*Tetrastigma hemsleyanum* flavones exert anti-hepatic carcinoma property both *in vitro* and *in vivo*

Yangyang Liu (刘阳阳)\(^a\), Yonglu Li (李永璐)\(^a,\dagger\), Wen Chen (陈文)\(^a\), Xiang Ye (叶翔)\(^a\), Ruoyi Jia (贾若一)\(^a\), Lushuang Yu (俞露霜)\(^a\), Qiong Tang (唐琼)\(^a\), Pengcheng Tu (屠鹏程)\(^a\), Yong Jiang (蒋勇)\(^b\), Qiang Chu (楚强)\(^c,\ast\), Xiaodong Zheng (郑晓冬)\(^a,\ast\)

\(^\dagger\) These authors contributed equally to this work.

\(^a\) Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-food Processing, Fuli Institute of Food Science, College of Biosystems Engineering and Food Science, National Engineering Laboratory of Intelligent Food Technology and Equipment, Key Laboratory for Agro-Products Postharvest Handling of Ministry of Agriculture, Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou 310058, China.

\(^b\) Shanghai Zhengyue Enterprise management Co., Ltd. 19th Floor, Block B, Xinchengkonggu Building, NO.388 Zhongjiang Road, Putuo District, Shanghai 600062, China)

\(^c\) State Key Laboratory of Silicon Materials, School of Materials Science and Engineering, Zhejiang University, Hangzhou, 310058, China

\(^\ast\) Corresponding author,

Qiang Chu, E-mail: 0619363@zju.edu.cn

Postal address: State Key Laboratory of Silicon Materials, School of Materials Science and Engineering, Zhejiang University, Hangzhou, 310058, China

© The Author(s) 2021. Published by Oxford University Press on behalf of Zhejiang University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Xiaodong Zheng, E-mail: fs319zju@126.com

Postal address: Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310058, China.
Abstract:

*Tetrastigma hemsleyanum* has been regarded as an anticancer food in China. However, its corresponding mechanisms remains unclear. Thus, in this study, the antitumor activity of flavones-rich fraction of root of *Tetrastigma hemsleyanum* (FRTH) was investigated *in vitro* and *in vivo*. The results indicated that FRTH could inhibit the proliferation and migration of HepG2 cells *in vitro* by PI3K/AKT pathway. FRTH could increase the ROS level and change the mitochondrial membrane potential (MMP) in HepG2 cells. In addition, FRTH treatment (300, 600 mg/kg BW) significantly suppressed tumor growth on HepG2 tumor-bearing nude mice. Besides, immunohistochemistry assays and western blotting revealed that FRTH enhanced the expression level of Bax/Bcl-2, cytochrome C, Caspase-3, caspase-9, Cleaved-caspase-3, and downregulated the expression level of CD31, ki67 and VEGF in HepG2 tumor-bearing mice. Our study suggests *Tetrastigma hemsleyanum* as a promising candidate medicine for liver cancer treatment.

**Key words:** *Tetrastigma hemsleyanum*; HepG2; flavone; antitumor; apoptosis
1. Introduction

Though many strategies and drugs have been developed to conquer cancer, hepatocellular carcinoma (HCC), one of the most prevalent life-threatening cancers, is still a leading cause of high cancer-related mortality all over the world due to the early diagnosis difficulty (Bray et al., 2018). A study has predicted that a 35% increase of liver cancer cases would be observed from 2005 to 2030 in 30 countries (Valery et al., 2018). As liver cancer is a kind of noncommunicable disease and a major cause of death (Chen et al., 2018), it is still full of challenges performing hepatectomy, while patients usually suffer from recurring.

Besides, HCC occurs in hepatocytes often accompanied with oxidative stress, inflammation and underlying liver diseases which may further lead to patients’ agony (Petrick et al., 2019). Liver cancer is one of the most devastating cancers as many patients were usually diagnosed at late stages with less possibility in curative effects. Liver cancer incidence is increasing in many countries, whereas the treatments for anti-liver cancer still remain unsatisfied.

Recently, clinical cancer therapies are mainly divided as surgical resection, radiotherapy, chemotherapy, biotherapy and traditional Chinese medical herbal treatments. Compared with radiotherapy and chemotherapy, traditional Chinese medical herbal treatments have attracted tremendous attention due to their advantages in preventing tumor metastasis (Ancuceanu et al., 2004; Qiu et al., 2014). Traditional Chinese medicine has been widely used for thousands of years in the prevention and treatment of various diseases in China. Specifically, traditional Chinese medicine has been adopted in cancer therapy due to its diverse advantages including outstanding bioactivities, easy availability, lower costs and fewer side effects. Many compounds derived from natural Chinese medicines have been proved to have excellent antitumor activity, such as taxol (Zhu et al., 2019), vinblastine (Nobre et al., 2019), vincristine (Taghizadehghalehjoughi et al., 2019) and
hydroxycamptothecin (Guo et al., 2019). Seeking for potentially active natural compounds from traditional Chinese medicines might provide possible candidates for cancer therapy.

*Tetrastigma hemsleyanum,* Diels & Gilg belongs to the genus *Tetrastigma* Planch, family *Vitaceae*. As a main component of Hua Shi Xuan Fei mixture (Approval number of Zhejiang medicine, Z20200026000), *Tetrastigma hemsleyanum* has been approved by Zhejiang Provincial Drug Administration for clinical treatment of COVID-19 (Ji et al., 2021). Besides, it has been reported to exert significant inhibitory effects against proliferation of HepG2 cells (Peng et al., 2016), Hela cells (Xiong et al., 2015), MDA-MB-435 cells (Lin et al., 2016), A549 cells (Liu et al., 2019). A series of reports have already proved the excellent antitumor effects of *Tetrastigma hemsleyanum*. However, various studies have only focused on the anti-cancer abilities of *Tetrastigma hemsleyanum* at *in vitro* level, whereas less research have paid attention to *Tetrastigma hemsleyanum*’s *in vivo* efficiency in nude mice. In this study, the flavones-rich fraction of *Tetrastigma hemsleyanum*’s root (FRTH) was obtained by extraction and purification, and its anti-HCC abilities were further investigated both at *in vitro* and *in vivo* levels.

2. Methods and materials

2.1. Material and reagent

The root of *Tetrastigma hemsleyanum* was obtained from Lishui Zhejiang, China. Trypsin-EDTA 0.25%, Fetal bovine serum (FBS) and High glucose Dulbecco’s modified Eagle Medium (DMEM) medium were obtained from Wisent. Saline were provided by Hangzhou minsheng pharmaceutical co. LTD. Deionized water was purified by a Milli-Q system. 5-fluorouracil (5-Fu) was obtained from Aladin. Ethanol was supplied by Sinopharm Chemical Reagent Co.,Ltd. Hoechst 33258 solution (bis-benzimide), DCFH-DA (2′,7’-dichlorodihydrofluorescein diacetate) and Propidium Iodide (PI) were obtained from Sigma.
Premiary antibodies Bax, Bcl-2, Caspase-3, Caspase-9, cytochrome C, Cleaved-caspase-3 and β-actin were obtained from abcam. PI3K, p-PI3K, AKT, p-AKT, Ki67, Rock1, Mlc-2 were obtained from affinity Biosciences LTD. All other reagents used for analysis were of analytical grade.

2.2. Extract preparation and HPLC-MS analysis

The root of *Tetrastigma hemsleyanum* was ground into powder which was then passed through 60 mesh sieves. The powder of root of *Tetrastigma hemsleyanum* (5.0 kg) was extracted with 50 L water at 55 °C for 90 min. After centrifugation for 10 min at 4 °C at 4000 g, the supernatant was collected and the residue was extracted repeatedly for three times. Subsequently, the supernatant was combined and evaporated at 55 °C by an evaporator. The concentrated solution was subsided with alcohol at the final concentration of 80% to remove polysaccharide. The supernatant was collected and evaporated at 55 °C again. Then the flavones were enriched with AB-8 macroporous resin which was soaked in 1 M NaOH, 1 M HCl, ethanol in sequence for 6 h, and washed with deionized water for three times before it was used. Briefly, the macroporous resin was loaded into a chromatographic column with the diameter of 2.5 cm and height of 100 cm. Subsequently 300 mL concentrations were slowly loaded onto the chromatographic column. Then two times volume of purified water were flushed through the macroporous resin. Subsequently, 80% ethanol(v/v) containing 1% formic acid as eluant component were flushed through macroporous resin slowly and the eluant was collected and evaporated at 55 °C. The concentrations were freeze-dried and stored at -80 °C before use. The dried flavones-rich fraction of root of *Tetrastigma hemsleyanum* (FRTH) was resolved in deionized water for cell experiments and in saline for animal experiments. FRTH were analyzed using UPLC-ESI-QTOF-MS² according to previous study (Li *et al.*, 2019).
Waters UPLC (Waters Corp., Milford, MA, USA), ACQUITY UPLC HSS SB-C8 column (4.6 mm × 250 mm; 5 μm, Waters Corp.) was used in all the chromatographic experiments. The mobile phases were acetonitrile (A) and 0.1% formic acid-water (B). The linear gradient programs were as follows, 0-1 min, 95% B, 1-21 min, 95-85% B, 21-46 min, 85-75% B, 46-56 min, 75-95% B, 56-60 min, 95% B. Sample injection volume was 10 μL. Column oven temperature was 40°C. Flow rate was 0.8 mL/min. The UV detector was set at 360 nm.

Mass Spectrometry : AB TripleTOF 5600⁺ System (AB SCIEX, Framingham, USA). The optimal MS conditions: negative ion mode: source voltage was −4.5 kV, and the source temperature was 550°C. Positive ion mode: source voltage was +5.5 kV, and the source temperature was 600°C. The pressure of Gas 1 (Air) and Gas 2 (Air) were set to 55 psi. The pressure of Curtain Gas (N₂) was set to 35 psi. Maximum allowed error was set to ± 5 ppm. Declustering potential (DP), 100 V; collision energy (CE), 10 V. For MS/MS acquisition mode, the parameters were almost the same except that the collision energy (CE) was set at 40 ± 20 V, ion releasedelay (IRD) at 67, ion release width (IRW) at 25. The IDA-based auto-MS² was performed on the 8 most intense metabolite ions in a cycle of full scan (1 s). The scan range of m/z of precursor ion and product ion were set as 100 – 1500 Da and 50 – 1500 Da. The exact mass calibration was performed automatically before each analysis employing the Automated Calibration Delivery System.

2.3. Cytotoxicity

HepG2 cell line was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. The cells were cultured with DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 °C in an atmosphere of 5% CO₂.
Cytotoxicity was detected with MTT assay according to previous study (Liu et al., 2019) with slight modification. Briefly, $6 \times 10^3$ HepG2 cells per well were seeded in 96-well plates and cultured in the incubator. The cells were treated with FRTH (12.5, 25, 50, 75, 100, 150, 200, 300 $\mu$g/mL). After 24 h, MTT reagent was added with the final concentration of 0.5 mg/mL and the plates were incubated for 4 h. Subsequently, DMSO was added to each well after the supernatant was removed. The absorbance was measured by a microplate reader at a wavelength of 570 nm.

2.4 Scratching assay

To determine the influence of FRTH on the migration of HepG2 cells, scratching assay were performed according to previous study (Li, et al., 2020). Briefly, HepG2 cells were seeded onto a 6-well plate with $2 \times 10^5$ cells per well for 24 h. A sterile toothpick was used to scratch a single layer of cells on the bottom. The cells were washed with PBS and subsequently the cells were treated with FRTH (100, 150, 200 $\mu$g/mL). the cells were photographed with a Nikon microscope after incubation for 48 h.

2.5 Clonogenic assay

Clonogenic assay was performed to evaluate the influence of FRTH on the clone formation of HepG2 cells. Briefly, HepG2 cells were seeded onto plate and incubated with FRTH for 14 days. Then the clones were fixed with 4% paraformaldehyde, stained with crystal violet and photographed after washed with PBS.

2.6 Fluorescent probes staining

Fluorescent probes DCFH-DA, DHE and RH123 were used to determine the production of intracellular ROS level, $O_2^\bullet$ level and cellular mitochondrial membrane potential respectively according to previous study (Chu et al., 2019) with slight modifications. Briefly,
HepG2 cells were treated with FRTH for 24h and followed by incubating with fluorescent probe at 37°C for 20 min. Then the fluorescent probe solution was removed and the cells were washed with PBS twice and phenol red free DMEM was added. Then the cells were observed with a fluorescence microscope. The results were expressed as mean fluorescence intensity calculated by image analysis software Image Pro Plus6.0.

2.7. Immunofluorescence assay

HepG2 cells were seeded onto a six-well plate and incubated with FRTH for 12 h. Then cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The fixative solution was removed, washed with PBS, permeabilized. Subsequently, 5% BSA was used for blocking and incubated with primary antibody (Cleaved-caspase-3, β-tubulin) and secondary antibody. 5 μg/mL DAPI was added after washed with TBST. Finally, the images were captured with fluorescence microscope.

2.8. Animals and treatments

Twenty-five 5-weeks old male BALB/C nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were allowed to acclimatize to the laboratory conditions for three days before the actual experiment were performed. All the mice were allowed to take in water and food freely. The room temperature and relative humidity were 24 ± 2°C and 60 ± 5%. The experimental procedures were performed according to the guidelines issued by the Laboratory Animal Welfare and Ethics committee of Zhejiang University, China (Permission No. ZJU20190136).

Mice were randomly assigned into five groups (five mice per group):

Group I, model group (saline);

Group II, fluorouracil, 30 mg/kg BW per day (5-Fu);
Group III, low dose group, 100 mg/kg BW per day (FRTH);

Group IV, middle dose group, 300 mg/kg BW per day (FRTH);

Group V, high dose group, 600 mg/kg BW per day (FRTH);

A total of $2 \times 10^6$ HepG2 cells in 0.1ml DMEM without FBS were respectively injected into the right flank of mice. After 7 days, when the tumor volume reached about 80 mm$^3$, mice were randomly assigned administrated as described above according to the size of tumor. The food consumptions of each group were recorded every 3 days. Fifteen days later, the blood was collected, subsequently the mice were sacrificed and tumors from each group were surgically excised, wiped, weighed and photographed. Data were presented as mean standard error of the mean (SEM).

2.9. Weight and organ index calculation.

The body weights of the mice were measured and recorded every 3 days. At day 15 after intragastric administration, the blood of mice was collected and then mice were sacrificed. After the mice was sacrificed, the spleen, liver and kidney were aseptically removed and weighed, and the liver and kidney indexes were calculated as follows:

Liver index = liver weight (mg)/body weight (g);

Kidney index = kidney weight (mg)/body weight (g).

2.10. H&E staining and immunochemical analyses

After subcutaneous xenograft tumor, liver, spleen and kidney were collected, a portion of the tissue of all harvested organs and tumor tissues were dissected and subsequently they were subjected to 4% paraformaldehyde, paraffin embedding and sectioning and lastly hematoxylin and eosin (H&E) staining.
For immunochemical analyses, the slides were washed in PBS for three times and blocked with 5% bovine serum albumin (BSA). Subsequently, the slides were washed in PBS again and incubated at 4 °C overnight with rabbit anti-rabbit Bax primary antibody. Horseradish peroxidase (HRP) goat anti-rabbit was used as a secondary antibody. The tumor slides were stained with diaminobenzidine (DAB) and subsequently the nuclei were subjected to hematoxylin counterstaining. Immunohistochemical images were captured at 200× with a microscope. The expression levels of Caspase-3, CD31, Ki67 and VEGF in tumor tissue were determined with the same methods of Bax.

2.11. Western blotting assay

The protein of tumor was lysed in a WB/IP lysis buffer. The samples were separated via 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk and incubated with primary antibodies (Bax, Bcl-2, Caspase-3, Cleaved-caspase-3, cytochrome C, Caspase-9, β-Actin) at 4 °C overnight, then the membranes were washed with TBST for three times, followed by incubation with second antibodies for 1.5 h at room temperature. Finally, the proteins were visualized with enhanced chemiluminescence. β-actin was used as loading control and densitometry was analyzed with Image J software.

The HepG2 cells were lysed in a WB/IP lysis buffer and primary antibodies (PI3K, p-PI3K, AKT, p-AKT, Bax, Bcl-2, Ki67, Mlc-2, Rock1, Cylin D1, β-Actin) were used to detect the expression levels of proteins with the same method.

2.12. Statistical analysis

All measurements were performed in triplicate and all data were analyzed by SPSS 22.0 statistical software (IBM Corporation, Armonk, NY, USA) and Graphpad Prism version 6.
The paired test was used for the parametric test to compare the means between two related groups. One-way ANOVA with Duncan test was used for the parametric test to compare intergroup. \( p \) values of 0.05 were considered as a statistically significant level.

### 3. Results and discussions

#### 3.1. Characterization of the extracts of FRTH

Many ingredients were detected and identified from the root of *Tetrastigma hemsleyanum* including catechin, resveratrol, kaempferol, quercetin, orientin, Procyanidin dimmer, rutin, kaempferol-3-xylosylglucose-7-rhamnoside (Sun et al., 2015), quercetin-3-O-glucoside (isoquercitrin) (Xia et al., 2018), kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside (astragalin), alkaloids (Wang et al., 2018), tachioside, isotachioside (Liu et al., 2015), vitexin, isovitexin (Sun et al., 2013) and polysaccharide (Ru et al., 2019).

In present study, the major components in FRTH were detected by a UPLC-MS/MS system with a DAD detector. As a result, the chromatogram of FRTH was presented in Figure 1A. According to the retention time in sequence. The major constituents in FRTH were identified with MS/MS data (Figure 1B and Table 1) as follows: rutin (1), quercetin-3-O-glucoside (isoquercitrin) (2), kaempferol-3-O-rutinoside (3), and kaempferol-3-O-glucoside (astragalin) (4). More data about the chemical constituents in *Tetrastigma hemsleyanum* could also be found in our previous study (Li et al., 2019).

#### 3.2. FRTH induces apoptosis and inhibits migration in HepG2 cells.

To evaluate the anti-proliferative effects of FRTH, MTT assay was performed. As Figure 1C showed, 5-Fu (50 \( \mu \)g/mL) markedly inhibited the cell proliferation of HepG2 cells as 5-Fu-treated cell viability was only 70% of the control. Moreover, FRTH could
significantly inhibit the cell proliferation from 25 to 300 μg/mL concentration. The cell viability declined with the FRTH dose increasing. In addition, the IC50 value of the inhibition rate of FRTH in HepG2 cells was 137.2 ± 7.1 μg/mL. Besides, the expression level of apoptosis executive protein Cleaved-capase-3 upregulated after FRTH treatment for 6 h (supplementary Figure 1A). According to Figure 1D and Figure 1E, scratch width in FRTH treated group was obviously larger than control group indicating that FRTH suppressed migration of HepG2 cells. Cytoplasmic skeleton participates in the cell migration process. Microtubule is a major component of cytoplasmic skeleton. In this study, the intensity of β-tubulin decreased after FRTH treatment for 6 h which indicated that FRTH broke the balance of the cytoskeleton (supplementary Figure 1B).

Figure 1F suggested that the numbers of clones decreased after FRTH treatment indicating that FRTH could suppressed the clone formation of HepG2 cells. Figure 1G demonstrated that the intensity of HepG2 cells obviously restrained in FRTH treated group by contrast with control group, while the morphology of cells was also altered after FRTH intervention. According to Figure 1H, the number of bright blue dots increased in HepG2 cells after exposure to FRTH compared with control group, suggesting nuclear damage caused by FRTH. An obvious enhancement of red fluorescence intensity was found after FRTH exposure for 24 h, which represented that the number of dead cells increased in FRTH treated group compared with control group in Figure 1I. The results of Hoechst 33258 and PI staining assays indicated that FRTH could cause nuclear damage and cell apoptosis in dose dependent manner.

3.3. FRTH induces the loss of mitochondrial membrane potential (MMP)

The dysfunction of mitochondrial may affect the ROS production level. In this study, mitochondrial membrane potential was detected with RH123 fluorescent probe. Figure 2A
and 2D showed that mean RH123 fluorescence intensities significantly increased in FRTH groups compared with control group. The results indicated that FRTH might trigger a change of mitochondrial membrane potential, induce mitochondrial dysfunction, and thereby trigger ROS accumulation.

3.4. FRTH triggers intracellular ROS and $O_2^•$ accumulation of HepG2

Many chemotherapeutics could trigger ROS generation to cytotoxic levels in targeting cancer cells (Conklin, 2004). ROS may lead to DNA damage in cancer. Over production of ROS could activate the apoptotic effectors which contains caspase family proteins, Bcl-2 family proteins and cytochrome C (D'Arcy, 2019). In the intrinsic apoptotic pathways, excessive ROS could interfere with the function of caspase. Over production of $O_2^•$ may induce damage to mitochondrial membrane which results in a release of cytochrome C and subsequently it may cause caspase-dependent cytosolic signaling events (Su et al., 2019). The release of cytochrome C may induce an increasing production of ROS (Moloney et al., 2018). In view of this, therapies used to elevate ROS production showed potentially effective strategy in cancer therapies. Previous studies have indicated that some natural products can induce cancer cell death through the alteration of ROS level in cancer cells. Ginsenoside Rh4 could activate ROS accumulation in colorectal cancer cells (Wu et al., 2018) and polyunsaturated fatty acids could induce ovarian cancer cell death by activating ROS generation (Tanaka et al., 2017). Excessive accumulation of ROS can lead to oxidative stress and induce cancer cell apoptosis (Volpe et al., 2018). In this study, intracellular ROS accumulation in HepG2 cells was assessed by DCFH-DA fluorescent probe staining. Figure 2B illustrated the production level of ROS after exposure to different concentrations of FRTH for 24 h. Mean DCFH-DA fluorescence intensities of FRTH treated groups were significantly higher than control group in Figure 2E, which indicated that FRTH could increase the production level of ROS in HepG2 cells. The superoxide $O_2^•$ was measured with DHE.
fluorescent probe. Figure 2C demonstrated the production level of superoxide \( \text{O}_2^- \cdot \) in HepG2 cells with different treatment. An obvious elevation of mean DHE fluorescence intensity can be seen in HepG2 cells after exposure to FRTH for 24 h in Figure 2F. The results revealed that FRTH could enhance the \( \text{O}_2^- \cdot \) level in HepG2 cell. In agreement with our results, reports have revealed that cisplatin could cause a mitochondrial-dependent ROS response which could promote the cytotoxic effect on nuclear DNA damage against cancer cells (Marullo et al., 2013).

3.5. FRTH inhibited PI3K/AKT pathway in HepG2 cells

PI3K/AKT pathway could regulate cell proliferation and apoptosis in cancer cells. Phosphorylation of PI3K can cause the phosphorylation of downstream AKT upregulating the Cyclin D1 to promote cell proliferation (Revathidevi et al., 2019). Thus, inhibition of PI3K/AKT pathway is regarded as a target for cancer thereby. Cyclin D1 plays a key role in cell division and regulates cell cycle. The downregulated of Cyclin D1 could suppress the cell proliferation (Kim et al., 2015). In addition, molecular switch Bax/Bcl-2 enhanced can induce Caspase-3 activated which formed apoptotic executor Cleaved-caspase-3 resulting cell apoptosis (Li et al., 2020). To investigate the mechanism by which FRTH inhibited proliferation of HepG2, western blotting was performed to detect PI3K/AKT pathway. As Figure 2G and 2H suggested, the ratio of p-PI3K/PI3K, p-AKT/AKT significantly decreased and the expression level of Cyclin D1 and proliferation biomarker Ki67 reduced after FRTH treatment. Besides, Bax/Bcl-2 and Cleaved-caspase-3 were upregulated intervened by FRTH (Figure 2I and 2J). The results indicated that FRTH inhibited cell proliferation and induce cell apoptosis relating to PI3K/AKT pathway.

Rho associated protein kinase 1 (Rock1), plays an important role in cell migration and invasion. Overexpression of Rock1 is closely related to the metastasis of many tumors (Croft...
et al., 2004). Previous study confirmed that suppression of PTEN/PI3K/FAK pathway may suppress cell migration and invasion via downregulated Rock1 (Hu et al., 2019). The phosphorylation of myosin light chain 2 (MLC-2) is a key process in inducing stress fiber contraction, which contributes to contractile force generation driving disassembly of epithelial cell-cell junctions at the rear of migrating cells (Sturge et al., 2006; J. Sun et al., 2013). As Figure 2I and 2J exhibited, FRTH downregulated the expression level of Mlc-2 and Rock1. The results indicated that FRTH suppressed the cell migration is closely related with the downregulated Mlc-2 and Rock1.

3.6. FRTH exhibits anti-tumor activities in HepG2-tumor bearing mice

A tumor model with HepG2 cells was constructed to evaluate the anti-tumor effect of FRTH in HepG2 tumor-bearing nude mice. The bodyweights of mice were observed to evaluate the effect of FRTH on the growth status. As shown in Table 2, no obvious difference was observed among the initial body weights of all mice. Whereas the final weight of 5-Fu group is lower than model group and FRTH treated group. However, the daily food consumption of all the three FRTH treatment groups (100, 300 or 600 mg/kg BW) were similar to model group but higher than 5-Fu group. The bodyweight and daily food consumption treated by 5-Fu all implied side-effect on mice. Nevertheless, no side-effect were observed in FRTH treatment group judging by bodyweight and food consumption.

The tumors were harvested after mice sacrificing and as Figure 3A manifested the tumor sizes in FRTH treatment groups (300 or 600 mg/kg BW) were smaller than model group. As shown in Table 2 and Figure 3B, no obvious difference was observed between FRTH treatment group (100 mg/kg BW) and model group. However, when dose of FRTH reached 300 or 600 mg/kg BW, the tumor weights were significantly lower than model group (p <
0.05), and 300, 600 mg/kg BW of FRTH could inhibit the tumor growth with inhibition rate of 27.0%, 28.7%, respectively.

To evaluate the influence of FRTH on organs of mice, the liver and kidney tissues of HepG2 bearing mice were harvested. As Figure 3C illustrated, no significant difference of liver index was observed among the 5-Fu treated group and model group ($p < 0.05$). However, the kidney index of 5-Fu treated group was a slightly higher than model group ($p < 0.05$) as manifested in Figure 3D, which may attribute to the final bodyweight loss caused by 5-Fu. Meanwhile, the kidney indexes and liver indexes of FRTH-treated groups were similar to model group ($p < 0.05$). H&E staining method was conducted to examine the morphological change in organs and tumor tissues. No obvious morphological difference was observed in liver and kidney in FRTH- treated groups compared with model group according to Figure 3E. Hepatic lobe and hepatic cord were clear in model group and FRTH groups judging by histological examination of liver in Figure 3E. The structure of renal tubules and glomeruli is normal in kidney based on the histological examination with microscope in Figure 3E. These results indicated that FRTH had not induce damage to the normal organs and could serve as a safe agent for anti-HCC prevention and treatment.

3.7 FRTH down-regulated proliferations-related proteins in tumor

At the same time, the in vivo antitumor effect of FRTH and its mechanism were detected by morphological change and expression level of proliferations related proteins in tumor bearing mice. The antitumor efficacy of FRTH on tumor bearing mice was also shown histologically. As Figure 4A demonstrated, the tumor tissue in model group were made up of high density of cells with atypical mitosis. However, the cancer cell density in FRTH treated group were lower compared with model group. In addition, FRTH treated groups exhibited a
loose cellular arrangement and larger apoptotic regions than model group, which suggested that FRTH exerted antiproliferation activity in tumor cells.

To explore the antitumor mechanism of FRTH in vivo, immunohistochemical analysis were performed to assess the expression level of proliferations-related proteins in vivo. The expression level of angiogenic marker CD31 which was considered to be associated with larger tumor size (Cristina et al., 2010) and was detected with immunohistochemical method. The results in this research indicated that there is a correlation between tumor size and CD31 expression. The expression level of CD31 was downregulated after FRTH treated compared with model group in vivo (Figure 4A, 4E). Ki67 was considered to be a cancer cell proliferation biomarker (Healey et al., 2017). As Figure 4A and 4F exhibited, the positive expression cells in FRTH group has a lower degree compared with model group which indicated that FRTH down-regulated the expression level in vivo. VEGF exhibited important effect on tumor angiogenesis. It has been reported that Panax notoginseng saponins can regulate VEGF to suppress the progression of esophageal squamous cell carcinoma (Chen et al., 2020). According to Figure 4A and 4D, the expression level of VEGF was higher in model group which contributed the tumor growth. However, FRTH could significantly suppress the expression level of VEGF, indicating that FRTH could inhibit tumor angiogenesis. These results confirmed that FRTH could exhibit antitumor activity via inhibiting the proliferation of HepG2 in vivo.

3.8 FRTH up-regulated apoptosis-related proteins in tumor

Usually, the antitumor effects could be triggered by cell apoptosis. As a highly structured and orchestrated process, apoptosis plays a key role in regulating cell number for the growth and homeostasis of tissues by eliminating aged, damage and unwanted cells. Apoptotic-related pathways usually insists intrinsic pathway and extrinsic pathway (D’Arcy,
Intrinsic pathway of apoptosis can be initiated by the cell itself when it detects damage via a number of intracellular sensors. Extrinsic pathway of apoptosis result from interaction between a cell of the immune system and a damaged cell. The initiation of the two pathways of apoptosis is dependent on the activation of cysteine-aspartic proteases named as caspases (Yin et al., 2020).

Mitochondria plays an important role in intrinsic mitochondria pathway of apoptosis. Meanwhile, the production of reactive oxygen species (ROS) is always related to mitochondria (Yang, et al., 2018). Recent study has shown that ROS can change mitochondrial membrane potential and further brings out the change of expression level of B-cell lymphoma-2 (Bcl-2) family proteins (Zhang et al., 2019). Some of these proteins (such as Bax) are pro-apoptotic, while others (such as Bcl-2) are anti-apoptotic. The balance between pro-apoptotic member Bax and anti-apoptotic member Bcl-2 determined the fate of cell apoptotic. Over production of Bax may lead to cell apoptosis while excess of Bcl-2 may make cell resilient (Siddiqui et al., 2015). As an anti-apoptotic protein, Bcl-2 can regulate cell apoptosis either by binding to apoptosis-activating factors or by suppressing the release of cytochrome C from mitochondria into cytosol, while pro-apoptotic Bax has the opposite function (Ren et al., 2020). The expression level of Bax/Bcl-2 plays a key role in cell apoptosis. When the expression level of Bax/Bcl-2 enhanced, it may lead to that mitochondria releases pro-apoptotic proteins, such as cytochrome C which induces the formation of apoptosome. Subsequently, apoptosome motivates initiation of caspase-9 (Gao et al., 2020). Caspase-9 is a mediator caspase which involved in the intrinsic pathway of apoptosis and lead to the activation of downstream Caspase-3. Caspase-3 is a important regulator of apoptosis in caspase family. Caspase-3 is silence before it was activated by proteolytic cleavage of a certain subunit and formed activated caspase-3 (Cleaved-caspase-3). Finally, Cleaved-caspase-3 induces cell apoptosis (Crawford et al., 2011).
It has been reported that *Tetrastigma hemsleyanum* could inhibit the proliferation of cancer cells via affecting the expression level of caspase and Bcl-2 family proteins (Sun *et al.*, 2018). To better understand the mechanism that FRTH induces apoptosis, we demonstrated in this study that an enhanced expression level of Bax/Bcl-2 triggered the unbalance of mitochondrial membrane potential specifically resulted in the apoptosis, the subsequent cytochrome C release and excites Caspase-9 which further induces activation of Caspase-3. As a result, the executor cleaved-caspase-3 leads to cell apoptosis.

In present study, western blotting assay were performed to assess the expression level of apoptosis-related proteins in xenograft tumor. As Figure 5A-D showed, the anti-apoptotic protein Bcl-2 were downregulated after FRTH treatment, while the pro-apoptotic protein Bax were upregulated. Meanwhile, the ratio of Bax/Bcl-2 which is a ‘molecular switch’ was also enhanced in FRTH treated groups. In addition, the expression level of pro-apoptotic protein, cytochrome C, Caspase-9, Caspase-3 and Cleaved-caspase-3 elevated in FRTH treated groups according to Figure 5E-H. Besides, immunohistochemical analysis also indicated that FRTH upregulated Bax and caspase-3 according to Figure 4A-C. Based on these results, we could deduce that FRTH could trigger apoptosis of HepG2 *in vivo* via downregulating proliferated proteins and upregulating apoptotic proteins in mitochondrial caspase-dependent pathway of apoptosis.

**4. Conclusion**

In this study, rutin, kaempferol-3-O-rutinoside, astragalin and isoquercitrin were detected in purified extracts of *Tetrastigma hemsleyanum*. To explore the anti-HCC ability of *Tetrastigma hemsleyanum*, further *in vitro* and *in vivo* research were carried out. The results indicated that FRTH could cause DNA damage, enhance the ROS and O$_2^{-}$• level *in vitro*. In addition, antiproliferative activity of FRTH against HepG2 cell may be related with the
downregulated PI3K/AKT pathway in vitro. The findings on HepG2 tumor bearing mice revealed that FRTH could suppress the tumor growth via upregulated expression of Bax/Bcl-2, cytochrome C, Caspase-9, Caspase-3, cleaved-caspase-3 and downregulated expression of VEGF, Ki67, CD31. In summary, the findings in present research indicated that the root of Tetrastigma hemsleyanum exhibited antitumor effect in vitro and in vivo. In addition, to confirm the antitumor mechanism of FRTH, cell signal pathway activator or inhibitor can be used in further study. Therefore, the root of Tetrastigma hemsleyanum exerted potential value in further cancer therapy.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgement

This research was based on Functional food research and development of Tetrastigma hemsleyanum funded by Shanghai Zhengyue Enterprise management Co., Ltd. and the Science and Technology Department of Zhejiang Province (No.2018C02045).

CrediT authorship contribution statement

Yangyang Liu: Data curation, Writing - original draft, Writing – review & editing, Methodology, Investigation, Software; Yonglu Li: Supervision, Formal analysis, Writing - review & editing; Xiang Ye: Investigation, Validation; Wen Chen: Investigation, Resources; Ruoyi Jia: Visualization; Lushuang Yu: Investigation; Qiang Tang: Investigation; Pengcheng Tu: Investigation; Qiang Chu: Validation, Methodology, Writing - review & editing; Yong Jiang: Resources; Xiaodong Zheng: Conceptualization, Supervision, Funding acquisition.
References:

Ancuceanu, R. V., & Istudor, V. (2004). Pharmacologically active natural compounds for lung cancer. *Alternative medicine review: a journal of clinical therapeutic*, 9(4), 402-419.

Bray, F., Ferlay, J., Soerjomataram, I., *et al.* (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA-A CANCER JOURNAL FOR CLINICIANS*, 68(6), 394-424.

Chen, W., Sun, K., Zheng, R., *et al.* (2018). Cancer incidence and mortality in China, 2014. *Chinese Journal of Cancer Research*, 30(1), 1-12.

Chen, X., Lv, Z., Zhang, C., *et al.* (2020). Panax notoginseng saponins regulate VEGF to suppress esophageal squamous cell carcinoma progression via DVL3-mediated Wnt/beta-catenin signaling. *RSC Advances*, 10(6), 3256-3265.

Chu, Q., Jia, R., Chen, M., *et al.* (2019). *Tetrastigma hemsleyanum* tubers polysaccharide ameliorates LPS-induced inflammation in macrophages and Caenorhabditis elegans. *International Journal of Biological Macromolecules*, 141, 611-621.

Conklin, K. A. (2004). Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integrative Cancer Therapies*, 3(4), 294-300.

Crawford, E. D., & Wells, J. A. (2011). Caspase Substrates and Cellular Remodeling. In R. D. Kornberg, C. Raetz, J. E. Rothman, & J. W. Thorner (Eds.), *Annual Review of Biochemistry*, 80, 1055-1087.

Cristina, C., Ines Perez-Millan, M., Luque, G., *et al.* (2010). VEGF and CD31 Association in Pituitary Adenomas. *Endocrine Pathology*, 21(3), 154-160.

Croft, D. R., Sahai, E., Mavria, G., *et al.* (2004). Conditional ROCK activation in vivo induces tumor cell dissemination and angiogenesis. *Cancer Research*, 64(24), 8994-9001.
D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biology International*, 43(6), 582-592.

Gao, J., Tian, X., Yan, X., et al. (2020). Selenium Exerts Protective Effects Against Fluoride-Induced Apoptosis and Oxidative Stress and Altered the Expression of Bcl-2/Caspase Family. *Biological Trace Element Research*, 199(2), 682–692.

Guo, H., Li, F., Qiu, H., et al. (2019). Chitosan-Based Nanogel Enhances Chemotherapeutic Efficacy of 10-Hydroxycamptothecin against Human Breast Cancer Cells. *International Journal of Polymer Science*, 2019(1914976)

Healey, M. A., Hirko, K. A., Beck, A. H., et al. (2017). Assessment of Ki67 expression for breast cancer subtype classification and prognosis in the Nurses' Health Study. *Breast Cancer Research and Treatment*, 166(2), 613-622.

Hu, C., Zhou, H., Liu, Y., et al. (2019). ROCK1 promotes migration and invasion of non-small-cell lung cancer cells through the PTEN/PI3K/FAK pathway. *International journal of oncology*, 55(4), 833–844.

Ji, T., Ji, W. W., Wang, J., et al. (2021). A comprehensive review on traditional uses, chemical compositions, pharmacology properties and toxicology of *Tetrastigma hemsleyanum*. *Journal of Ethnopharmacology*, 264, 113247.

Kim, M. K., Park, G. H., Eo, H. J., et al. (2015). Tanshinone I induces cyclin D1 proteasomal degradation in an ERK1/2 dependent way in human colorectal cancer cells. *Fitoterapia*, 101, 162-168.

Li, Y., Chu, Q., Liu, Y., et al.. (2019). Radix Tetrastigma flavonoid ameliorates inflammation and prolongs the lifespan of Caenorhabditis elegans through JNK, p38 and Nrf2 pathways. *Free Radical Research*, 53(5), 562-573.

Li, Y., Feng, X., Yu, X., et al. (2020). Radix Tetrastigma flavonoids inhibit the migration and promote the apoptosis of A549 cells both in vitro and in vivo. *Journal of Functional Foods*, 72, 104076.

Li, Y., Feng, X., Zhang, Y., et al. (2020). Dietary flavone from the *Tetrastigma hemsleyanum* vine triggers human lung adenocarcinoma apoptosis via autophagy. *Food & Function*, 11(11), 9776-9788.
Lin, Z., Chen, L., Qiu, Q., et al. (2016). Isolation and identification of antiproliferative compounds from the roots of *Tetrastigma hemsleyanum* against MDA-MB-435S cell lines. *Pakistan Journal of Pharmaceutical Sciences*, 29(4), 1171-1175.

Liu, H., He, L., Huang, R., et al. (2015). Chemical Constituents of the Rhizomes of *Tetrastigma hemsleyanum*. *Chemistry of Natural Compounds*, 51(6), 1077-1079.

Liu, P., Pu, J., Zhang, J., et al. (2019). Bioinformatic analysis of miR-4792 regulates *Radix Tetrastigma hemsleyani* flavone to inhibit proliferation, invasion, and induce apoptosis of A549 cells. *OncoTargets and Therapy*, 12, 1401-1412.

Liu, Y., Ye, X., Li, Y., et al. (2019). The influence of the extraction method on bioactivity of the root of *Tetrastigma hemsleyanum*. *Food Science & Nutrition*, 7(11), 3644-3653.

Marullo, R., Werner, E., Degtyareva, N., et al. (2013). Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. *PloS one*, 8(11), e81162.

Moloney, J. N., & Cotter, T. G. (2018). ROS signalling in the biology of cancer. *Seminars in Cell & Developmental Biology*, 80, 50-64.

Nobre, L., Pauck, D., Golbourn, B., et al. (2019). Effective and safe tumor inhibition using vinblastine in medulloblastoma. *Pediatric blood & cancer*, 66(6), e27694.

Volpe, C., Villar-Delfino, P. H., Dos Anjos, P., et al. (2018). Cellular death, reactive oxygen species (ROS) and diabetic complications. *Cell death & disease*, 9(2), 119.

Peng, X., Zhang, Y., Wang, J., et al. (2016). Ethylacetate extract from *Tetrastigma hemsleyanum* induces apoptosis via the mitochondrial caspase-dependent intrinsic pathway in HepG2 cells. *Tumor Biology*, 37(1), 865-876.

Petrick, J. L., & McGlynn, K. A. (2019). The Changing Epidemiology of Primary Liver Cancer. *Current Epidemiology Reports*, 6(2), 104-111.

Qiu, X., & Jia, J. (2014). Research advances on TCM anti-tumor effects and the molecular mechanisms. *Journal of Cancer Research and Therapeutics*, 101(5), 8-13.
Ren, Z., Chen, S., Seo, J., et al. (2020). Mitochondrial dysfunction and apoptosis underlie the hepatotoxicity of perhexiline. *Toxicology in Vitro, 69*. 104987

Revathidevi, S., & Munirajan, A. K. (2019). Akt in cancer: Mediator and more. *Seminars in cancer biology, 59*, 80–91.

Ru, Y., Chen, X., Wang, J., Guo, L., Lin, Z., Peng, X., & Qiu, B. (2019). Polysaccharides from *Tetrastigma hemsleyanum* Diels et Gilg: Extraction optimization, structural characterizations, antioxidant and antihyperlipidemic activities in hyperlipidemic mice. *International Journal of Biological Macromolecules, 125*, 1033-1041.

Siddiqui, W. A., Ahad, A., & Ahsan, H. (2015). The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. *Archives of toxicology, 89*(3), 289-317.

Sturje, J., Wienke, D., & Isacke, C. M. (2006). Endosomes generate localized Rho-ROCK-MLC2-based contractile signals via Endo180 to promote adhesion disassembly. *The Journal of cell biology, 175*(2), 337–347.

Su, E., Chu, Y., Chueh, F., et al. (2019). Bufalin Induces Apoptotic Cell Death in Human Nasopharyngeal Carcinoma Cells through Mitochondrial ROS and TRAIL Pathways. *American Journal of Chinese Medicine, 47*(1), 237-257.

Sun, J., Zhang, D., Zheng, Y., et al. (2013). Targeting the metastasis suppressor, NDRG1, using novel iron chelators: regulation of stress fiber-mediated tumor cell migration via modulation of the ROCK1/pMLC2 signaling pathway. *Molecular pharmacology, 83*(2), 454–469.

Sun, Y., Hui, Q., Chen, R., et al. (2018). Apoptosis in human hepatoma HepG2 cells induced by the phenolics of *Tetrastigma hemsleyanum* leaves and their antitumor effects in H22 tumor-bearing mice. *Journal of Functional Foods, 40*, 349-364.

Sun, Y., Li, H., Hu, J., et al. (2013). Qualitative and Quantitative Analysis of Phenolics in *Tetrastigma hemsleyanum* and Their Antioxidant and Antiproliferative Activities. *Journal of Agricultural and Food Chemistry, 61*(44), 10507-10515.
Sun, Y., Qin, Y., Li, H., et al. (2015). Rapid characterization of chemical constituents in *Radix Tetrastigma*, a functional herbal mixture, before and after metabolism and their antioxidant/antiproliferative activities. *Journal of Functional Foods*, 18(A), 300-318.

Taghizadehghalejoughi, A., Sezen, S., Hacimuftuoglu, A., et al. (2019). Vincristine combination with Ca\(^{2+}\) channel blocker increase antitumor effects. *Molecular Biology Reports*, 46(2), 2523-2528.

Tanaka, A., Yamamoto, A., Murota, K., et al. (2017). Polyunsaturated fatty acids induce ovarian cancer cell death through ROS-dependent MAP kinase activation. *Biochemical and Biophysical Research Communications*, 493(1), 468-473.

Valery, P. C., Laversanne, M., Clark, P. J., et al. (2018). Projections of primary liver cancer to 2030 in 30 countries worldwide. *Hepatology*, 67(2), 600-611.

Wang, C. Y., Jang, H., Han, Y. et al. (2018). Alkaloids from *Tetrastigma hemsleyanum* and Their Anti-Inflammatory Effects on LPS-Induced RAW264.7 Cells. *Molecules*, 23(6), 1445.

Wu, Q., Deng, J., Fan, D., et al. (2018). Ginsenoside Rh4 induces apoptosis and autophagic cell death through activation of the ROS/JNK/p53 pathway in colorectal cancer cells. *Biochemical Pharmacology*, 148, 64-74.

Xia, G., Li, S., & Zhou, W. (2018). Isoquercitrin, ingredients in *Tetrastigma hemsleyanum* Diels et Gilg, inhibits hepatocyte growth factor/scatter factor-induced tumor cell migration and invasion. *Cell Adhesion & Migration*, 12(5), 464-471.

Xiong, Y., Wu, X., & Rao, L. (2015). *Tetrastigma hemsleyanum* (Sanyeqing) root tuber extracts induces apoptosis in human cervical carcinoma HeLa cells. *Journal of Ethnopharmacology*, 165, 46-53.

Yin, F., Zhou, H., Fang, Y., et al. (2020). Astragaloside IV alleviates ischemia reperfusion-induced apoptosis by inhibiting the activation of key factors in death receptor pathway and mitochondrial pathway. *Journal of Ethnopharmacology*, 248(112319)

Zhang, X., Yang, P., Luo, X., et al. (2019). High olive oil diets enhance cervical tumor growth in mice: transcriptome analysis for potential candidate genes and pathways. *Lipids in Health and Disease*, 18(1), 76.
Zhu, L., & Chen, L. (2019). Progress in research on paclitaxel and tumor immunotherapy. *Cellular & Molecular Biology Letters, 24*(40)
Table 1. Characterization of chemical constitution of FRTH by UPLC-ESI-TOF-MS.

| Peak No | Retention time (min) | Measured (m/z) | Calculated (m/z) | Formula | Error (ppm) | Major fragment ions (m/z) | Identification |
|---------|----------------------|----------------|------------------|---------|-------------|--------------------------|----------------|
| 1       | 36.51                | 609.1458       | 609.1461         | C_{27}H_{30}O_{1}       | -0.5         | 255.0286 (7), 271.0253 (12), 300.0276 (78), 301.0352 (44), 609.1525 (100) | Rutin          |
| 2       | 38.92                | 463.0875       | 463.0882         | C_{26}H_{28}O_{1}       | -1.5         | 151.0025 (7), 243.0288 (6), 255.0289 (16), 271.0237 (24), 300.0266 (100), 301.0345 (38), 463.0893 (18) | Quercetin-3-O-glucoside |
| 3       | 41.3                 | 593.1508       | 593.1512         | C_{27}H_{30}O_{1}       | -0.7         | 227.0339 (7), 229.0496 (6), 255.0287 (15), 257.0450 (7), 284.0316 (52), 285.0395 (100), 593.1573 (44) | Kaempferol-3-O-rutinoside |
| 4       | 44.0                 | 447.0927       | 447.0933         | C_{21}H_{27}O_{1}       | -1.3         | 227.0337 (40), 255.0287 (58), 256.0380 (9), 284.0313 (100), 285.0394 (42), 447.0938 (36) | Astragalin     |
Table 2. Effect of FRTH on body weight, tumor weight, food consumption and tumor growth rate in HepG2 tumor-bearing mice.

| Parameter          | FRTH (mg/kg BW) |
|--------------------|------------------|
|                    | 100              | 300              | 600              |
| Tumor weight (g)   | 1.587 ± 0.311\(^a\) | 0.157 ± 0.090\(^b\) | 1.480 ± 0.319\(^a\) | 1.159 ± 0.164\(^b\) | 1.132 ± 0.414\(^a\) |
| Inhibition rate (%)| -                | 90.1\(^a\)       | 6.7\(^a\)       | 27.0\(^a\)       | 28.7\(^a\)       |
| Food consumption (g)| 3.01 ± 0.25\(^a\) | 2.22 ± 0.29\(^b\) | 3.28 ± 0.20\(^a\) | 3.06 ± 0.17\(^b\) | 3.03 ± 0.92\(^a\) |
| Original weight (g)| 15.257 ± 1.289\(^a\) | 15.075 ± 1.002\(^a\) | 14.080 ± 0.968\(^a\) | 14.117 ± 1.561\(^a\) | 14.283 ± 1.055\(^a\) |
| Final weight (g)   | 18.316 ± 1.861\(^b\) | 13.300 ± 1.131\(^c\) | 18.720 ± 1.668\(^b\) | 17.350 ± 1.923\(^b\) | 17.033 ± 2.404\(^b\) |
| Weight change(g)   | 3.059 ± 1.531\(^b\) | -1.775 ± 1.273\(^b\) | 4.640 ± 2.342\(^b\) | 3.233 ± 1.401\(^b\) | 2.750 ± 1.471\(^b\) |

The results are indicated as mean ± standard error mean of mice for each group.

Values with different letters (a, b, c) represents for significant difference (p < 0.05).
Figure 1. The chromatograms of FRTH and cytotoxicity effect in HepG2 cells. (A) The base peak chromatograms of FRTH detected at wavelength of 360 nm; (B) The MS/MS data of major flavones in FRTH; (C) MTT assays, HepG2 cells were treated with FRTH at different concentrations; (D) Scratch width of HepG2 cells with FRTH intervention; (E) Migration ability of HepG2 cells with FRTH intervention; (F) Clone formation after FRTH treatment (G) Light microscopic features of HepG2 cells with and without FRTH intervention; (H) Hoechst 33258 staining in HepG2 cells; (I) Dead
HepG2 cells with PI staining. Different letters (a, b, c) represents for significant difference (p < 0.05) according to one-way ANOVA test.
Figure 2. Effect of FRTH on oxidative damage in HepG2 cells. (A) RH123 staining for mitochondrial membrane potential (MMP); (B) DCF-DA staining for ROS; (C) DHE staining for $O_2^{•-}$; (D)-(F) were quantitative data of (A)-(C), the images were quantified by Image-Pro Plus 6.0; (G) Intensity of bands of PI3K/AKT protein; (H) Protein levels corrected by β-actin. (I) Intensity of bands of apoptosis -migration-related proteins; (J) Apoptosis- migration -related proteins levels corrected by β-actin. Different letters (a, b, c) represents for significant difference ($p < 0.05$) according to one-way ANOVA test.
Figure. 3. Effects of FRTH on HepG2 tumor bearing mice. (A) photographs of tumors obtained from HepG2 tumor bearing mice; (B) Tumor weight. (C) liver index; (D) kidney index; (E) morphological changes in kidney, liver (100×); Different letters (a, b, c) represents for significant difference ($p < 0.05$) according to one-way ANOVA test.
Figure. 4. Effects of FRTH on morphological changes and expression of apoptosis-related protein. (A) morphological changes in tumors for H&E and immunohistochemical staining; (B) The expression level of Bax; (C) The expression level of Caspase-3; (D) The expression level of VEGF; (E) The expression level of CD31; (F) The expression level of Ki67. Different letters (a, b, c) represents for significant difference ($p < 0.05$) according to one-way ANOVA test.

Figure. 5. Protein levels and intensity of bands of cytochrome C, Bax, Bcl-2, cytochrome C, Caspase-9, Caspase-3 and β-actin. (A) Protein levels and intensity of bands of Bax, Bcl-2, Caspase-3, cytochrome C, Caspase-9, Cleaved-caspase-3β-actin;
(B) Protein levels and intensity of bands of Bax corrected by β-actin; (C) Protein levels and intensity of bands of Bcl-2 corrected by β-actin; (D) Ratio of Protein levels and intensity of bands of Bax/Bcl-2 corrected by β-actin; (E) Protein levels and intensity of bands of cytochrome C corrected by β-actin; (F) Protein levels and intensity of bands of Caspase-9 corrected by β-actin; (G) Protein levels and intensity of bands of Caspase-3 corrected by β-actin; (H) Protein levels and intensity of bands of Cleaved-caspase-3 corrected by β-actin; Different letters (a, b, c) represents for significant difference ($p < 0.05$) among different groups according to one-way ANOVA test.
Research Highlights

1. Flavones-rich fraction of root of *Tetrastigma hemsleyanum* (FRTH) could inhibit HepG2 cell growth through PI3K/AKT pathway *in vitro*.

2. FRTH could enhance the ROS level and change the mitochondrial membrane potential in HepG2 cells.

3. The antitumor activities of FRTH were investigated in HepG2 tumor-bearing nude mice. FRTH could suppress the tumor growth *in vivo*.

4. FRTH could upregulate the expression level of Bax/Bcl-2 and Caspase-3, Caspase-9, Cleaved-caspase-3 and cytochrome C.

5. FRTH could downregulate the expression level of CD31, Ki67 and VEGF.