Chinese Herbal Medicines 14 (2022) 449–458

Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

# Tea and *Citrus maxima* complex induces apoptosis of human liver cancer cells via PI3K/AKT/mTOR pathway *in vitro*

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ARTICLE INFO

*Article history:*
Received 8 May 2021
Revised 8 July 2021
Accepted 5 September 2021
Available online 22 June 2022

*Keywords:*
apoptosis
*Citrus maxima* cv. Tomentosa
liver cancer
signaling pathway
tea

ABSTRACT

*Objective:* In this study, black tea and *Citrus maxima* (BT-CM), yellow tea and *C. maxima* (YT-CM), green tea and *C. maxima* (GT-CM) as subjects, the active ingredient content and antioxidant activity of three tea and *C. maxima* (T-CM) were analyzed. The effects of three T-CMs on apoptosis of liver cells *in vitro* and its mechanism were further explored.

*Methods:* National standard method and HPLC were used for active ingredient analysis. MTT, cell flow cytometry and Western blot were used to analyze the effects of three T-CMs on cell proliferation, apoptosis, and its underlying molecular mechanism.

*Results:* The content of tea polyphenols, free amino acids, ratio of polyphenols and amino acids, ester catechins, non-ester catechins and caffeine in YT-CM and GT-CM was significantly higher than that of BT-CM. The *in vitro* antioxidant capacity of YT-CM and GT-CM was also significantly stronger than that of BT-CM. Three T-CMs had the effects of inhibiting proliferation, arresting cell cycle and inducing apoptosis in HepG2 and Bel7402 cells, especially YT-CM and GT-CM. Western blot analysis showed three T-CMs activated PI3K/AKT/mTOR signaling pathway and regulated the expression levels of apoptosis-related proteins Bax, Bcl-2 and Caspase-3/9. YT-CM and GT-CM had better ability to change the signal pathway than BT-CM.

*Conclusion:* In short, T-CMs, which combined different degrees of fermentation tea with *C. maxima*, were rich in nutrients and biologically active substances. T-CMs, especially YT-CM and GT-CM, are healthy drinks that help to prevent and treat liver cancer.



## 1. Introduction

Tea belongs to the *Camellia* genus in the classification of plants, and is a drink made from the leaves of the *Camellia* tea tree. China is the origin of the world's tea trees. It has a long history of drinking tea in Chinese history and is the best natural health drink for human beings (Wang et al., 2014; Zhu, Huang, & Tu, 2006). Tea contains more than 450 kinds of organic compounds, more than 15 kinds of inorganic minerals and essential nutrients (Yue et al., 2014). Tea has an effect on the prevention and treatment of hardening of blood vessels, hypertension, atherosclerosis, radiation damage, tumor and immune disorder (Baeza, De Castro, Arranz, & De la Fuente, 2010; Tamaya et al., 2010; Zhou et al., 2004). Studies have shown that tea had strong anti-oxidation and free radical scavenging ability, and was mainly related to its high antioxidant content, such as gallic acid, polysaccharides or polyphenols (Haque et al., 2006; Hsu et al., 2012). Tea can be divided into: non-fermented tea (such as green tea), light fermented tea (such as yellow tea) and whole fermented tea (such as black tea) (Liu, 2018). The antioxidant activity of tea differs from the fermentation degrees, indicating that the antioxidant and free radical scavenging properties of tea are closely related to its processing technology.

In recent years, the number of cancer patients has gradually increased, and even became the first cause of death in developed countries and the second cause of death in developing countries. In particular, the incidence of liver cancer in developing countries is higher than in developed countries (Wu et al., 2018). Tea and its functional ingredients have shown anti-cancer effects in many animal model experiments. Tea kills cancer cells and inhibits their development, on the other hand, it can improve the antioxidant activity and enhance cancer resistance. In chemical-induced rat

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https://doi.org/10.1016/j.chmed.2021.09.015
1674-6384/

models, tea components inhibited the occurrence and development of colon and oral cancer and promoted cancer cell apoptosis (Sengupta, Ghosh, & Das, 2003; Srinivasan, Sabitha, & Shyamaladevi, 2004). Tea-derived extracts such as tea polyphenols, (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC) or theaflavins significantly reduced the incidence of lung cancer in mice (Yang et al., 1997). Tea polyphenols and catechins had strong antioxidant activity and played an important role in inhibiting the development of tumors. In a mouse lung cancer model, a suitable dose of EGCG inhibited the production of reactive oxygen species in the liver and kidney of mice (Jung et al., 2007). Therefore, the anti-oxidative stress of tea components has positive significance to inhibit tumorigenesis.

*Citri Grandis Exocarpium* is a medicinal fruit produced in Guangdong, China. In recent years, the development and application of *Citrus maxima* cv. Tomentosa has received extensive attention from Chinese medicine. Pharmacological and clinical studies of the effective chemical constituents of *C. maxima* are receiving attention due to their potential immunomodulatory effects (Deng, Wang, Lin, Xiao, & Shuai, 2013; Wang, Li, & Xie, 2014). In this study, we prepared black tea and *C. maxima* (BT-CM), yellow tea and *C. maxima* (YT-CM), green tea and *C. maxima* (GT-CM) by loading Yinghong No. 9 black tea, yellow tea and green tea into the peel of *C. maxima*. Then the active ingredients, antioxidant capacity *in vitro* and the anticancer effects were analyzed. Our aim is to provide a natural and safe health drink that helps prevent and treat liver.

## 2. Materials and methods

### 2.1. Cells and reagents

BT-CM, YT-CM and GT-CM were produced by Tea Research Institute of Guangdong Academy of Agricultural Sciences and Guangdong Kaili Biochemical Science & Technology Co., ltd. (Guangzhou, China). Human liver cancer cells HepG2 and Bel7402 were purchased from the Cell Resource Center of the Shanghai Academy of Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS (Hangzhou Sijiqing, Hangzhou China). Forinol (Beijing Solarbio, Beijing, China), gallic acid (Tianjin Damao Chemical, Tianjin, China), catechin standard (Shanghai Shunbo Bio, Shanghai, China), MTT (Beijing MYM Bio, Beijing, China), Cell Cycle and Apoptosis Detection Kit (Beyotime Biotechnology, C1052, Shanghai, China), and Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology, C1062L, Shanghai, China) were purchased.

### 2.2. Preparation of tea extract

The pulverized tea and *C. maxima* (T-CM) coarse powder was weighed and added to a 90 °C water bath for 30 min at W/V = 1:20. The tea soup was then repeatedly extracted three times. The tea was concentrated by rotary evaporation to 1/10 of the original volume, and then lyophilized to obtain the T-CM aqueous extract.

### 2.3. Determination of biochemical components in tea extracts

The content of tea polyphenols was determined by GB/T 31740.2–2015 "Method for the determination of tea polyphenols and catechins". The free amino acid content was determined by GB/T 8314–2013 "Determination of the total amount of free amino acids in tea". The soluble sugar content was detected by an anthrone-sulfuric acid colorimetric method. The total amount of theaflavins, thearubigins, and the theaflavins was measured by a system colorimetric method.

### 2.4. Determination of catechin monomer by HPLC

The column was an Agilent ZORBAX Eclipse XDB-$C_{18}$ column (150 mm × 4.6 mm, 5 μm). The detection wavelength was 280 nm and the detection temperature was 28 °C. Mobile phase A was an aqueous solution containing 0.5% acetic acid, 1% acetonitrile and 2% methanol. Mobile phase B was an aqueous solution containing 0.5% acetic acid, 10% acetonitrile and 20% methanol. When eluting, within 30 min, phase A was from 72.5% to 20%, and phase B was from 27.5% to 80%. After 30 min, within 5 min, phase A recovered from 20% to 72.5%, and phase B recovered from 80% to 27.5%. The flow rate was 1.0 mL/min, and injection volume was 10 μL. The external standard method was used to quantify by peak area.

### 2.5. Determination of hesperidin by UPLC

The ultra-liquid chromatograph was an Agilent 1290 Ultra High Performance Liquid Chromatograph with an Agilent XDB-$C_{18}$ (1.8 μm, 2.1 mm × 100 mm). The mobile phase A phase was chromatographically pure acetonitrile and chromatographically pure methanol. Phase B was ultrapure water. The gradient concentration was 78 min, the column temperature was 30 °C, the injection volume was 5 μL, and the flow rate was 0.3 mL/min. When eluting, in phase 0–4, phase A was from 2.5% to 5%, and phase B was from 95% to 90%. Within 4–30 min, phase A was from 5% to 15%, and phase B was from 90% to 70%. Within 30–46 min, phase A was from 15% to 22.5%, and phase B was from 70% to 55%. Within 46–70 min, phase A was from 22.5% to 45%, and phase B was from 55% to 10%. Within 70–78 min, phase A was from 45% to 2.5%, and phase B was restored from 10% to 95%.

### 2.6. Determination of ferric ion reducing antioxidant power (FRAP)

A tea sample solution having a gradient concentration of 200, 150, 100, 50, 20 μg/mL was prepared. Pipette 40 μL of the sample and add 200 μL of FRAP working solution (containing 300 mmol/L pH 3.6 acetate buffer, 10 mmol/L TPTZ solution, 20 mmol/L $FeCl_3$ solution). The sample was mixed and reacted in the dark for 10 min, and its absorbance was measured at 595 nm. Finally, the FRAP value was calculated from the standard curve.

### 2.7. Detection of DPPH, ABTS and NADH/PMS/NBT superoxide radicals in vitro

For the DPPH test, 60, 30, 10, 5, 2.5 μg/mL tea solutions were prepared, and vitamin C was used as a positive control. DPPH solutions (100 μL) were mixed with 100 μL tea solution and left in the dark for 30 min. The DPPH value was measured by the absorbance at 510 nm. For the ABTS and NADH/PMS/NBT assays, the tea solution was set to 0.25, 0.2, 0.15, 0.1, 0.05, 0.025 mg/mL. A total of 100 μL of the test solution was added to 3 mL of the ABTS application solution, and the ABTS value was calculated by measuring the absorbance at 734 nm. The sample solution (50 μL) was added to 50 μL of NADH + 50 μL of PMS + 50 μL of NBT reaction system. The ADH/PMS/NBT values were calculated by measuring the absorbance at 545 nm.

### 2.8. MTT cell proliferation assay

The log phase cells ($5 \times 10^4$ cells/mL) were collected in 96-well plates overnight in a 5% $CO_2$ incubator at 37 °C. In the experimental group, 100 μL of a given concentration of T-CM aqueous extract

was added and cultured for 24 h. Then, 10 μL of MTT (5 mg/mL) was added to each well for 3 h. The culture solution was aspirated, 150 μL of DMSO was added to each well, and incubated for 10 min in a 37 °C incubator. The OD value was measured at a wavelength of 490 nm using a microplate reader and cell viability was calculated.

### 2.9. Cell cycle assay

The cells were set as control group, BT-CM group, YT-CM group and GT-CM group, and treated for 48 h. The cells were collected and fixed in 700 μL ethanol at − 20 °C for 12 h. Then, 0.5 mL of propidium iodide (PI) staining solution was added to the cells, and the bath was kept at 37 °C for 30 min in the dark. Finally, a red fluorescent signal was detected by a flow cytometer at an excitation wavelength of 488 nm. The data was processed using ModFit LT software.

### 2.10. Cell apoptosis assay

The cells were set as control group, BT-CM group, YT-CM group and GT-CM group, and treated for 48 h. A total of ($10 \times 10^4$) resuspended cells were fixed with 195 μL of buffer, 5 μL of Annexin V-FITC, and 10 μL of PI staining solution and incubated at room temperature for 20 min. The FITC and PE signals were then detected by flow cytometry. The data was analyzed by Flowjo software.

### 2.11. Western blot

Protein samples were obtained after the cells were lysed. The total protein in the cells was transferred to the PVDF membrane after SDS-PAGE electrophoresis. The PVDF membrane was blocked for 1 h and then incubated overnight at 4 °C with the corresponding diluted primary antibody. The membrane was washed three times with TBST and then reacted with the corresponding secondary antibody for 50 min. The protein signals were shown by the ECL chromogenic solution.

### 2.12. Statistical analysis

All data are expressed as mean ± standard deviation (Mean ± SD). Data analysis was performed using Graph Pad Prism 7.0 software. One-way ANOVA analysis was performed between the three tea samples. Different lowercase letters in the same figure indicated significant differences at $P<0.05$.

## 3. Results

### 3.1. Active ingredient content in aqueous extracts of tea and C. Maxima

The results of the active ingredients in aqueous extracts of the three tea and *C. maxima* (T-CM) were shown in Table 1. The contents of tea polyphenols (TP), amino acids (AA) and the ratio between TP and AA (TP/AA) in aqueous extracts of YT-CM and GT-CM were significantly higher than those in BT-CM. The contents of flavonoids and soluble sugar in BT-CM, YT-CM and GT-CM decreased in turn. In addition, the content of theabrownins in BT-CM was significantly higher than that in YT-CM and GT-CM.

The detection of monomeric components such as catechins, hesperidin, gallic acid (GA) and caffeine (CAFF) in aqueous extracts of the three T-CMs were shown in Table 2. The contents of total catechins, esterfied catechins, and non-ester catechins in YT-CM and GT-CM were significantly higher than those in BT-CM. Among them, the contents of esterfied catechins (including (-)-epigallocatechin gallate (EGCG), gallocatechin gallate (GCG) and (-)-epicatechin-3-gallate (ECG)) and non-ester catechins (C) were sequentially decreased in YT-CM, GT-CM and BT-CM. Hesperidin and GA had the highest content in BT-CM among the three T-CMs, while CAFF had the lowest content in BT-CM.

**Table 1**
Biochemical components in aqueous extracts of BT-CM, YT-CM and GT-CM (means ± SD, $n = 3$).

| Components* | BT-CM | YT-CM | GT-CM |
|---|---|---|---|
| TP (%) | 19.50 ± 0.93$^b$ | 38.85 ± 0.64 $^a$ | 37.50 ± 0.79 $^a$ |
| AA (%) | 3.84 ± 0.10$^b$ | 4.97 ± 0.10 $^a$ | 4.84 ± 0.20 $^a$ |
| TP/AA | 5.08 ± 0.11$^b$ | 7.69 ± 0.12 $^a$ | 7.96 ± 0.44 $^a$ |
| Flavonols (%) | 1.75 ± 0.09 $^a$ | 1.63 ± 0.23 $^a$ | 1.53 ± 0.09 $^a$ |
| Solube sugar (%) | 24.11 ± 0.65 $^a$ | 20.79 ± 0.04$^b$ | 18.62 ± 0.25$^c$ |
| TFs (%) | 0.12 ± 0.02 $^a$ | 0.11 ± 0.02 $^a$ | 0.10 ± 0.02 $^a$ |
| TRs (%) | 2.00 ± 0.33 $^a$ | 2.21 ± 0.30 $^a$ | 2.17 ± 0.15 $^a$ |
| TBs (%) | 3.36 ± 0.13 $^a$ | 1.61 ± 0.13$^b$ | 1.62 ± 0.07$^b$ |

Values represent. The result of using different lowercase letters in the same line means that the difference is significant at the 5% level.
* TP, tea polyphenols; AA, amino acid; TFs, theaflavins; TRs, thearubigins; TBs, theabrownins.

**Table 2**
Detection of components in aqueous extract of BT-CM, YT-CM and GT-CM (means ± SD, $n = 3$).

| Components* | Contents (mg/g) | | |
|---|---|---|---|
| | BT-CM | YT-CM | GT-CM |
| EGCG | 4.45 ± 0.09$^c$ | 37.27 ± 1.07 $^a$ | 33.35 ± 0.37$^b$ |
| GCG | 1.81 ± 0.09$^c$ | 9.34 ± 0.11 $^a$ | 8.15 ± 0.26$^b$ |
| ECG | 8.11 ± 0.12$^c$ | 57.00 ± 0.88 $^a$ | 49.93 ± 0.04$^b$ |
| CG | 1.27 ± 0.08$^b$ | 5.95 ± 0.28 $^a$ | 5.51 ± 0.09 $^a$ |
| GC | – | 4.33 ± 0.10 $^a$ | 3.76 ± 0.01$^b$ |
| EGC | 6.75 ± 0.96$^b$ | 13.31 ± 0.41 $^a$ | 13.51 ± 1.02 $^a$ |
| C | 2.19 ± 0.07$^c$ | 11.40 ± 0.44 $^a$ | 10.11 ± 0.51$^b$ |
| EC | 5.11 ± 0.15$^b$ | 24.48 ± 0.68 $^a$ | 23.84 ± 0.76 $^a$ |
| Hesperidin | 6.26 ± 0.12 $^a$ | 5.51 ± 0.17$^b$ | 5.18 ± 0.11$^b$ |
| GA | 10.64 ± 0.10 $^a$ | 6.19 ± 0.12$^c$ | 7.31 ± 0.01$^b$ |
| CAFF | 53.71 ± 0.48$^b$ | 56.70 ± 0.79 $^a$ | 56.65 ± 0.24 $^a$ |
| Esterfied catechins | 15.65 ± 0.22$^c$ | 108.41 ± 1.48 $^a$ | 96.94 ± 0.74$^b$ |
| Non-ester catechins | 14.05 ± 0.82$^c$ | 53.51 ± 0.87 $^a$ | 50.36 ± 0.43$^b$ |
| Total catechins | 29.69 ± 0.60$^c$ | 161.46 ± 1.97 $^a$ | 146.93 ± 0.08$^b$ |

* EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; GA, gallic acid; CAFF, Caffeine. The result of using different lowercase letters in the same line means that the difference is significant at the 5% level.

### 3.2. In vitro antioxidant capacity of tea and citrus maxima

In the total antioxidant capacity measurement, the ferric ion reducing antioxidant power (FRAP) of YT-CM and GT-CM was significantly higher than that of BT-CM (Fig. 1A). In the $IC_{50}$ values of the reagents for scavenging oxygen free radicals, the $IC_{50}$ values of YT-CM and GT-CM scavenging DPPH free radicals (Fig. 1B), ABTS (Fig. 1C) and NADH-NBT-PMS (Fig. 1D) were significantly lower than BT-CM. The ability of YT-CM and GT-CM to scavenge free radicals was significantly higher than that of BT-CM, and YT-CM had the strongest effect of clearing ABTS and NADH-NBT-PMS superoxide anion.

### 3.3. Inhibition of liver cancer cell proliferation by tea and C. Maxima

MTT results showed that the three T-CMs significantly inhibited the proliferation of HepG2 cells (Fig. 2A) and Bel7402 cells (Fig. 2B). BT-CM treatment of HepG2 cells and Bel7402 cells pre-

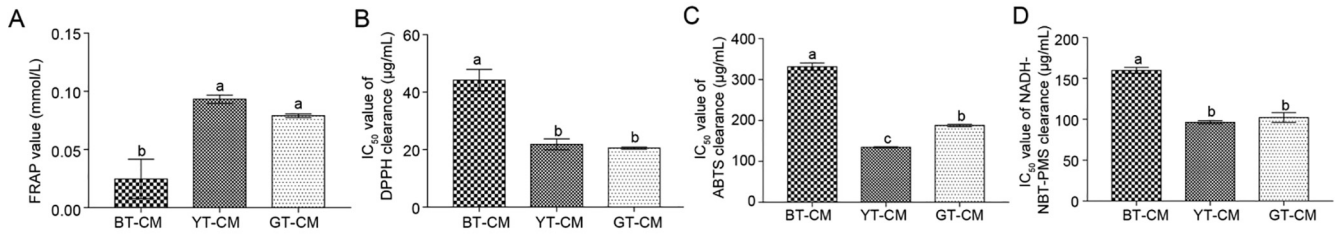


**Fig. 1.** *In vitro* antioxidant capacity of three T-CMs. A, $Fe^{3+}$ concentration reduced to $Fe^{2+}$ under treatment of a 20 μg/mL T-CM sample was used as the measurement result of FRAP. B, The ability of three T-CMs to scavenge DPPH free radicals was expressed as the $IC_{50}$ values of the tea samples for DPPH free radical scavenging. C, The ability of three T-CMs to scavenge ABTS free radicals was expressed as the $IC_{50}$ values of the tea samples for ABTS clearance. D, The ability of the three T-CMs to clear NADH-NBT-PNS was expressed as the $IC_{50}$ values of the tea samples for NADH-NBT-PNS clearance. Values were expressed as Mean ± SD, which were repeated three times independently, and a, b and c represented $P < 0.05$.

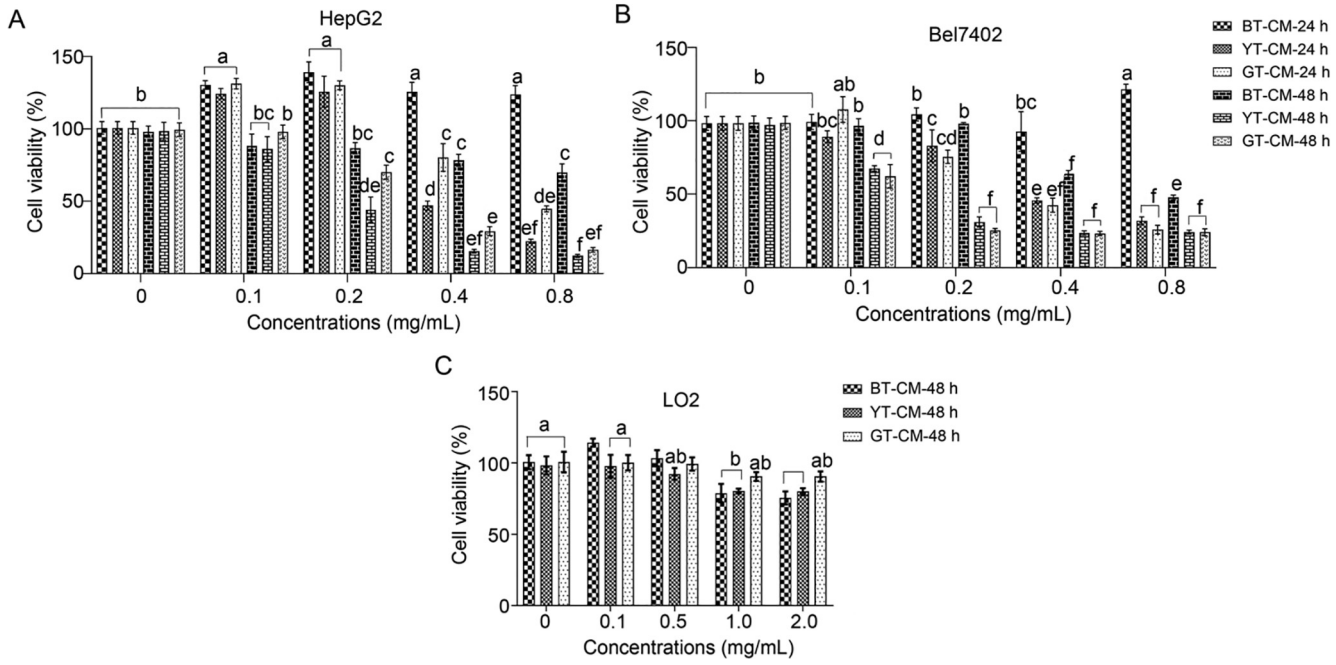


**Fig. 2.** Inhibition of proliferation of liver cancer cells by three T-CMs. A, HepG2 cells were treated with three T-CMs at concentrations of 0.0, 0.1, 0.2, 0.4, and 0.8 mg/mL for 24 h and 48 h, respectively, and then subjected to MTT assays. The 0.0 mg/mL treatment group was used as a blank control (100% cell activity). B, MTT assays of Bel7402 cells treated with three T-CMs for 24 h and 48 h were performed. C, Normal human liver cells LO2 were treated with three T-CMs at a dose range of 0.0 to 2.0 mg/mL for 48 h, then tested for MTT. Each result was expressed by Mean ± SD with four replicates and the lower case letters indicated $P < 0.05$.

sented obviously dose-dependent for 48 h. While YT-CM or GT-CM treatment of HepG2 cells and Bel7402 cells also appeared significantly dose-dependent for 24 h and 48 h. Then, the inhibition of cell viability showed obviously time dependence in HepG2 cells and Bel7402 cells when treated with three T-CMs for 24 h and 48 h. Among them, the cell survival rate in the BT-CM treatment was the highest among the three T-CMs, which indicated that the inhibition effect of BT-CM in liver cancer cells was significantly lower than that of YT-CM or GT-CM. In addition, compared with GT-CM, YT-CM had a higher inhibitory effect in HepG2 cells and a lower inhibitory effect in Bel7402 cells. What's more, three T-CMs didn't present significant cytotoxicity at 2.0 mg/mL when treated with normal liver cells LO2 for 48 h (Fig. 2C).

### 3.4. Cell cycle blockage of liver cancer cells by tea and C. Maxima

In the cell cycle assays, the proportion of S phase of HepG2 cells increased significantly as the concentration of the three T-CM treatments increased (Fig. 3A and B). This indicated that the three T-CMs had a significant retardation effect on the S phase of HepG2 cells. The proportion of G2/M phase of Bel7402 cells increased significantly as the concentration of the three T-CM treatments increased (Fig. 3C and D). Therefore, the three T-CMs had a significant blockage effect on the G2/M phase of Bel7402 cells.

### 3.5. Apoptosis of liver cancer cells under tea and C. maxima treatment

The results of cell apoptosis induced by three T-CMs in HepG2 and Bel7402 cells were shown in Fig. 4. As the concentration of the three T-CMs treatments increased, the apoptotic rates of HepG2 (Fig. 4A) and Bel7402 cells (Fig. 4C) increased significantly. The proportion of apoptotic cells showed that at the same concentration, GT-CM had the strongest apoptotic effect on HepG2 cells (Fig. 4B), and YT-CM had the greatest degree of apoptosis promotion for Bel7402 cells (Fig. 4D), followed by BT-CM.

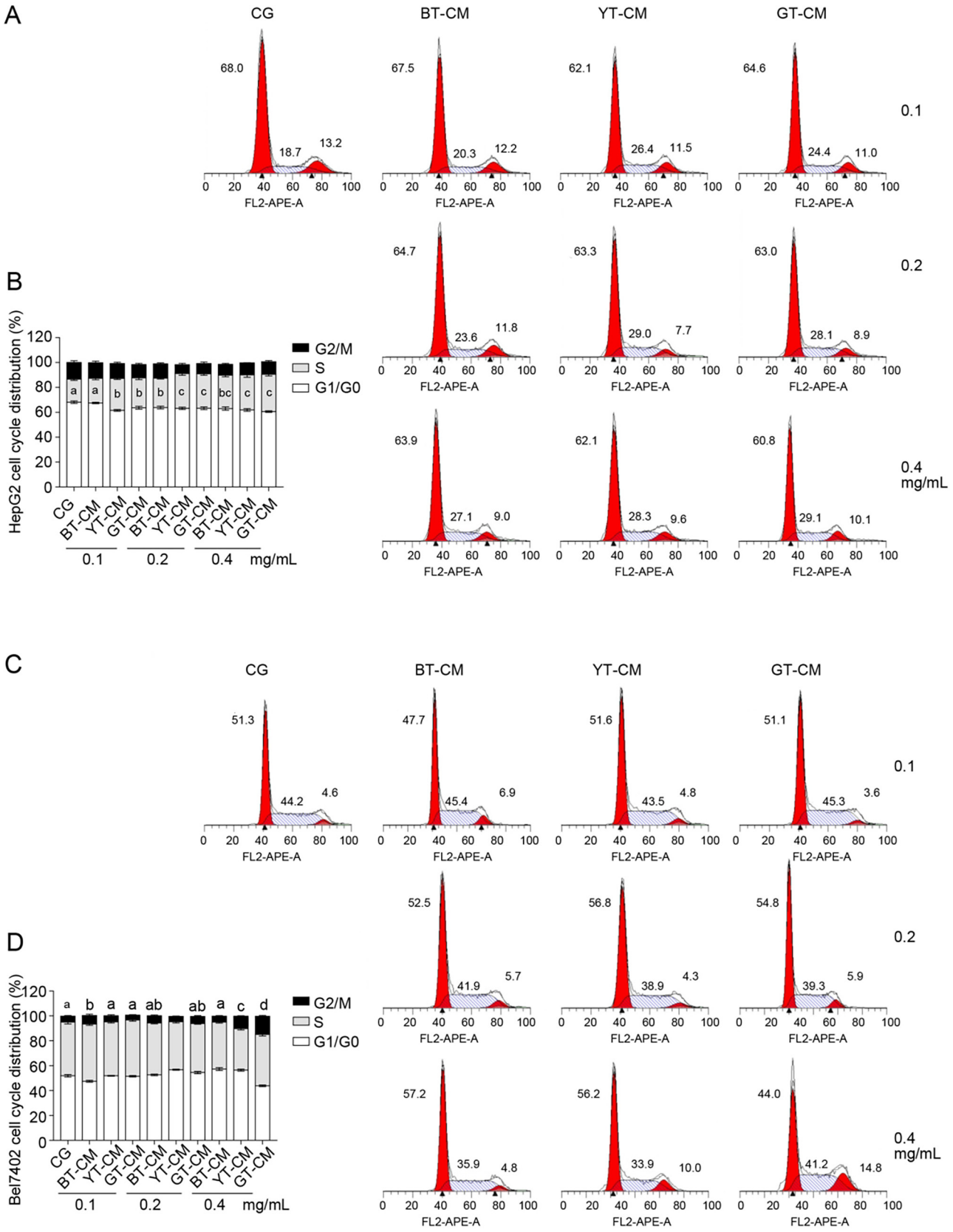


**Fig. 3.** Blocking effect of three T-CMs on cell cycle of liver cancer cells. A, B, HepG2 cells were treated with three T-CMs at concentrations of 0.1, 0.2, and 0.4 mg/mL for 48 h, respectively, and treated with 0.0 mg/mL as control group (CG). Each set of samples was tested by flow cytometry and the ratio of each cell cycle was obtained using ModFit LT software. GraphPad Prism 7.0 software was used to calculate cell cycle distribution. C, D, Results of flow cycle detection and cell cycle distribution of Bel7402 cells treated with three T-CMs. Each result was expressed by Mean ± SD with four replicates and the lower case letters indicated $P < 0.05$.

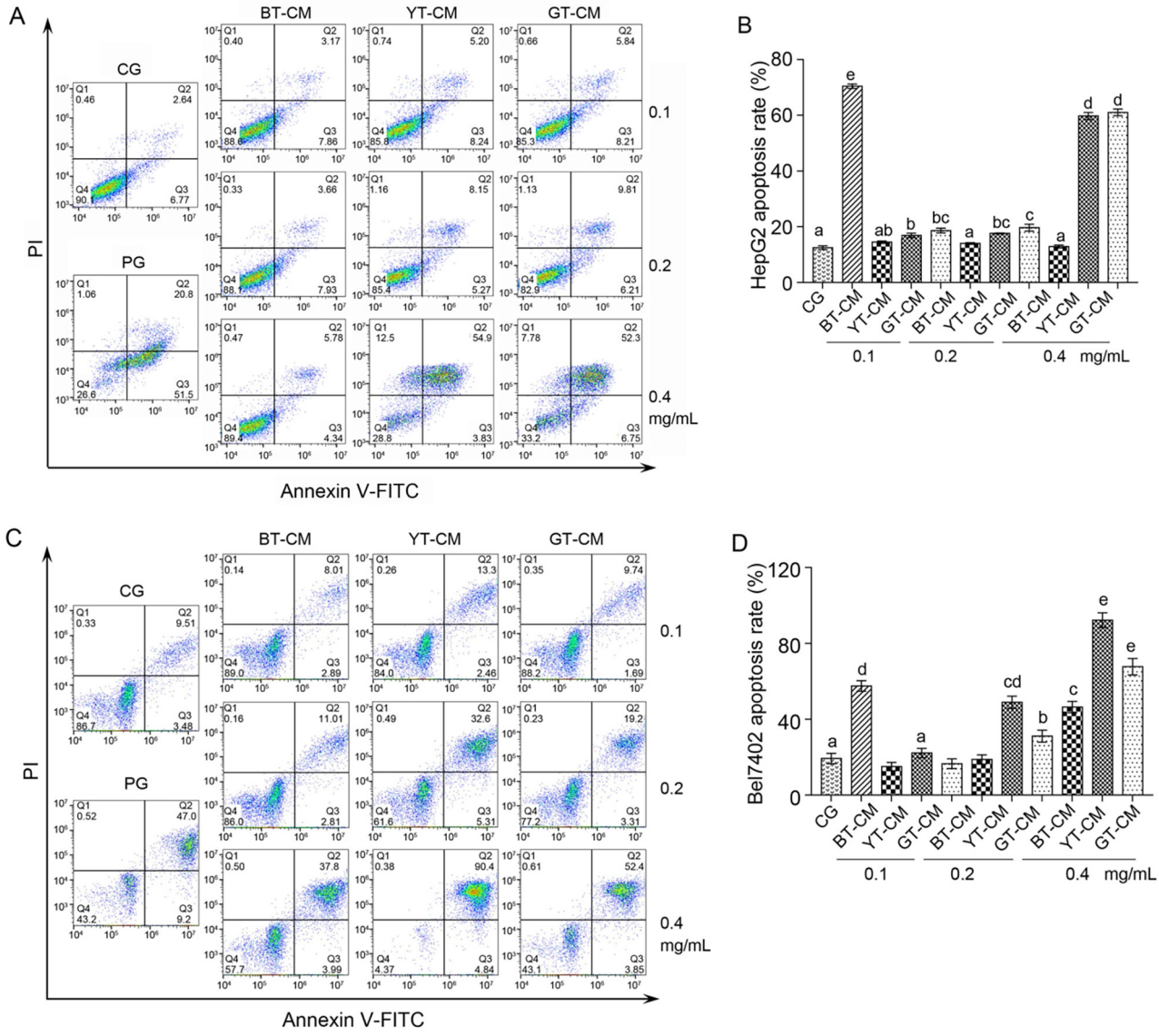


**Fig. 4.** Three T-CMs induced apoptosis in HepG2 (A, B) andBel7402 (C, D) cells. A, HepG2 cells were treated with three T-CMs at concentrations of 0.1, 0.2, and 0.4 mg/mL for 48 h. The treatment group with 0.0 mg/mL reagents was used as the control group (CG), and 10 μmol/L Pt was used as the positive drug group (PG). Apoptosis assays were then performed and the cells were detected by flow cytometry. B, GraphPad Prism 7.0 software was used to count the apoptotic rates shown in A. C, Bel7402 cells were treated with three T-CMs at concentrations of 0.1, 0.2, and 0.4 mg/mL for 48 h. Apoptosis assays were then performed and the cells were detected by flow cytometry. D, GraphPad Prism 7.0 software was used to count the apoptotic rates shown in C. Each result was expressed by Mean ± SD with four replicates and the lower case letters indicated $P < 0.05$.

### *3.6. Regulation of PI3K/AKT/mTOR pathway in liver cancer cells by tea and C. Maxima*

Next, we examined the changes in PI3K/AKT/mTOR signaling pathways in three T-CM-treated HepG2 cells by Western blot (Fig. 5A). Statistical analysis of phosphorylated protein expression showed that all three T-CMs down-regulated the ratio of p-PI3K/PI3K (Fig. 5B), p-AKT/AKT (Fig. 5C), and p-mTOR/mTOR in HepG2 cells (Fig. 5D). Especially at the treatment concentration of 0.2 mg/mL, the inhibition of the signaling pathway was more significant in the YT-CM and GT-CM treatment groups than in the BT-CM. The results of three T-CMs regulating the PI3K/AKT/mTOR signaling pathway in Bel7402 cells were shown in Fig. 5E. Similarly, the ratio of p-PI3K/PI3K (Fig. 5F), p-AKT/AKT (Fig. 5G), and p-mTOR/mTOR (Fig. 5H) in Bel7402 cells was significantly down-regulated. The results indicated that T-CM inhibited liver cancer cell proliferation and induced apoptosis by regulating PI3K/AKT/mTOR signaling pathway.

### *3.7. Regulation of apoptosis-related proteins in liver cancer cells by tea and C. Maxima*

We examined the expression of apoptosis-related proteins in HepG2 cells after three T-CM treatments by Western blot (Fig. 6A). Expression analysis of apoptosis-related proteins revealed that three T-CM significantly upregulated the ratio of Bax/Bcl-2 (Fig. 6B), Cyto c/Actin (Fig. 6C), Cleaved Caspase-9/Actin (Fig. 6E), and Cleaved Caspase-3/Actin (Fig. 6G) under concentration dependent. The changes in these apoptotic proteins were more pronounced in the YT-CM and GT-CM groups compared to the BT-CM group. The protein levels of full length Caspase-9/Actin (Fig. 6D) and full length Caspase-3/Actin (Fig. 6F) were significantly down-regulated under the three T-CM exposures.

Western blot assays in Bel7402 cells were also performed (Fig. 7A). Bax/Bcl-2 (Fig. 7B), Cyto c/Actin (Fig. 7C), cleaved Caspase-9/Actin (Fig. 7E), and cleaved Caspase-3/Actin (Fig. 7G) in Bel7402 cells after T-CM treatment were also significantly

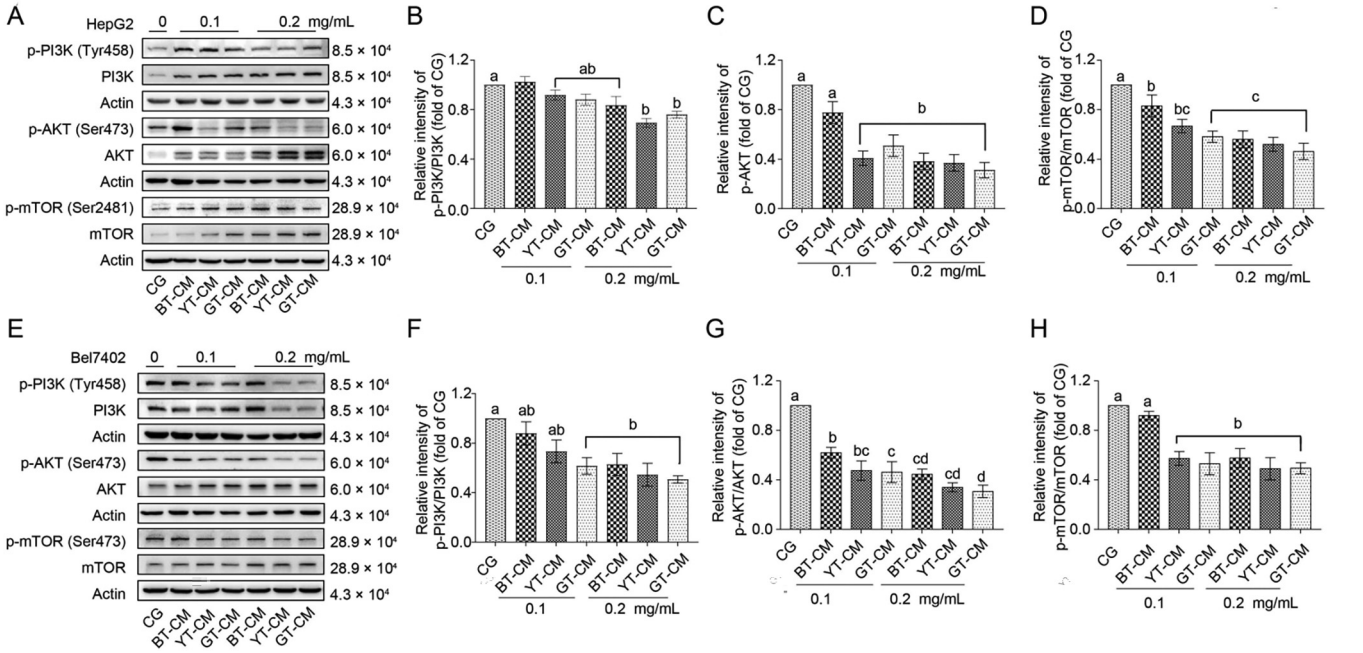


**Fig. 5.** Three T-CMs inhibited PI3K/AKT/mTOR protein phosphorylation levels in liver cancer cells. A, Protein expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in HepG2 cells after three T-CM treatments was performed by Western blot. B – D, The ratio of p-PI3K/PI3K, p-AKT/AKT, and p-mTOR/mTOR in HepG2 cells by a under grayscale analysis was counted. E, Protein expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in Bel7402 cells after three T-CM treatments was performed by Western blot. F – H, The ratio of p-PI3K/PI3K, p-AKT/AKT, and p-mTOR/mTOR in Bel7402 cells by a under grayscale analysis was counted. Each result was expressed by Mean ± SD with four replicates and the lower case letters indicated $P < 0.05$.

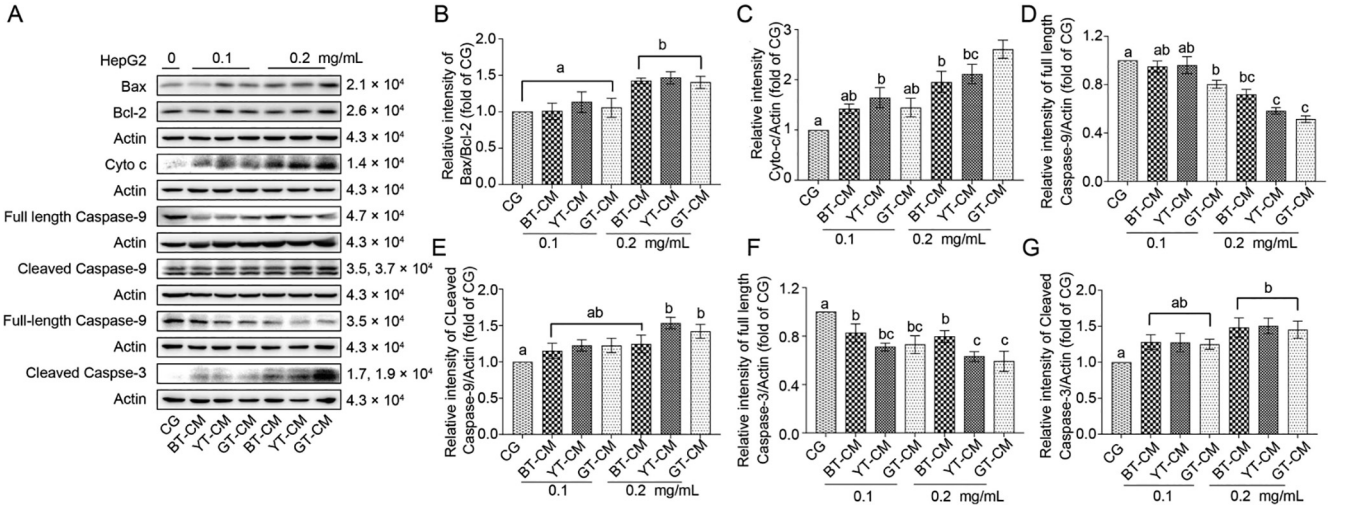


**Fig. 6.** Three T-CMs regulated expression level of apoptosis-related proteins in HepG2 cells. A, Protein expression of Bax, Bcl-2, Cyto c, full length Caspase-9, cleaved Caspase-9, full length Caspase-3, cleaved Caspase-3 and Actin in HepG2 cells after three T-CM treatments was performed by Western blot. B – G, The ratio of Bax/Bcl-2, Cyto c/Actin, full length Caspase-9/Actin, cleaved Caspase-9/Actin, full length Caspase-3/Actin, cleaved Caspase-3/actin by a under grayscale analysis was counted. Each result was expressed by Mean ± SD with four replicates and the lower case letters indicated $P < 0.05$.

upregulated. The expression levels of full length Caspase-9/Actin (Fig. 7D) and full length Caspase-3/Actin (Fig. 7F) were significantly down-regulated under the three T-CM exposures.

## 4. Discussion

The contents of the fresh tea leaves are basically the same, but the finished tea made by different processing techniques has changes in the contents. Our research found that YT-CM, GT-CM and BT-CM have different active ingredients and contents due to different fermentation processes. Polyphenol oxidase was killed during the processing of non-fermented tea (green tea) and light-fermented tea (yellow tea) (Chan, Lim, & Chew, 2007). Therefore the tea polyphenols were retained more in green tea and yellow tea.

Studies have shown that *Citri Grandis Exocarpium* also has antioxidant and scavenging free radicals (Narotzki, Levy, Aizenbud, & Reznick, 2013). Although the content of hesperidin in the three T-CMs was not much different, the component ratio of polyphenols was very different in them (Table 2). Polyphenols are the main substances in the antioxidant and scavenging free radicals in tea. Among them, EGCG has the strongest antioxidant

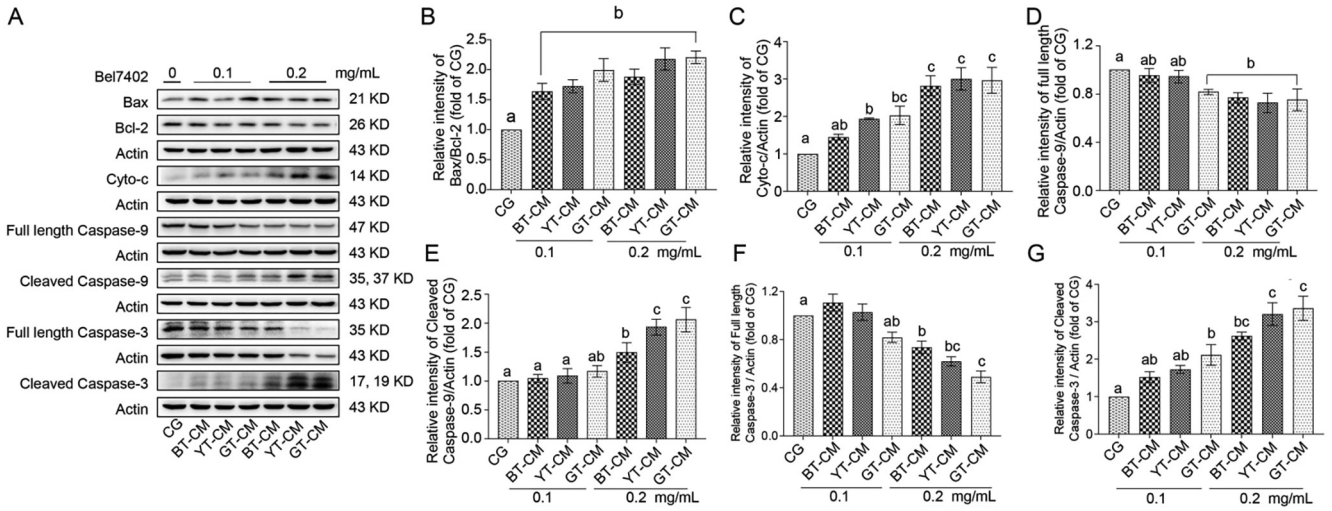


**Fig. 7.** Three T-CMs regulated expression level of apoptosis-related proteins in Bel7402 cells. A, Protein expression of Bax, Bcl-2, Cyto c, full length Caspase-9, cleaved Caspase-9, full length Caspase-3, cleaved Caspase-3 and Actin in Bel7402 cells after three T-CM treatments was performed by Western blot. B – G, The ratio of Bax/Bcl-2, Cyto c/Actin, full length Caspase-9/Actin, cleaved Caspase-9/Actin, full length Caspase-3/Actin, cleaved Caspase-3/Actin by a under grayscale analysis was counted. Each result was expressed by Mean ± SD with four replicates and the lower case letters indicated $P < 0.05$.

effect. The higher the content of tea polyphenols is, the stronger the ability to resist oxidation and scavenge free radicals is (Camouse, Hanneman, Conrad, & Baron, 2005; Mo, 2011). This may be the difference in the ability to resist oxidation and scavenge free radicals between YT-CM, GT-CM and BT-CM. This is consistent with the higher tea polyphenol content in YT-CM and GT-CM in this study.

The functional role of tea in preventing cancer is closely related to the substances such as tea polyphenols. Tea polyphenols, caffeine and other substances promote tumor cell apoptosis, inhibit tumor cell proliferation, angiogenesis and metastasis (Lu et al., 2006). Adding 0.5% tea polyphenol or 0.044% caffeine to the drinking water of tumor-bearing mice can inhibit the transformation of lung adenomas into malignant and the proliferation of malignant adenoma cells, while reducing c-Jun and phospho-Erk1/2 level (Davalli et al., 2012). Previous studies have reported the inhibitory effects of three teas on tumors. Drinking water supplemented with 0.1% green tea polyphenols significantly inhibited the deterioration and distant spread of prostate cancer in mice (Hudlikar et al., 2017). Black tea polyphenols inhibited the development of mouse lung cancer induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by regulating p38 and AKT (Kujawska et al., 2016). Yellow tea extract protected *N*-nitrosodiethylamine-induced rats from liver cancer progression (Zhao et al., 2017).

The ethanol extract of *Citri Grandis Exocarpium* has antispasmodic, antiasthmatic, analgesic and anti-inflammatory effects (Jiang et al., 2014). In the case of cancer research, the number of reports of *Citri Grandis Exocarpium* is relatively small. We combined *Citri Grandis Exocarpium* with tea to produce T-CMs and revealed the inhibitory effect of T-CMs on liver cancer cells. This indicates that *Citri Grandis Exocarpium* has potential therapeutic and preventive value for liver cancer.

Many studies (Akcakanat, Sahin, Shaye, Velasco, & Meric-Bernstam, 2008; Kawauchi, Ogasawara, Yasuyama, Otsuka, & Yamada, 2009; Le Grand et al., 2014; Leger et al., 2006) have found that cell cycle arrest was a very important feature that inhibits cancer cells proliferation. Zhong et al (2019) reported that THZ1, the cyclin-dependent kinase 7 (CDK7) inhibitor, had the activity of anti-cervical cancer, and its mechanism of action was to block cell cycle at the G2/M phase. We completely agree with that, and the same conclusion were also confirmed in our research. What's more, there was another investigate, which showed that the growth of breast cancer cells was suppressed by mitochondrial ncRNA, and its cell cycle was blocked at the S phase (Fitzpatrick et al., 2019). This finding was the same as our results that HepG2 cell cycle was treated with T-CMs in the study.

The currently known types of cell death mainly include apoptosis, autophagy, and necrosis. Apoptosis plays a very important role in the process of cell death. In the study of inhibiting the growth of tumor cells, induction of apoptosis is often essential, mainly because it is a process that induces suicide programmed cell death. Human endometrial cancer cells growth was restrained through inducing apoptosis, and presenting a concentration dependence by Juglone, which have been put forward by Zhang et al (2019). In addition, Zhou et al (2018) have deeply explored the pharmacological effect of histone deacetylase (HDAC) 1 and 2 in liver cancer, which confirmed that the inhibition of tumor cell proliferation was mainly related to the induction of apoptosis and cell cycle arrest. In our study, three T-CMs obviously induced apoptosis of two liver cancer cells HepG2 and Bel7402, and these results were similar to the above reports.

The mechanism may be related to strong antioxidant activity, inhibition of key enzyme activities and signaling pathways. The PI3K/AKT signaling pathway is an important pathway for transmitting extracellular signals into the nucleus, and plays an important regulatory role in various life activities such as cell proliferation, apoptosis, survival and metabolism. AKT is an important downstream mediator of PI3K promoter. After phosphorylation, AKT regulates the expression of various genes such as apoptosis, cell cycle and other genes by regulating its downstream targets mTOR, FOXO-1 and GSK-3 (Kawauchi et al., 2009; Leger et al., 2006). A number of studies have shown that elevated levels of free radicals in tumor cells led to inhibition of apoptosis through mediated mitochondrial dysfunction and promotion of the PI3K/AKT signaling pathway (Akcakanat et al., 2008). Increased levels of free radicals caused phosphorylation of AKT and mTOR, activated downstream target proteins, and ultimately inhibited endogenous apoptotic pathways (Le Grand et al., 2014). In our study, YT-CM, GT-CM and BT-CM inhibited the PI3K/AKT/mTOR signaling pathway by scavenging free radicals in liver cancer cells. Therefore, T-CMs-mediated inhibition of liver cancer cells is dependent on the inactivation of the PI3K/AKT/mTOR signaling pathway.

Inducing apoptosis is occurred mainly in mitochondria, where is a place with apoptosis proteins distribution and function. Bax and Bcl-2 are closely related to pro-apoptotic protein and anti-apoptotic protein, respectively (Keyvanloo Shahrestanaki, Bagheri, Ghanadian, Aghaei, & Jafari, 2019). So, the ratio of Bax/Bcl-2 is a considerable indicator to induce apoptosis. Upregulation of its ratio can stimulate Cyto-c secretion, and Caspase-9 activation, which will result in increased expression of cleaved Caspase-9 (Zhao et al., 2017). Then Caspse-3 is further activated, the expression of cleaved Caspase-3 will be upregulated (Zhao et al., 2017). After that, apoptosis will be accelerated. Norcantharidin (NCTD) has been determined to be cytotoxic in HepG2 cell by Chang et al (Chang, Zhu, Mei, Liu, & Luo, 2010). In-depth study of molecular mechanism revealed a close relationship with the mitochondrial apoptosis pathway, which was accomplished by adjusting certain proteins, including Bax, Bcl-2, Cyto-c, and Caspase-9/3 proteins. The same result also appeared in our research. The expression of the ratio of Bax/Bcl-2, Cyto-c, and Cleaved Caspase-9/3 proteins in HepG2 and Bel 7402 cells treated with three T-CMs was increased, while the full length Caspase-9/3 protein expression was decreased.

## 5. Conclusion

In conclusion, our study revealed a *in vitro* antioxidant activity of T-CMs and the activity of inducing apoptosis of liver cancer cells. The light-fermented YT-CM and non-fermented GT-CM had stronger antioxidant activity than the heavy-fermented BT-CM. Three T-CMs activations regulated the PI3K/AKT/mTOR signaling pathway and the expression levels of apoptosis-related proteins to exert their anti-hepatocarcinogenic effects. T-CMs, especially YT-CM and BT-CM, are good health drinks that help prevent and treat liver cancer.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The research was supported by the "14th Five-Year Plan" team-building projects of Guangdong Academy of Agricultural Sciences [202126TD]; Guangdong Basic and Applied Basic Research Foundation [2020A1515011266, 2021A1515010958]; Guangzhou Science and Technology Plan Projects [202002030202, 202102020047]; Key-Area Research and Development Program of Guangdong Province [2020B0202080003]; Innovation Fund projects of Guangdong Academy of Agricultural Sciences [202115, 202035]; Special fund for scientific innovation strategy-construction of high level Academy of Agriculture Science [R2019PY-JX004].

## References

Akcakanat, A., Sahin, A., Shaye, A. N., Velasco, M. A., & Meric-Bernstam, F. (2008). Comparison of Akt/mTOR signaling in primary breast tumors and matched distant metastases. *Cancer, 112*(11), 2352–2358.

Baeza, I., De Castro, N. M., Arranz, L., & De la Fuente, M. (2010). Soybean and green tea polyphenols improve immune function and redox status in very old ovariectomized mice. *Rejuvenation Research, 13*(6), 665–674.

Camouse, M. M., Hanneman, K. K., Conrad, E. P., & Baron, E. D. (2005). Protective effects of tea polyphenols and caffeine. *Expert Review of Anticancer Therapy, 5*(6), 1061–1068.

Chan, E. W. C., Lim, Y. Y., & Chew, Y. L. (2007). Antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia. *Food Chemistry, 102*(4), 1214–1222.

Chang, C., Zhu, Y., Mei, J., Liu, S., & Luo, J. (2010). Involvement of mitochondrial pathway in NCTD-induced cytotoxicity in human hepG2 cells. *Journal of Experimental & Clinical Cancer Research, 29*, 145–153.

Davalli, P., Rizzi, F., Caporali, A., Pellacani, D., Davoli, S., Bettuzzi, S., ... D'Arca, D. (2012). Anticancer activity of green tea polyphenols in prostate gland. *Oxidative Medicine and Cellular Longevity, 2012*(3), 984219–984236.

Deng, S. D., Wang, L. J., Lin, L., Xiao, F. X., & Shuai, O. (2013). Comparison on UPLC and HPLC fingerprints of flavonoids in *Citri Grandis Exocarpium*. *Chinese Traditional and Herbal Drugs, 44*(9), 1195–1198.

Fitzpatrick, C., Bendek, M., Briones, M., Farfán, N., Silva, V., Nardocci, G., ... Burzio, V. (2019). Mitochondrial ncRNA targeting induces cell cycle arrest and tumor growth inhibition of MDA-MB-231 breast cancer cells through reduction of key cell cycle progression factors. *Cell Death & Disease, 10*(6), 423–431.

Haque, A. M., Hashimoto, M., Katakura, M., Tanabe, Y., Hara, Y., & Shido, O. (2006). Long-term administration of green tea catechins improves spatial cognition learning ability in rats. *The Journal of Nutrition, 136*(4), 1043–1047.

Hsu, F. L., Huang, W. J., Wu, T. H., Lee, M. H., Chen, L. C., Lu, H. J., ... Lin, M. H. (2012). Evaluation of antioxidant and free radical scavenging capacities of polyphenolics from pods of *Caesalpinia pulcherrima*. *International Journal of Molecular Sciences, 13*(5), 6073–6088.

Hudlikar, R. R., Venkadakrishnan, V. B., Kumar, R., Thorat, R. A., Kannan, S., Ingle, A. D., ... Mahimkar, M. B. (2017). Polymeric black tea polyphenols (PBPs) inhibit benzo(a)pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone-induced lung carcinogenesis potentially through down-regulation of p38 and Akt phosphorylation in A/J mice. *Molecular Carcinogenesis, 56*(2), 625–640.

Jiang, K., Song, Q., Wang, L., Xie, T., Wu, X., Wang, P., ... Wang, T. (2014). Antitussive, expectorant and anti-inflammatory activities of different extracts from *Exocarpium Citri Grandis*. *Journal of Ethnopharmacology, 156*, 97–101.

Jung, J. Y., Han, C. R., Jeong, Y. J., Kim, H. J., Lim, H. S., Lee, K. H., ... Kim, W. J. (2007). Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neuroscience Letters, 411*(3), 222–227.

Kawauchi, K., Ogasawara, T., Yasuyama, M., Otsuka, K., & Yamada, O. (2009). Regulation and importance of the PI3K/Akt/mTOR signaling pathway in hematologic malignancies. *Anti-cancer Agents in Medicinal Chemistry, 9*(9), 1024–1038.

Keyvanloo Shahrestanaki, M., Bagheri, M., Ghanadian, M., Aghaei, M., & Jafari, S. (2019). Centaurea cyanus extracted 13-O-acetylsolstitialin A decrease Bax/Bcl-2 ratio and expression of cyclin D1/Cdk-4 to induce apoptosis and cell cycle arrest in MCF-7 and MDA-MB-231 breast cancer cell lines. *Journal of Cellular Biochemistry, 120*(10), 18309–18319.

Kujawska, M., Ewertowska, M., Adamska, T., Ignatowicz, E., Gramza-Michalowska, A., & Jodynis-Liebert, J. (2016). Protective effect of yellow tea extract on N-nitrosodiethylamine-induced liver carcinogenesis. *Pharmaceutical Biology, 54*(9), 1891–1900.

Le Grand, M., Rovini, A., Bourgarel-Rey, V., Honore, S., Bastonero, S., Braguer, D., & Carre, M. (2014). ROS-mediated EB1 phosphorylation through Akt/GSK3beta pathway: Implication in cancer cell response to microtubule-targeting agents. *Oncotarget, 5*(10), 3408–3423.

Leger, B., Cartoni, R., Praz, M., Lamon, S., Deriaz, O., Crettenand, A., ... Russell, A. P. (2006). Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. *The Journal of Physiology, 576*(3), 923–933.

Liu, D. (2018). Effect of Fuzhuan brick-tea addition on the quality and antioxidant activity of skimmed set-type yoghurt. *International Journal of Dairy Technology, 70*, 22–33.

Lu, G., Liao, J., Yang, G., Reuhl, K. R., Hao, X., & Yang, C. S. (2006). Inhibition of adenoma progression to adenocarcinoma in a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis model in A/J mice by tea polyphenols and caffeine. *Cancer Research, 66*(23), 11494–11501.

Mo, T. (2011). The change and regulation mechanism of key components during tea processing. *Journal of Chinese Institute of Food Science & Technology, 11*(9), 176–180.

Narotzki, B., Levy, Y., Aizenbud, D., & Reznick, A. Z. (2013). Green tea and its major polyphenol EGCG increase the activity of oral peroxidases. *Advances in Experimental Medicine and Biology, 756*(756), 99–104.

Sengupta, A., Ghosh, S., & Das, S. (2003). Tea can protect against aberrant crypt foci formation during azoxymethane induced rat colon carcinogenesis. *Journal of Experimental & Clinical Cancer Research, 22*(2), 185–191.

Srinivasan, P., Sabitha, K. E., & Shyamaladevi, C. S. (2004). Therapeutic efficacy of green tea polyphenols on cellular thiols in 4-nitroquinoline 1-oxide-induced oral carcinogenesis. *Chemico-Biological Interactions, 149*(2–3), 81–87.

Tamaya, K., Matsui, T., Toshima, A., Noguchi, M., Ju, Q., Miyata, Y., ... Tanaka, K. (2010). Suppression of blood glucose level by a new fermented tea obtained by tea-rolling processing of loquat (*Eriobotrya japonica*) and green tea leaves in disaccharide-loaded sprague-dawley rats. *Journal of The Science of Food and Agriculture, 90*(5), 779–783.

Wang, R., Li, Q., & Xie, Q. (2014). Study on the quality of commercial *Exocarpium Citri Grandis*. *Chinese Journal of Pharmaceutical Analysis, 34*(4), 669–672.

Wang, Y. S., Xu, Y. J., Gao, L. P., Yu, O., Wang, X. Z., He, X. J., ... Xia, T. (2014). Functional analysis of flavonoid 3',5'-hydroxylase from tea plant (*Camellia sinensis*): Critical role in the accumulation of catechins. *BMC Plant Biology, 14*(1), 347.

Wu, J., Yang, S., Xu, K., Ding, C., Zhou, Y., Fu, X., ... Li, L. (2018). Patterns and trends of liver cancer incidence rates in eastern and southeastern asian countries (1983–2007) and predictions to 2030. *Gastroenterology, 154*(6), 1719–1728.

Yang, G. Y., Liu, Z., Seril, D. N., Liao, J., Ding, W., Kim, S., ... Yang, C. S. (1997). Black tea constituents, theaflavins, inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice. *Carcinogenesis, 18* (12), 2361–2365.

Yue, Y., Chu, G. X., Liu, X. S., Tang, X., Wang, W., Liu, G. J., ... Zhang, Z. Z. (2014). TMDB: A literature-curated database for small molecular compounds found from tea. *BMC Plant Biology, 14*(1), 243–250.

Zhang, Y., Zhang, F., Zhang, Y., Thakur, K., Zhang, J., Liu, Y., ... Wei, Z. (2019). Mechanism of juglone-induced cell cycle arrest and apoptosis in ishikawa human endometrial cancer cells. *Journal of Agricultural and Food Chemistry, 67* (26), 7378–7389.

Zhao, Y., Jing, Z., Lv, J., Zhang, Z., Lin, J., Cao, X., ... Mao, W. (2017). Berberine activates caspase-9/cytochrome c-mediated apoptosis to suppress triple-negative breast cancer cells *in vitro* and *in vivo*. *Biomedicine & Pharmacotherapy, 95*, 18–24.

Zhong, S., Zhang, Y., Yin, X., & Di, W. (2019). CDK7 inhibitor suppresses tumor progression through blocking the cell cycle at the G2/M phase and inhibiting transcriptional activity in cervical cancer. *OncoTargets and Therapy, 12*, 2137–2147.

Zhou, B., Pan, J., Dai, F., Zhao, C. Y., Zhang, L. P., Wei, Q. Y., ... Liu, Z. L. (2004). Redifferentiation of human hepatoma cells induced by green tea polyphenols. *Research on Chemical Intermediates, 30*, 626–635.

Zhou, H., Cai, Y., Liu, D., Li, M., Sha, Y., Zhang, W., ... Huang, A. (2018). Pharmacological or transcriptional inhibition of both HDAC1 and 2 leads to cell cycle blockage and apoptosis via p21Waf1/Cip1 and p19INK4d upregulation in hepatocellular carcinoma. *Cell Proliferation, e12447*.

Zhu, Y., Huang, H., & Tu, Y. (2006). A review of recent studies in China on the possible beneficial health effects of tea. *International Journal of Food Science and Technology, 41*(4), 333–340.