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Extraction of total triterpenoids from raspberry fruit and evaluation of their effects on human hepatocellular carcinoma cells

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Abstract

In this study, the total triterpenoids of raspberry fruit (TTRF) were prepared, and their effects on human hepatocellular carcinoma SMMC-7721 cells were investigated. SMMC-7721 cells were treated with TTRF with concentration of 0 (control), 50, 100, 200 and 400 μg/mL, respectively. The cell proliferation, cycle and apoptosis were detected. The reactive oxygen species (ROS) in cells and the expression levels of B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-2 associated X (Bax) in cells were determined. Results showed that, TTRF could obviously inhibit the proliferation of SMMC-7721 cells, arrest most cells in S and G2/M phases, and promote their apoptosis. In addition, TTRF could increase the ROS level, down-regulate the Bcl-2 protein expression and up-regulate the Bax protein expression in cells. In conclusion, cucurbitacin I has inhibitory effect on growth of SMMC-7721cells. The action mechanism may be related to its increasing ROS level and regulating of Bcl-2 and Bax expression in cells.

Keywords: total triterpenoids; raspberry fruit; live cancer; SMMC-7721.

Practical Application: This study has provided a basis for preparation of total triterpenoids from raspberry fruit and their further development and utilization.

1 Introduction

Raspberry (*Rubus idaeus* L.) is wild Rosaceae plant widely distributed in Europe, Asia and North America area. The raspberry fruit is a important product of raspberry, and is a potential source of bioactive compounds (Krivokapić et al., 2021). The raspberry fruit has pleasant color and tastes delicate. It is rich in carbohydrates, proteins, amino acids and other nutrients and trace elements (Skrovankova et al., 2015). In addition, the raspberry fruit contents polyphenols (Basu et al., 2018), polysaccharides (Yang et al., 2015) and terpenes (He et al., 2020), and has good antioxidant (Baby et al., 2018), anti-aging (Shukitt-Hale et al., 2017), anti-diabetic (Noratto et al., 2017), anti-inflammatory (Jean-Gilles et al., 2012), and anticancer (Baby et al., 2018) effects. Liver cancer is a common malignant tumor in human. In the past few years, the morbidity and mortality of liver cancer are increasing. It is a serious threat to human life and health (Anwanwan et al., 2020). Therefore, improving the survival rate and quality of life of patients with liver cancer has been one of the focuses of research in recent years. At present, most early liver cancer has no obvious symptoms. It often has metastasized or spread when found by doctors, so the patients have lost the best opportunity of surgical treatment (Li et al., 2021). Some current chemotherapeutic drugs not only kill tumor cells, but also cause great damage to normal cells of the body. More ever, many chemotherapeutic drugs can lead the drug resistance (Vempati & Malla, 2020). Therefore, exploring drugs from natural plants that are effective in treatment of liver cancer and have little adverse reactions may be a research hotspot recently (Zhou et al., 2016). In this study, the total triterpenoids of raspberry fruit (TTRF) were prepared, and their effects on human hepatocellular carcinoma SMMC-7721 cells were investigated. The objective was to provide an experimental basis for the clinical application of TTRF to treatment of liver cancer.

2 Materials and methods

2.1 Preparation of TTRF

Raspberry fruit (obtained from Lishui, Zhejiang, China) was dried and smashed. The powder was placed in the extraction kettle, followed by adding 8 times (volume to mass) of 90% ethanol-water solution. The heat-refluxing extraction was performed for 3 h. After filtrating, the extraction solution was obtained, followed by rotary evaporation to remove ethanol. Then, 5% sodium hydroxide solution was added to adjust pH to 7. After filtrating, the solution was obtained. The solution was loaded on AB-8 macroporous resin column, followed by gradient elution. The target elution solution was collected. After concentrating by rotary evaporation and drying, the crude product was obtained. The crude product was loaded on silica gel column, followed by elution with chloroform-methanol-water in different proportions. The elution solution was collected. After concentrating by rotary evaporation and drying, the final product was obtained. The content of TTRF was 75.23% determined by acetic anhydride-sulfuric acid (Liebermann Burchard) method.

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2.2 Culture of SMMC-7721 cells

Human hepatocellular carcinoma SMMC-7721 cells (provided by the Laboratory of Lishui University, Lishui, China) were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin at 37 °C and with 5% CO_2. The cells were digested with 0.25% trypsin for passage. The cells in logarithmic growth stage were taken for following experiments.

2.3 Detection of cell proliferation

MTT assay was used for detection of cell proliferation. SMMC-7721 cells in logarithmic growth stage were inoculated into the 96-well plates, with $5\times10^3-1\times10^4$ cells/well. After the cells adhered to the wall completely, the medium was discarded. TTRF was added to the well, and the final TTRF concentration was 0 (control), 50, 100, 200 and 400 μg/mL, respectively. The blank group only added with medium was set. Each group was set up with 5 repetitions. The mixture was incubated for 24, 48 and 72 h, respectively. A 5 mg/mL MTT solution was added to each well, followed by incubation for 4 h. A 150 μL DMSO solution was added to each well, followed by oscillation for 10 min. The optical density (OD) of each well was measured at the wavelength of 492 nm in microplate reader. The cell proliferation inhibition rate was calculated as follows: proliferation inhibition rate (Equation 1)

$$(\%) = \left[\left(OD_{treatment} - OD_{blank}\right) / \left(OD_{control} - OD_{blank}\right)\right] \times 100\% \quad (1)$$

2.4 Detection of cell cycle

The cell cycle was detected by flow cytometry. SMMC-7721 cells in logarithmic growth stage were treated with 0 (control), 50, 100, 200 and 400 μg/mL TTRF for 24 h, respectively. The cells were collected, and digested with trypsin without EDTA. Then, the cells were fixed with 75% ethanol over night. After centrifuging at 2000 rpm for 5 min, the cells were collected. The cells were washed wash PBS twice, followed by passing through the 300-mesh cell sieve. After centrifuging, the supernatant was discarded. A 500 μL prepared propidium iodide solution was added to the cells, followed by incubation at 4 °C for 30 min. The wells were detected in the flow cytometer. The proportion of cells in different phases was analyzed.

2.5 Detection of cell apoptosis

The cell apoptosis was detected by flow cytometry. SMMC-7721 cells in logarithmic growth stage were treated with 0 (control), 50, 100, 200 and 400 μg/mL TTRF for 24 h, respectively. The cells were collected, and digested by trypsin without EDTA. After centrifuging at 2000 rpm for 5 min, the cells were collected. The cells were washed wash PBS twice, followed by passing through the 300-mesh cell sieve. After centrifuging, the supernatant was discarded. The cells were re-suspended in 500 μL Binding Buffer. A 5 μL annexin V-FITC and 5 μL propidium iodide were added to the suspension, followed by incubation for 10 min at room temperature. The wells were detected in the flow cytometer, and the apoptosis rate of cells was calculated.

2.6 Detection of reactive oxygen species in cells

The content of reactive oxygen species (ROS) in cells was detected by DCFH-DA probe under fluorescence. SMMC-7721 cells were inoculated into 96-well plates for 24 h. The supernatant was discarded, and the cells were washed with PBS for three times. The new culture medium containing 4 μmol/L H_2O_2 was added, and the blank group was added with culture medium not containing H_2O_2. After incubation for 24 h, the cells were washed with PBS for three times, and 0 (control), 50, 100, 200 and 400 respectively μg/mL TTRF was added, respectively. After incubation for 12 h, 40 μL of 1 mmol/L DCFH-DA was added to each group (final concentration DCFH-DA was 20 μmol/L). After incubation for 30 min, the supernatant was discarded, and the cells were washed with PBS twice. The fluorescence intensity of each group was detected in flow cytometer.

2.7 Western blotting

The expression levels of B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-2 associated X (Bax) in cells were determined using western blotting. SMMC-7721 cells were treated with 0 (control), 50, 100, 200 and 400 respectively μg/mL TTRF for 24 h, respectively. The cells were collected. RIPA cell lysate was used to extract total protein from cells. After centrifugation at 12000 r/min for 10 min, the supernatant was obtained. The protein concentration was measured by BCA method. The sample was subjected to 10% SDS-PAGE. The isolated protein was transferred to PVDF membrane. After washing the membrane with TBST, the blocking solution containing 5% skimmed milk powder was added, followed by reaction at room temperature for 2 h. The membrane was mixed with primary antibody (rabbit anti-human β-actin, Bcl-2, Bax), followed by incubation overnight at 4 °C. After washing with TBST, the membrane was incubated with secondary antibody (horseradish peroxidase-labeled IgG) at room temperature for 1 h. After washing with TBST, the membrane was developed using DAB method, followed by photographing. The gray value of protein bands was analyzed by ImageJ software. The relative expression level of the target protein was calculated.

2.8 Statistical analysis

Data were presented as the mean ± standard deviation. The statistical analysis was performed using SPSS statistical software (version 21.0). The single factor analysis of variance was used for comparison among different groups. $P < 0.05$ indicated the statistically significant difference.

3 Results

3.1 Effects of TBST on proliferation of SMMC-7721 cells

As shown in Figure 1, TBST with different concentration and different treatment time could obviously inhibit the proliferation of SMMC-7721 cells. The proliferation inhibition rate increased with the increase of TBST concentration and increase of treatment time, respectively. When the concentration of TBST was 400 μg/ml and the treatment time was 72 h, the

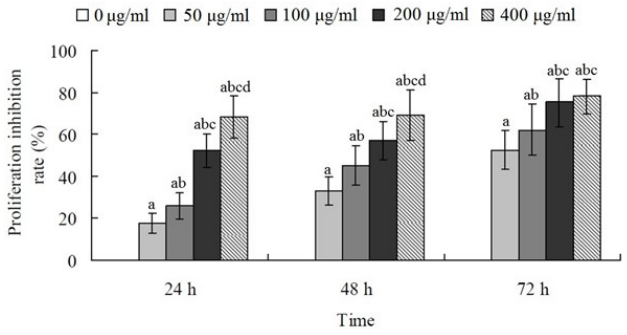

Figure 1. Effects of TBST on proliferation of SMMC-7721 cells (n = 5). (A) P < 0.05 compared with 0 μg/mL TBST group; (B) P < 0.05 compared with 50 μg/mL TBST group; (C) P < 0.05 compared with 100 μg/mL TBST group; (D) P < 0.05 compared with 200 μg/mL TBST group. TBST: total triterpenoids of raspberry fruit.

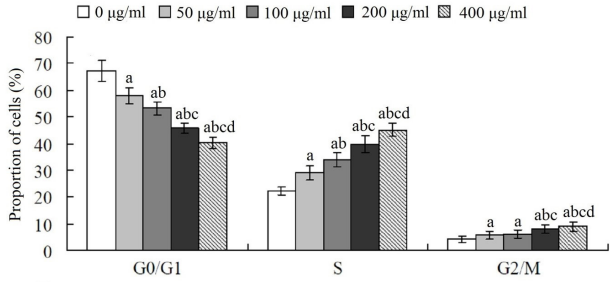

Figure 2. Effects of TBST on cycle of SMMC-7721 cells (n = 5). (A) P < 0.05 compared with 0 μg/mL TBST group; (B) P < 0.05 compared with 50 μg/mL TBST group; (C) P < 0.05 compared with 100 μg/mL TBST group; (D) P < 0.05 compared with 200 μg/mL TBST group. TBST: total triterpenoids of raspberry fruit.

proliferation inhibition rate was (76.67 ± 9.20)%, which was the highest among different groups.

3.2 Effects of TBST on cycle of SMMC-7721 cells

After treated with TBST for 24 h, the cycle of SMMC-7721 cells had greatly changed. With the increase of TBST concentration from 0 to 400 μg/mL, the proportion of SMMC-7721 cells in G0/G1 phase gradually decreased, and the proportion of cells in S phase and G2/M phase gradually increased, respectively (Figure 2).

3.3 Effects of TBST on apoptosis of SMMC-7721 cells

Figure 3 showed that, with the increase of TBST concentration and treatment time, the apoptosis rate of SMMC-7721 cells gradually increased, respectively. After SMMC-7721 cells were treated with 400 μg/mL TBST for 72 h, the apoptosis rate was (31.38 ± 5.02)%, which was the highest among different groups.

3.4 Effects of TBST on ROS level in SMMC-7721 cells

After treated with TBST for 24 h, the fluorescence intensity of ROS in SMMC-7721 cells greatly changed. With the increase of TBST concentration from 0 to 400 μg/mL, the fluorescence intensity of ROS gradually increased. There were significant differences among five groups (Figure 4).

3.5 Effects of TBST on Bcl-2 and Bax protein expressions in SMMC-7721 cells

Figure 5 showed that, after treated with TBST for 24 h, the relative expression level of Bcl-2 protein in SMMC-7721 cells gradually decreased with the increase of TBST concentration from 0 to 400 μg/mL. The expression level of Bax protein in SMMC-7721 cells gradually increased with the increase of TBST concentration from 0 to 400 μg/mL (Figure 5).

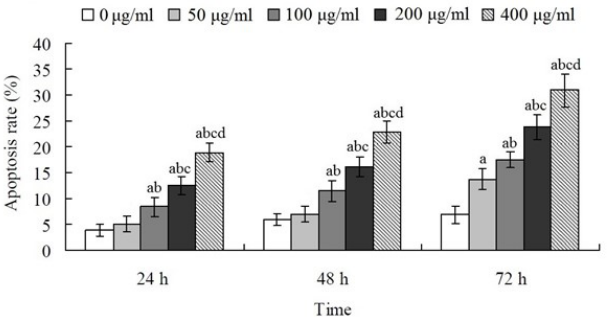

Figure 3. Effects of TBST on apoptosis of SMMC-7721 cells (n = 5). (A) P < 0.05 compared with 0 μg/mL TBST group; (B) P < 0.05 compared with 50 μg/mL TBST group; (C) P < 0.05 compared with 100 μg/mL TBST group; (D) P < 0.05 compared with 200 μg/mL TBST group. TBST: total triterpenoids of raspberry fruit.

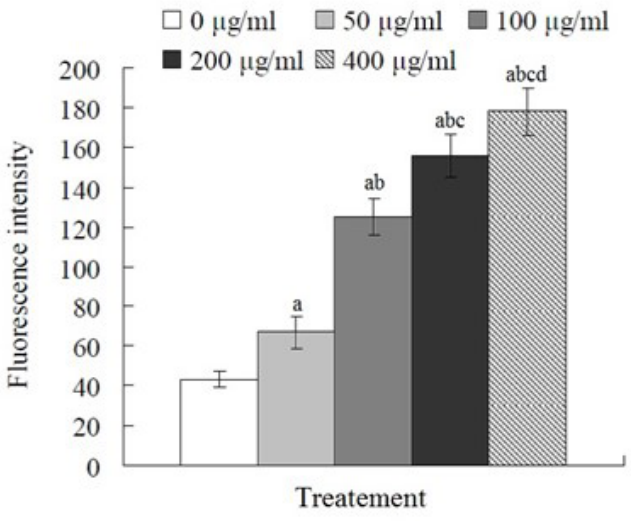

Figure 4. Effects of TBST on ROS level in SMMC-7721 cells (n = 5). (A) P < 0.05 compared with 0 μg/mL TBST group; (B) P < 0.05 compared with 50 μg/mL TBST group; (C) P < 0.05 compared with 100 μg/mL TBST group; (D) P < 0.05 compared with 200 μg/mL TBST group. TBST: total triterpenoids of raspberry fruit; ROS: reactive oxygen species.

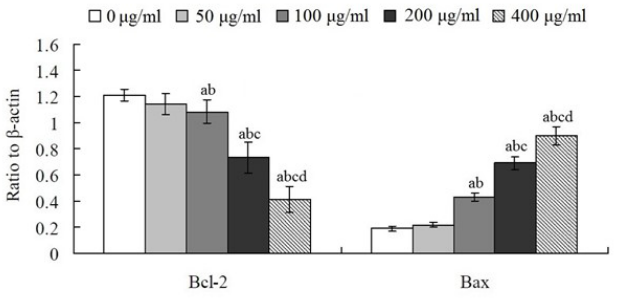

Figure 5. Effects of TBST on Bcl-2 and Bax protein expressions in SMMC-7721 cells (n = 5). (A) $P < 0.05$ compared with 0 μg/mL TBST group; (B) $P < 0.05$ compared with 50 μg/mL TBST group; (C) $P < 0.05$ compared with 100 μg/mL TBST group; (D) $P < 0.05$ compared with 200 μg/mL TBST group. TBST: total triterpenoids of raspberry fruit; Bcl-2: B-cell lymphoma-2; Bax: B-cell lymphoma-2 associated X.

4 Discussion

It is found that the some extracts from plants play an important role in the treatment of cancer (Huang et al., 2020; Zheng et al., 2020; Zhao et al., 2021). In addition, some food processing products exhibit the anticancer activity (Gaspar-Pintiliescu et al., 2020; Rafiq et al., 2020; Balthazar et al., 2021). However, some anticancer medicines may have different degrees of adverse reactions (Tewari et al., 2019). Therefore, looking for safer and more effective drugs from plants is still the trend of research and development of antitumor drugs. In this study, TTRF with 75.23% purity were prepared, and their effects on human hepatocellular carcinoma SMMC-7721 cells were investigated. Results showed that, TTRF can inhibit the proliferation of SMMC-7721 cells, promote their apoptosis, and arrest most cells in S and G2/M phases. This indicates that, TTRF has the inhibitory effect on growth of SMMC-7721 cells.

ROS are a normal part of cell metabolism and are necessary for cell signal transduction. ROS remain at a certain level in normal cells. When cells are exposed to exogenous hydrogen peroxide, due to the increased permeability of cell membrane, hydrogen peroxide will enter the cell through the cell membrane, and then decompose to produce reactive oxygen species such as oxygen ions, peroxides and oxygen-containing free radicals, resulting in the increase of ROS level in cells (Millare et al., 2020). With the increase of ROS level, the originally balanced redox level in cells was destroyed. When ROS reaches a certain value, it will lead to cell death after exceeding the cell's own clearance ability (Kang et al., 2019; Luo et al., 2019). In our study, the effects of TBST on ROS level in SMMC-7721 cells were investigated. Results showed that, after treated with TBST for 24 h, the fluorescence intensity of ROS in SMMC-7721 cells gradually increased with the increase of TBST concentration. Therefore, we believe that the mechanism of apoptosis induced by TBST may be to its increasing ROS level in cells.

Release of cytochrome C and cytochrome C-mediated apoptosis are regulated by multiple factors, of which the most obvious factor is the Bcl-2 family (Scorrano & Korsmeyer, 2003). Bcl-2 and Bax are important proteins in the Bcl-2 family and are most closely related to the apoptosis. Bax is an apoptotic protein located on the membrane of mitochondria. It is oligomerized to form a transmembrane channel, which increases membrane permeability and leads to a decrease in membrane potential (Lee et al., 2020). Bcl-2 is located in the endoplasmic reticulum. It can block Ca^{2+} from endoplasmic reticulum to cytoplasm and form heterodimer with Bax to prevent apoptosis (Jeong & Seol, 2008). This study investigated the effects of TBST on Bcl-2 and Bax protein expressions in SMMC-7721 cells. Results showed that, after treated with TBST for 24 h, the relative expression level of Bcl-2 protein in SMMC-7721 cells gradually decreased with the increase of TBST concentration, and the expression level of Bax protein gradually increased. This suggests that, TBST can regulate the Bcl-2 and Bax expressions, thus s promoting the apoptosis of SMMC-7721 cells.

5 Conclusion

In conclusion, TBST are obtained. They have inhibitory effect on the growth of human hepatocellular carcinoma SMMC-7721 cells. The mechanism may be related to its increasing ROS level and regulating of Bcl-2 and Bax expression in cells. This study may provide an experimental basis for further development and utilization of raspberry fruit and application of TBST to treatment of liver cancer.

Acknowledgements

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Conflict of interest

The authors declare that there is no conflict of interest.

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