, cell shrinkage, blebbing, nuclear fragmentation and formation of apoptotic bodies. In the present study, to determine whether apoptosis was involved in the cytotoxic effect induced by Ya'an Tibetan tea, morphological changes of apoptosis were observed by Hoechst 33258 dye and the amount of apoptosis was further confirmed by Annexin V/PI double staining with flowcytometry. The apoptotic cell population in HepG2 cells were significantly increased after treatment with Ya'an Tibetan tea. Caspase-3 which causes DNA fragmentation as well as DNA damage is known as a key protein in apoptosis process[12]. It can be activated by caspase-8 in extrinsic mitochondrial pathway while by caspase-9 in intrinsic mitochondrial pathway. Therefore Caspase-3, -8 and − 9 are three typical molecules explaining the mechanisms of cell apoptosis[3, 6]. The activity of these proteinases remarkably increased in HepG2 cells

when treated with Ya’an Tibetan tea, which is suggesting the involvement of both intrinsic and extrinsic pathways in the apoptosis process.

Paclitaxel is an anticancer drug originally derived from the pacific yew tree. It inhibits cell replication by binding to tubulin to enhance polymerization of microtubule bundles, which causes cell cycle arrest in the G2/M phase and induces cell apoptosis[8, 10, 15]. Paclitaxel has proved to be an effective chemotherapeutic drug to a series of tumors, such as ovarian, breast, gastric, and non-small cell lung cancer[10]. Though paclitaxel is also a common chemodrug for therapy of HCC patients[2], hepatoma cells are known to be highly resistant to it[21]. Due to drug resistance and the toxic effect of current chemotherapy strategies, combination treatment is frequently used in cancer therapy to enhance anticancer efficacy. Hence, to study the effect of Ya’an Tibetan tea on increasing the chemosensitivity of hepatocellular carcinoma, it was combined with paclitaxel on HCC cells. Our results revealed that compared with single use, the combination can significantly improve the growth inhibition rate and had significant effect on the colony-forming capacity of HepG2 cells. Meanwhile, Ya’an Tibetan tea significantly enhanced the pro-apoptotic effect of paclitaxel.

Conclusion

The extract of Ya'an Tibetan tea inhibited the proliferation of HepG2 cells by the induction of apoptosis and enhanced the paclitaxel-sensitization in hepatoma cells. It could be developed as a clinical therapeutic agent or chemotherapy-supplementary functional food for the clinical treatment of cancer. However, the active substances in Ya'an Tibetan tea extract should be explored separately to find out the direct effect on hepatoma cells and their underlying mechanisms.

Statements And Declarations

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Conflicts of interest/Competing interests: The authors declare no conflict of interest.

Availability of data and material: All data generated or analysed during this study are included in this published article.

Authors' contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lang He, Yu Yang, Siwei Deng, Guoqi Yu, Yuping Gong, Pei Yuan, Shujuan Cao and Haiyang Zhu. The first draft of the manuscript was written by Lang He, Junli Hao and Dan Wang. And all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval: Not applicable

Consent to participate: Not applicable

Consent for publication: Not applicable

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Figures

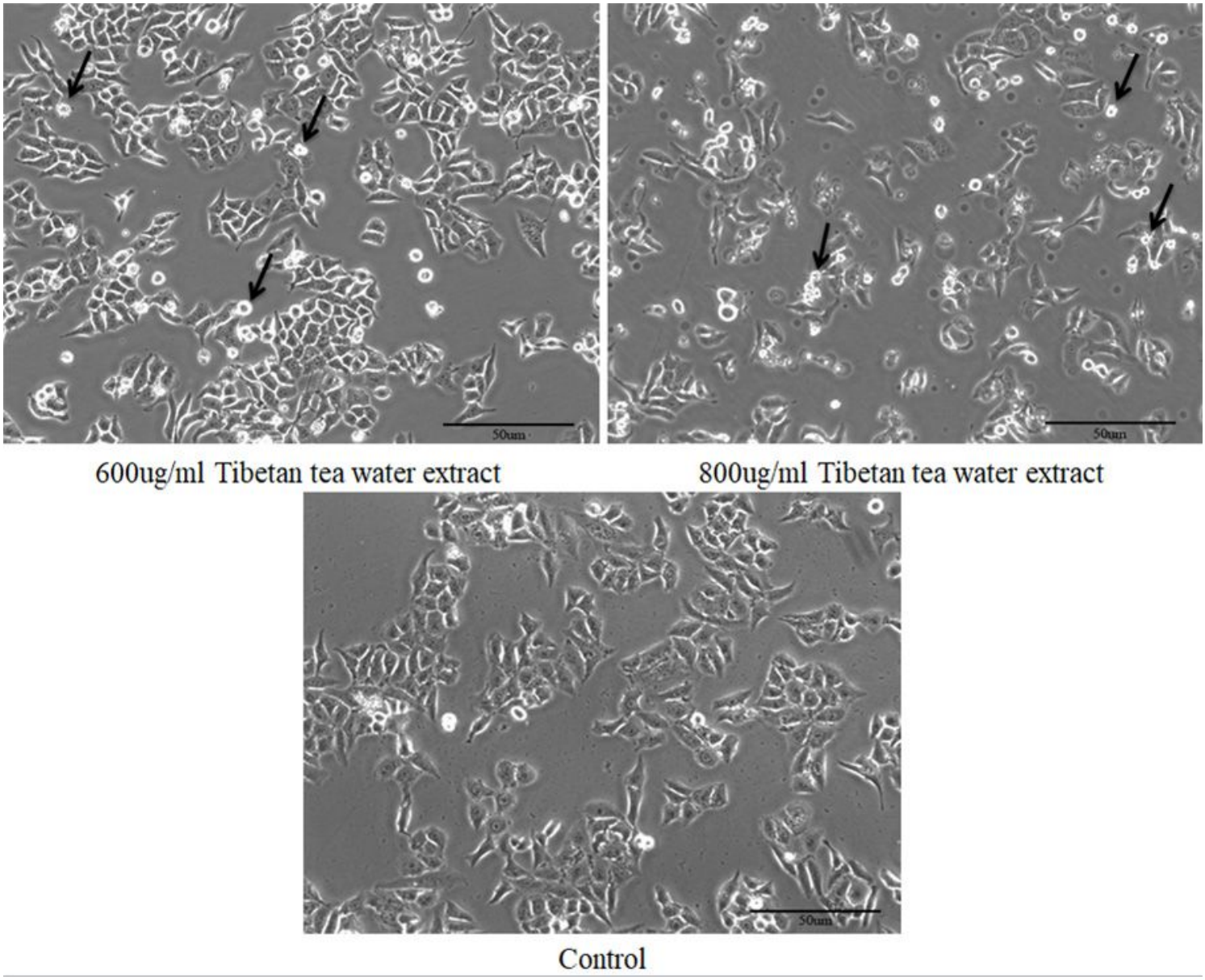

Figure 1

Morphological changes of HepG2 cells treated by different concentration of Tibetan tea water extract (100×)

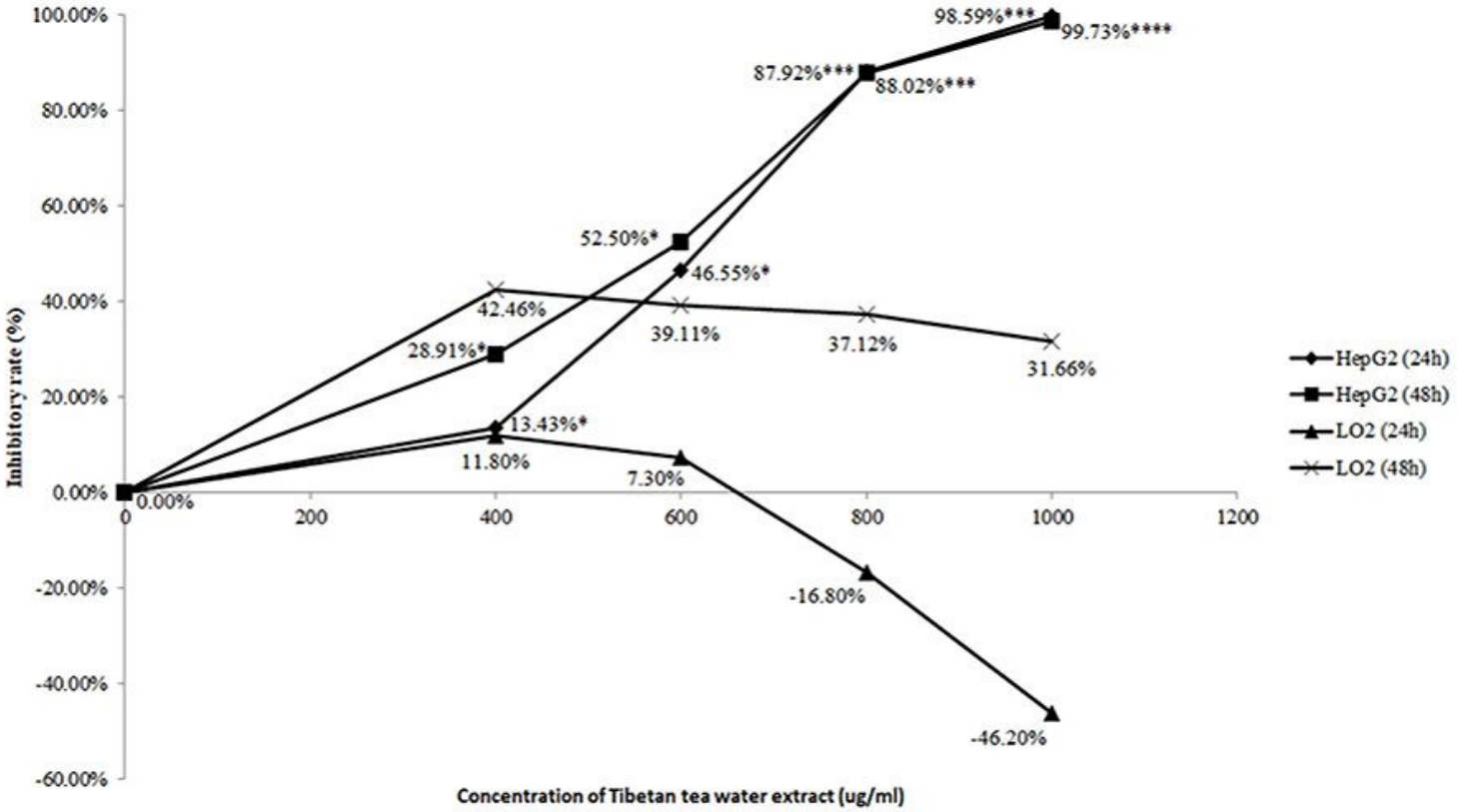

Figure 2

Tibetan tea water extract inhibits proliferation of HepG2 cells and LO2 cells determined by CCK-8 assay

Notes: Compared with control cells, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, **** $P < 0.00001$

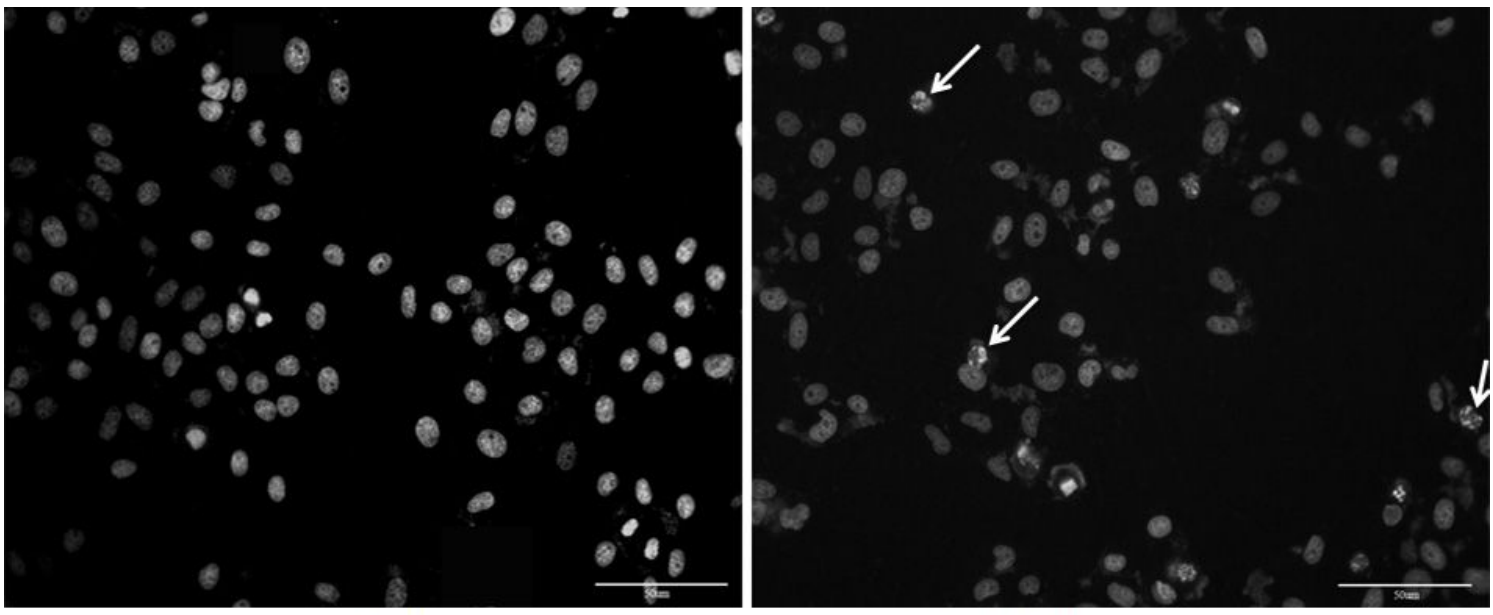

Figure 3

Fluorescent staining of nuclei in Tibetan tea water extract-treated HepG2 cells by Hoechst 33258 (200×)

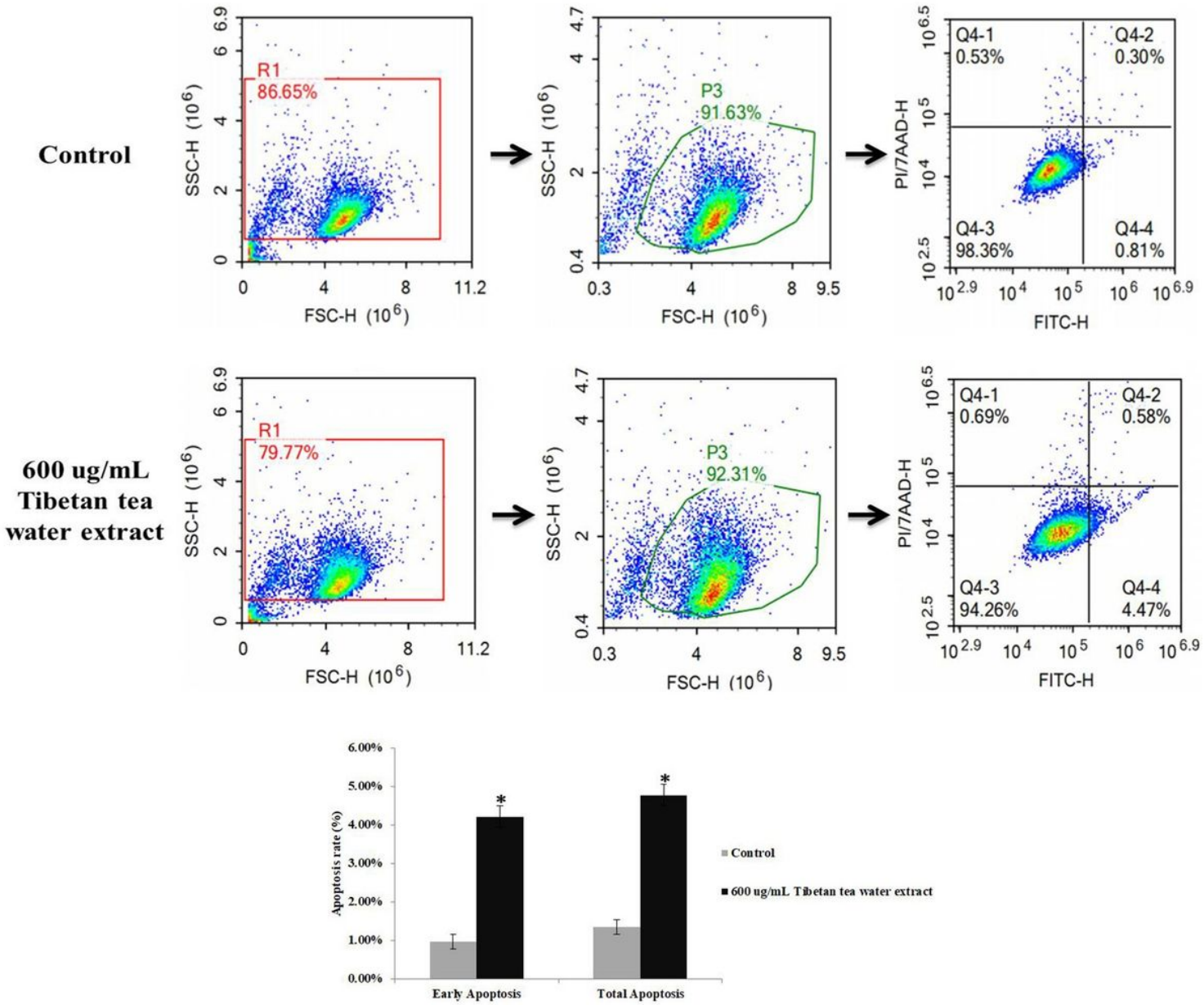

Figure 4

Flow cytometry analysis of untreated cells and Tibetan tea water extract-treated HepG2 cells at 24h

Notes: (A) HepG2 cells distribution at several phases. (B) The early and total apoptosis rate of HepG2 cells. As compared to untreated group (control) apoptosis, $*P < 0.05$ indicates significant differences; Cell death were assessed using Annexin V-FITC apoptosis marker and PI dye. Viable cells were considered Annexin V-FITC −/PI −; Early apoptotic cells were considered Annexin V-FITC +/PI −; Late apoptotic/dead

cells were identified as Annexin V-FITC +/PI +; Total apoptosis rate was the sum of the early and late apoptosis rates.

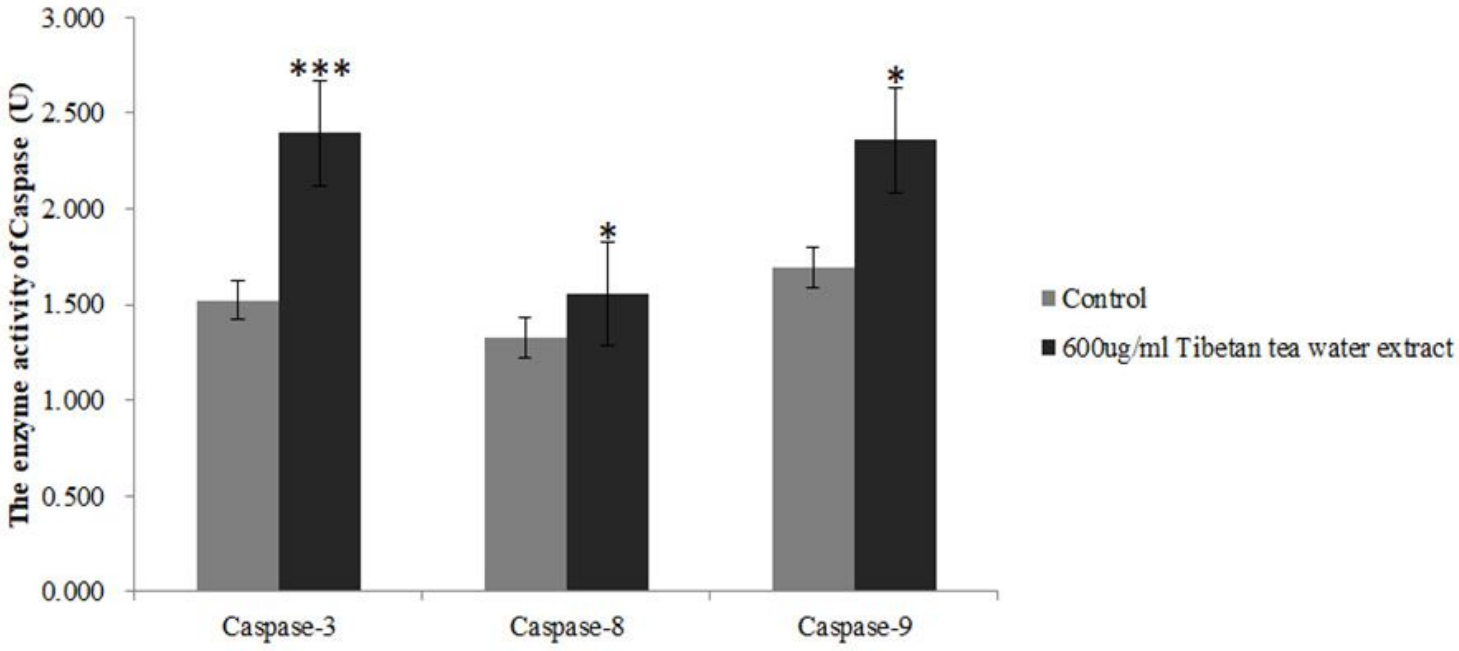

Figure 5

The enzyme activity of Caspase of HepG2 cells treated with Tibetan tea water extract

Notes: Data were presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

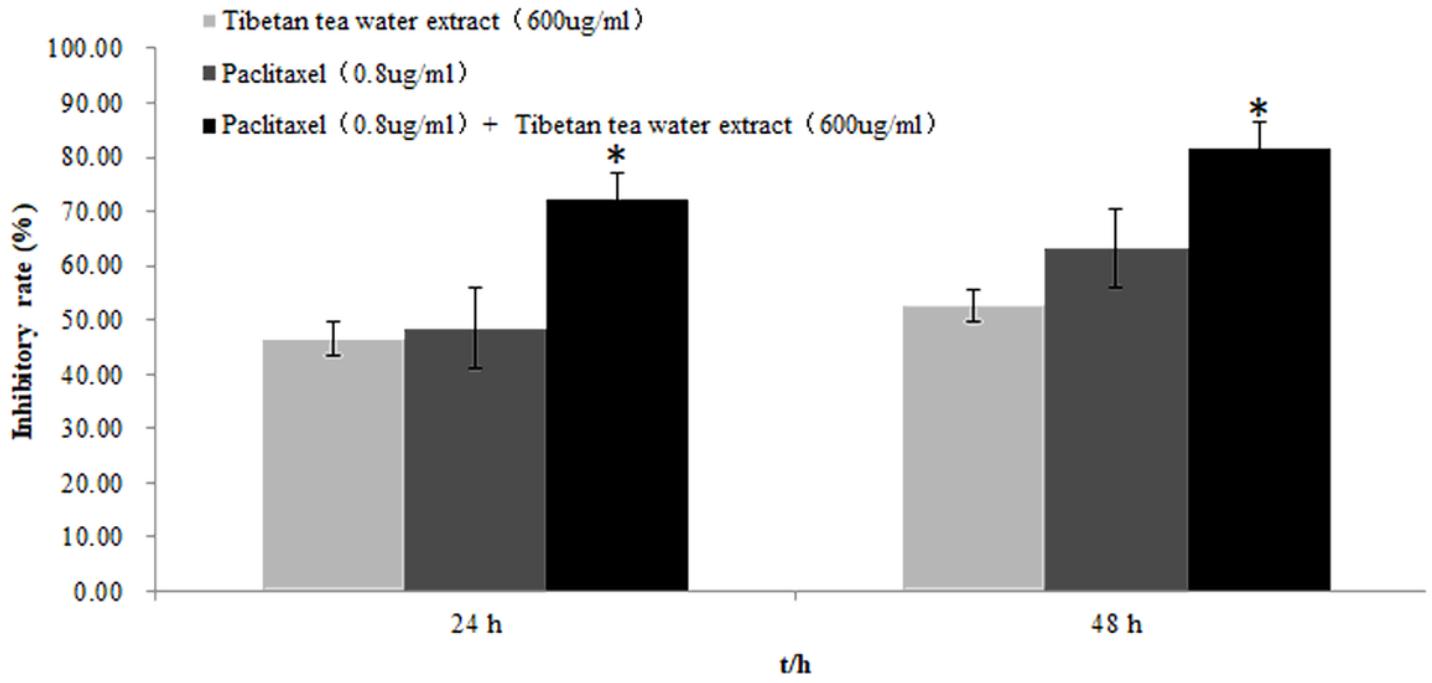

Figure 6

The inhibitory rate of Tibetan tea water extract combined with paclitaxel on HepG2 cells

Notes: Data were presented as mean ± SD. *P < 0.05 indicate significant differences as compared to paclitaxel-treated cells.

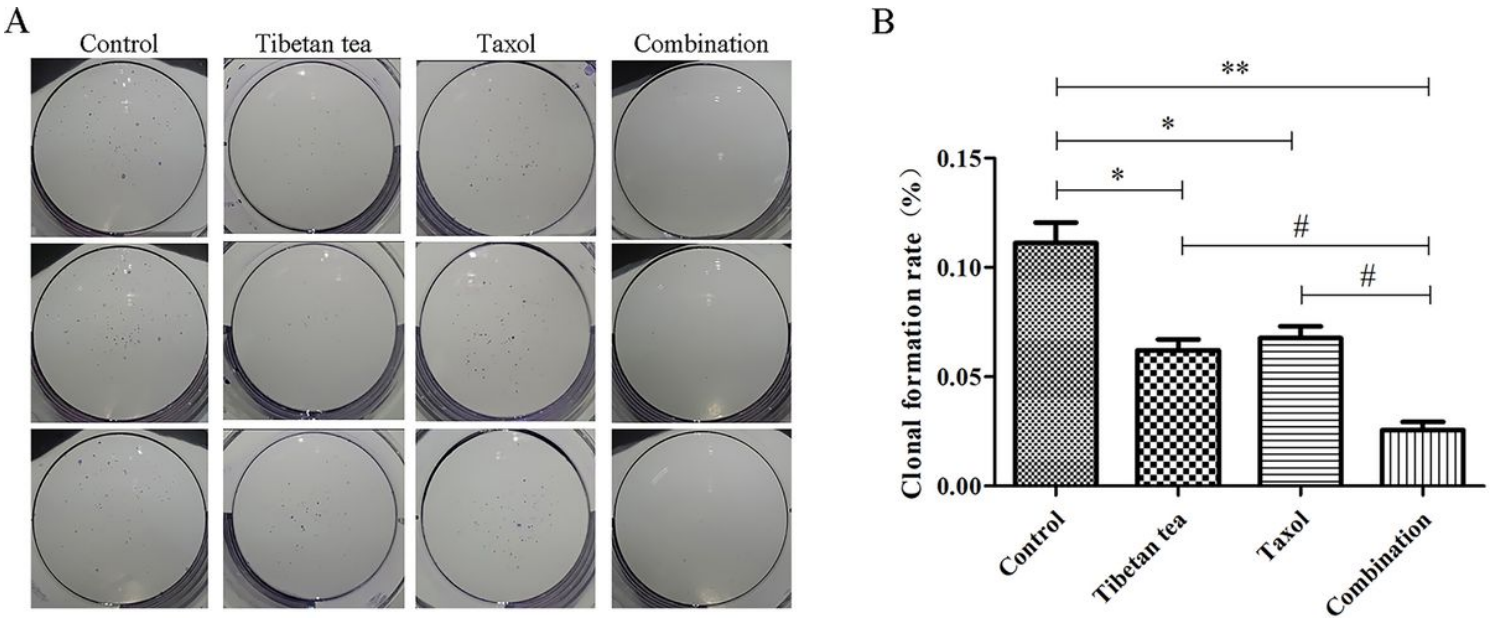

Figure 7

A. the clones of HepG2 cells were stained by crystal violet. **B.** Bar graphs presented the number of colonies generated from different groups. Notes: ∗P < 0.05, ∗∗P < 0.01 vs. control. #P < 0.05, combination vs. monotherapy.

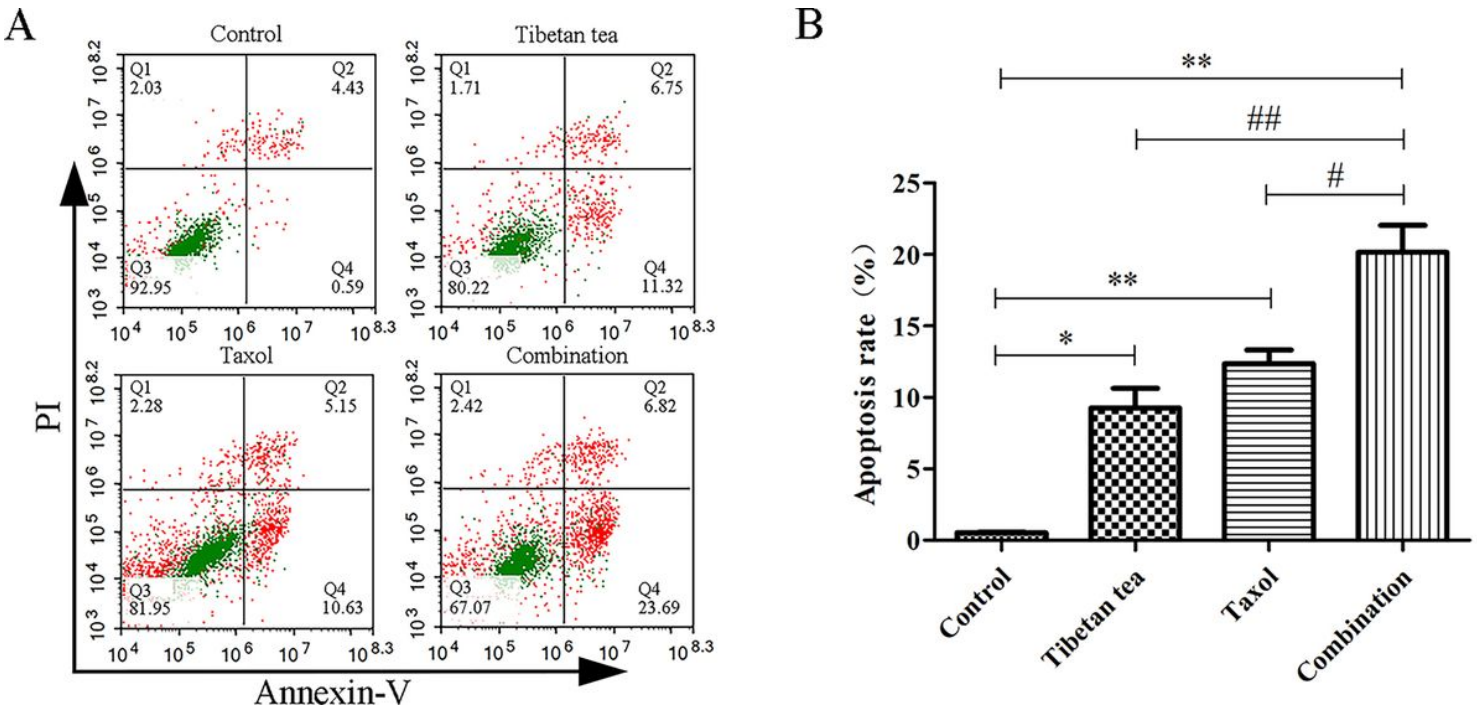

Figure 8

The apoptosis rates of HepG2 cells were detected by Annexin V/PI staining.

Notes: ∗P < 0.05, ∗∗P < 0.01 vs. control. #P < 0.05, ##P < 0.01 combination vs. monotherapy.