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Preparation of total triterpenoids from *Antrodia cinnamomea* fermentation mycelium and their in vitro inhibitory effects on hepatocellular carcinoma

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Abstract

Total triterpenoids from *Antrodia cinnamomea* fermentation mycelium (TT-ACFM) were prepared, and their in vitro inhibitory effects on hepatocellular carcinoma were investigated. Human hepatocellular carcinoma HepG2 cells were incubated with TT-ACFM with concentration of 0 (control), 12.5, 25, 50, 100 and 200 μg/mL, respectively. The proliferation of cell was detected using CCK-8 method. The apoptosis of cells was detected by flow cytometry. The migration and invasion of cells was determined using Transwell chamber. The level of reactive oxygen species (ROS) in cells was determined using DCFH-DA method. The expressions of matrix metalloproteinse-2 (MMP-2), matrix metalloproteinse-9 (MMP-9) and vascular endothelial growth factor (VEGF) protein in cells were detected by western blot assays. Results indicate that, TT-ACFM can not only inhibit the proliferation of HepG2 cells and promote their apoptosis, but also inhibit their invasion and invasion. The mechanism may be related to its increase of ROS and down-regulation of MMP-2, MMP-9 and VEGF expression in cells.

Keywords: *Antrodia cinnamomea*; total triterpenoids; mycelium; inhibitory; HepG2.

Practical Application: This study has provided a basis for preparation of total triterpenoids from *Antrodia cinnamomea* fermentation mycelium and their application to treatment of hepatocellular carcinoma.

1 Introduction

Antrodia cinnamomea is a valuable medicinal fungus which is mainly produced in Taiwan, China. *Antrodia cinnamomea* is mainly parasitic on *Cinnamomum kanehirae* trees which grow in a high-altitude area (Tsai et al., 2018). As the *Cinnamomum kanehirae* trees are highly specific, slow growing and rare, *Antrodia cinnamomea* is very precious. The artificial fermentation is a culture mode of *Antrodia cinnamomea*. It can simulate the growth environment of *Antrodia cinnamomea* and break the limitation in the scarcity of *Cinnamomum kanehirae* trees. The main components and metabolites of fermentation products are close to the fruiting body of natural *Antrodia cinnamomea* (Chiu & Hua, 2016; Meng et al., 2021). This may provide the possibility for the industrial production of *Antrodia cinnamomea* extracts. *Antrodia cinnamomea* contains a large number of active ingredients, including polysaccharides, flavonoids, triterpenoids, nucleic acids, immune proteins, etc. (Lu et al., 2013; Ganesan et al., 2019). Research has shown that, triterpenoids have multiple biological activities, and can inhibit the proliferation of many kinds of cancer cell (Patlolla & Rao, 2012; Li et al., 2020). Therefore, the triterpenoids of *Antrodia cinnamomea* may have important medicinal value. Hepatocellular carcinoma is one of the common malignant tumors with a high incidence (Konyn et al., 2021). In recent years, although the prognosis of hepatocellular carcinoma patients has been greatly enhanced with the improvement of diagnosis and treatment technology, the tumor is still prone to recurrence and metastasis, which greatly threatens the health of patients. Therefore, it is of great significance to find more effective drugs for the treatment of liver cancer. In this study, the total triterpenoids from *Antrodia cinnamomea* fermentation mycelium (TT-ACFM) were prepared, and their in vitro inhibitory effects on hepatocellular carcinoma were investigated. The objective was to provide a basis for preparation of TT-ACFM and their application to treatment of hepatocellular carcinoma.

2 Materials and methods

2.1 Preparation of TT-ACFM

An appropriate amount of TT-ACFM (produced in our laboratory) was placed in a round-bottom flask, followed by adding 12 times (mL: g) of 95% ethanol-water solution. The heat-reflux extraction was performed twice, 60 min each time. After filtering, the filtrates of two times were combined, followed by rotary evaporation to remove ethanol until no alcohol smell. After freeze drying, the crude extract was obtained. The crude extract was dissolved in water, and then loaded on AB-8 macroporous adsorption resin column. After gradient elution, the target elution solution was obtained. After vacuum concentration, the crude product was taken. The crude product was loaded on silica gel column, followed by elution. The elution solution was obtained. After vacuum concentration and drying, the final TT-ACFM product was prepared.

2.2 Cell culture

Human hepatocellular carcinoma HepG2 cells (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum at 37 °C and under 5% CO_2. The cells were

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passaged when the growth density reached 80%. The cells in logarithmic growth phase were taken for the next experiments.

2.3 Detection of cell proliferation

The inhibition of TT-ACFM on proliferation of HepG2 cells was detected using the Cell Counting Kit-8 (CCK-8) method. The cells with concentration of 2×10^3 cells/mL were inoculated into 96-well plates, 100 μL in each well. TT-ACFM was diluted with dimethyl sulfoxide (DMSO) into 1 mg/mL mother liquor. Different amount of TT-ACFM mother liquor was added to the wells. The final volume of each well was adjusted to 100 μL. In different treatment groups, the TT-ACFM concentration was 12.5, 25, 50, 100 and 200 μg/mL. At the same time, the control group (the same with treatment group except for adding TT-ACFM) and the blank group (the same with treatment group except for adding DMSO, TT-ACFM and cells) were set. Six repetitive wells were set for each group. The cells were cultured for 24, 48 and 72 h, respectively. After culture, a 10 μL CCK-8 reagent was added to each well. After 2.5 h of incubation, the optical density (OD) at 450 nm wavelength of the well was detected in the microplate reader. The proliferation inhibition rate of HepG2 cells was calculated as follows (Formula 1):

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

2.4 Detection of cell apoptosis

HepG2 cells with concentration of 2×10^3 cells/mL were inoculated into 96-well plate. After 12 h of culture, TT-ACFM was added to the wells. In different groups, the TT-ACFM concentration was 0 (control), 12.5, 25, 50, 100 and 200 μg/mL. Six repetitive wells were set for each group. The culture was performed for 24, 48 and 72 h, respectively. After washing the cells with phosphate buffered saline (PBS), 500 μL of binding buffer was added to suspend the cells. Then, 5 μL fluorescein isothiocyanate-labeled annexin-V and 5 μL propidium iodide were added, followed by incubation at room temperature for 15 min. Finally, the apoptosis of cells was detected in flow cytometer.

2.5 Determination of cell migration and invasion

HepG2 cells were treated with 0 (control), 12.5, 25, 50, 100 and 200 μg/mL TT-ACFM for 48 h, respectively. The cells were collected, and the concentration was adjusted to 8×10^5 cell/mL. The configured Matrigel glue was spread on the inner membrane of Transwell chamber. A 100 μL cell solution was added to the upper layer of chamber, while 600 μL medium containing 10% fetal bovine serum was added to the lower layer of chamber. Three chambers were set for each TT-ACFM concentration. The chamber was incubated for 24 h. Then, the chamber was taken out, followed by fixing with methanol and dyeing with crystal violet. After washing with water, the cells on the surface of membranes were wiped off using cotton swab dipped in normal saline. Three different fields of visual were randomly selected, and observed under the microscope. The number of cells passing through the membranes was counted. The invasion inhibition rate was calculated as follows: invasion inhibition rate (%) = (1 - number of invading cells in treatment group / number of invading cells in control group) × 100%. The procedure of cell migration experiment is basically the same with the invasion experiment, but the Matrigel glue was not spread on the inner membrane of Transwell chamber. The migration inhibition rate was calculated as follows: migration inhibition rate (%) = (1 - number of migrating cells in treatment group / number of migrating cells in control group) × 100%

2.6 Determination of reactive oxygen species (ROS) in cells

Level of ROS in HepG2 cells was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) method. HepG2 cell suspension was inoculated into 96-well plate for culture for 48 h. The cells were washed with PBS for two times. The new culture medium with 4 μmol/L H_2O_2 was added (the blank group was only added with medium). After 24 h of incubation, 0 (control), 12.5, 25, 50, 100 and 200 μg/mL TT-ACFM was added, respectively. After 12 h of incubation, 40 μL of 1 mmol/L DCFH-DA was added. After 30 min of incubation, the cells were washed with PBS twice. The fluorescence intensity of each well was determined under fluorescence microscope.

2.7 Western blot assays

HepG2 cells cells were treated with 0 (control), 12.5, 25, 50, 100 and 200 μg/mL TT-ACFM for 48 h, respectively. The cells were collected, and washed with PBS twice. The cells were lysated with RIPA. The total proteins were extracted by ultrasound, and the protein concentration was determined using the bicinchoninic acid kit. The sodium dodecyl sulfonate-polyacrylamide gel electrophoresis was performed to separate the proteins. The separated proteins were transferred to the PVDF membranes, followed by blocking with 5% skimmed milk powder at room temperature for 1 h. The membranes were incubated with the primary antibody of matrix metalloproteinse-2 (MMP-2), matrix metalloproteinse-9 (MMP-9) and vascular endothelial growth factor (VEGF), respectively, overnight at 4 °C, followed by incubating with the secondary antibody at 37 °C for 1 h. After washing with PBS for three times, the membranes were developed using enhanced chemiluminescence substrates. Finally, the bands on membranes were visualized and analyzed using Image J imaging system. β-actin was used as the internal reference. The relative expression level of target protein was calculated.

2.8 Statistical analysis

Data were presented as mean ± standard deviation. The single factor analysis of variance was performed using the SPSS 18.0 statistical software. The difference of comparison was statistically significant at $P < 0.05$.

3 Results

3.1 Effect of TT-ACFM on proliferation of HepG2 cells

As shown in Table 1, after treatment for 24, 48 and 72 h, TT-ACFM with different concentration could inhibit the proliferation of HepG2 cells. The proliferation inhibition rate of HepG2 cells increased with the increase of TT-ACFM concentration and prolonging of treatment time, respectively. When the TT-ACFM concentration

was 200 μg/mL and the treatment time was 72 h, the proliferation inhibition rate was the highest, which was (62.55 ± 7.51)%.

3.2 Effect of TT-ACFM on apoptosis of HepG2 cells

Table 2 showed that, the apoptosis rate of HepG2 cells increased with the increase of TT-ACFM concentration and prolonging of treatment time, respectively. When the TT-ACFM concentration was 200 μg/mL and the treatment time was 72 h, the apoptosis rate was (46.26 ± 5.42)%, which was the highest among different groups.

3.3 Effect of TT-ACFM on migration and invasion of HepG2 cells

After treatment for 48 h, TT-ACFM with different concentration could inhibit the migration and invasion of HepG2 cells. The migration and invasion inhibition rates of HepG2 cells increased with the increase of TT-ACFM concentration, respectively. When the TT-ACFM concentration was 200 μg/mL, the migration and invasion inhibition rates were (46.63 ± 5.77)% and (40.56 ± 6.67)%, respectively, which were the highest among different groups (Table 3).

3.4 Effects of TT-ACFM on fluorescence intensity of ROS in HepG2 cells

Effects of TT-ACFM on fluorescence intensity of ROS in HepG2 cells were shown in Figure 1. The fluorescence intensity in 0 μg/mL TT-ACFM group was the lowest. With the increase of TT-ACFM concentration, the fluorescence intensity gradually increased. When the TT-ACFM concentration was 200 μg/mL, the fluorescence intensity was (150.04 ± 7.21), the highest among six groups.

3.5 Effect of TT-ACFM on MMP-2, MMP-9 and VEGF protein expressions in HepG2 cells

Western blot assays showed that, after treatment for 48 h, the TT-ACFM could down-regulate the MMP-2, MMP-9 and VEGF protein expressions in HepG2 cells. The relative expression level of each protein decreased with the increase of TT-ACFM concentration. When the TT-ACFM concentration was 200 μg/mL, the relative expression level of each protein was the lowest (Table 4).

Table 1. Effect of TT-ACFM on proliferation of HepG2 cells (n = 6).

TT-ACFM (μg/mL)	Proliferation inhibition rate (%)		
	24 h	48 h	72 h
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12.5	4.82 ± 0.56^{a}	12.78 ± 1.65$^{a\#}$	23.48 ± 3.28$^{a\#\&}$
25	12.62 ± 2.34^{ab}	25.23 ± 3.15$^{ab\#}$	32.37 ± 4.41$^{ab\#\&}$
50	22.40 ± 3.66^{abc}	31.52 ± 4.42$^{abc\#}$	42.16 ± 6.67$^{abc\#\&}$
100	34.69 ± 5.21^{abcd}	41.10 ± 6.46$^{abcd\#}$	47.62 ± 6.82$^{abc\#\&}$
200	43.18 ± 4.10^{abcde}	52.22 ± 7.55$^{abcde\#}$	62.55 ± 7.51$^{abcde\#\&}$

$^{a}P < 0.05$ compared with 0 μg/mL TT-ACFM; $^{b}P < 0.05$ compared with 12.5 μg/mL TT-ACFM; $^{c}P < 0.05$ compared with 25 μg/mL TT-ACFM; $^{d}P < 0.05$ compared with 50 μg/mL TT-ACFM; $^{e}P < 0.05$ compared with 100 μg/mL TT-ACFM; $^{\#}P < 0.05$ compared with 24 h; $^{\&}P < 0.05$ compared with 48 h. TT-ACFM, total triterpenoids from *Antrodia cinnamomea* fermentation mycelium.

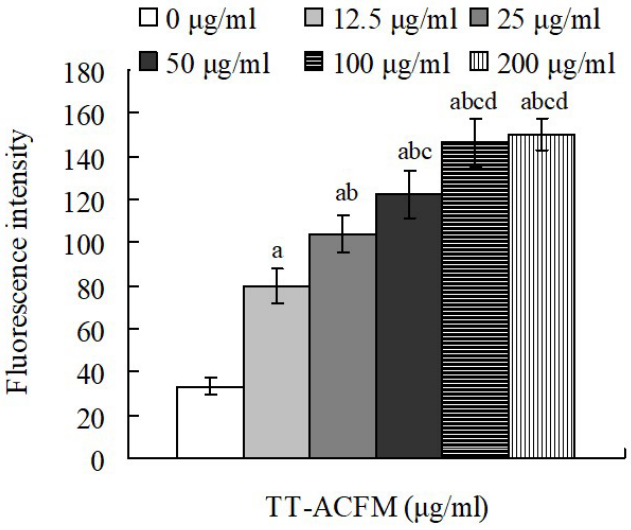

Figure 1. Effects of TT-ACFM on fluorescence intensity of reactive oxygen species in HepG2 cells (n = 6). $^{a}P < 0.05$ compared with 0 μg/mL TT-ACFM; $^{b}P < 0.05$ compared with 12.5 μg/mL TT-ACFM; $^{c}P < 0.05$ compared with 25 μg/mL TT-ACFM; $^{d}P < 0.05$ compared with 50 μg/mL TT-ACFM. TT-ACFM, total triterpenoids from *Antrodia cinnamomea* fermentation mycelium.

Table 2. Effect of TT-ACFM on apoptosis of HepG2 cells (n = 6).

TT-ACFM (μg/mL)	Apoptosis rate (%)		
	24 h	48 h	72 h
0	0.88 ± 0.12	2.13 ± 0.43$^{\#}$	1.67 ± 0.23$^{\#\&}$
12.5	4.03 ± 0.60^{a}	7.18 ± 1.20$^{a\#}$	12.04 ± 1.29$^{a\#\&}$
25	7.16 ± 1.12^{ab}	11.32 ± 2.90$^{ab\#}$	14.30 ± 2.18$^{a\#\&}$
50	15.37 ± 2.75^{abc}	19.84 ± 3.22$^{abc\#}$	27.21 ± 3.05$^{abc\#\&}$
100	21.55 ± 4.28^{abcd}	26.62 ± 4.51$^{abcd\#}$	36.52 ± 6.31$^{abcd\#\&}$
200	24.42 ± 3.15^{abcd}	32.33 ± 4.33$^{abcde\#}$	46.26 ± 5.42$^{abcde\#\&}$

$^{a}P < 0.05$ compared with 0 μg/mL TT-ACFM; $^{b}P < 0.05$ compared with 12.5 μg/mL TT-ACFM; $^{c}P < 0.05$ compared with 25 μg/mL TT-ACFM; $^{d}P < 0.05$ compared with 50 μg/mL TT-ACFM; $^{e}P < 0.05$ compared with 100 μg/mL TT-ACFM; $^{\#}P < 0.05$ compared with 24 h; $^{\&}P < 0.05$ compared with 48 h. TT-ACFM, total triterpenoids from *Antrodia cinnamomea* fermentation mycelium.

Table 3. Effect of TT-ACFM on migration and invasion of HepG2 cells (n = 6).

TT-ACFM (μg/mL)	Migration inhibition rate (%)	Invasion inhibition rate (%)
0	0.00 ± 0.00	0.00 ± 0.00
12.5	5.28 ± 1.05^{a}	4.44 ± 0.78^{a}
25	14.54 ± 2.38^{ab}	12.68 ± 1.93^{ab}
50	22.22 ± 4.05^{abc}	18.31 ± 3.55^{ab}
100	27.10 ± 3.39^{abc}	24.29 ± 4.23^{abc}
200	46.63 ± 5.77^{abcde}	40.56 ± 6.67^{abcde}

$^{a}P < 0.05$ compared with 0 μg/mL TT-ACFM; $^{b}P < 0.05$ compared with 12.5 μg/mL TT-ACFM; $^{c}P < 0.05$ compared with 25 μg/mL TT-ACFM; $^{d}P < 0.05$ compared with 50 μg/mL TT-ACFM; $^{e}P < 0.05$ compared with 100 μg/mL TT-ACFM. TT-ACFM, total triterpenoids from *Antrodia cinnamomea* fermentation mycelium.

Table 4. Effect of TT-ACFM on MMP-2, MMP-9 and VEGF protein expressions in HepG2 cells (n = 6).

TT-ACFM (μg/mL)	MMP-2/β-actin	MMP-9/β-actin	VEGF/β-actin
0	1.01 ± 0.09	0.88 ± 0.12	1.12 ± 0.10
12.5	1.07 ± 0.11	0.77 ± 0.07	1.04 ± 0.08
25	0.85 ± 0.10^{ab}	0.73 ± 0.06^{a}	0.92 ± 0.06^{ab}
50	0.52 ± 0.03^{abc}	0.46 ± 0.04^{abc}	0.56 ± 0.05^{abc}
100	0.49 ± 0.04^{abc}	0.38 ± 0.05^{abc}	0.47 ± 0.05^{abc}
200	0.37 ± 0.03^{abcde}	0.29 ± 0.02^{abcd}	0.41 ± 0.03^{abcd}

$^{a}P < 0.05$ compared with 0 μg/mL TT-ACFM; $^{b}P < 0.05$ compared with 12.5 μg/mL TT-ACFM; $^{c}P < 0.05$ compared with 25 μg/mL TT-ACFM; $^{d}P < 0.05$ compared with 50 μg/mL TT-ACFM; $^{e}P < 0.05$ compared with 100 μg/mL TT-ACFM. TT-ACFM, total triterpenoids from *Antrodia cinnamomea* fermentation mycelium; MMP-2, matrix metalloproteinse-2; MMP-9, matrix metalloproteinse-9; VEGF, vascular endothelial growth factor.

4 Discussion

Individualized program for comprehensive treatment is often advocated for hepatocellular carcinoma. According to the patient age, sex, location, and local and distant metastasis of hepatocellular carcinoma, the conventional surgical resection, chemotherapy, radiotherapy, laser microsurgery, photodynamic therapy and other methods are used alone or in combination. However, the 5-year survival rate of hepatocellular carcinoma is still low (Sarveazad et al., 2019). At present, the surgery combined with chemotherapy drugs is often used for the treatment of metastatic lesions of hepatocellular carcinoma. However, the effect of chemotherapy drugs cannot meet the expectations, and the side effects of chemotherapy drugs on normal cells are large (Winters et al., 2020). Therefore, it is an urgent task to find new drugs to treat the hepatocellular carcinoma in clinical and basic medical research. This study prepared TT-ACFM, and investigated the effect of TT-ACFM on the proliferation, apoptosis, migration and invasion of human hepatocellular carcinoma HepG2 cells. Results showed that, TT-ACFM with different concentration could inhibit the proliferation, migration and invasion of HepG2 cells and promote their apoptosis.

Apoptosis, also known as programmed cell death, is an orderly and autonomous active cell death process induced by external stimuli through a series of gene activation and regulation. Apoptosis plays an important role in cell development, tissue homeostasis, and defense against harmful and potentially dangerous cells. Mitochondrial signal pathways play a more important role in cell apoptosis (Shahar & Larisch, 2020). The characteristic of mitochondrial damage is that the loss of mitochondrial membrane potential increases the membrane permeability, and the apoptotic factors in mitochondria, such as cytochrome C, are released into the cytoplasm, forming apoptotic complexes in the cytoplasm and inducing the apoptosis (Qiao et al., 2019). ROS play an important role in the body. A lower level of ROS promotes the cellular proliferation, differentiation, tissue regeneration and metabolism, and the high concentrations of ROS induce cell apoptosis and necrosis by oxidative stress (Li et al., 2022). Results of this study showed that, with the increase of TT-ACFM concentration, the fluorescence intensity of ROS gradually increased. This suggests that, the mechanism of apoptosis induced by TT-ACFM may be related to the increased ROS in HepG2 cells.

Generally speaking, the tumor invasion and metastasis are a complex dynamic process. The tumor cells must overcome the histological barrier before invasion and metastasis. The barrier consists of basement membrane and extracellular matrix (Eble & Niland, 2019). In tumor cells, MMP-2 and MMP-9, members of MMPs family, belong to type IV collagenase. The main components of basement membrane and extracellular matrix are type IV collagen. When MMP-2 and MMP-9 are activated and over-expressed, they can decompose the type IV collagen, thus destroying the basement membrane and extracellular matrix. So, the histological barrier was destroyed, which promotes the invasion and metastasis of cancer cells (Lee et al., 2008). MMP-2 and MMP-9 are highly expressed in squamous cell carcinomas cells, and play an important role in the invasion and metastasis of cells (Monteiro-Amado et al., 2013). In our study, after treatment for 48 h, TT-ACFM could down-regulate the MMP-2 and MMP-9 protein expressions in HepG2 cells. This suggests that TT-ACFM may inhibit the invasion and metastasis of HepG2 cells by down-regulating the MMP-2 and MMP-9 expressions.

There is a large angiogenesis during the invasion and metastasis of tumor cells. VEGF, as one of the strongest factors to promote the angiogenesis, plays an important role in tumor invasion and metastasis. VEGF can increase the permeability of blood vessels, which is beneficial for tumor cells entering blood vessels and flowing to all parts of the body (Zhou et al., 2021). It is found that, the elevated VEGF expression may be a predictive biomarker for poor prognosis and adverse clinicopathological characteristics in patients with osteosarcoma (Zhang et al., 2021). Results of this study showed that, the baicalein could down-regulate the VEGF protein expression in HepG2 cells. This indicates that, the inhibition of HepG2 cell migration and invasion by TT-ACFM may be related to its down-regulating the VEGF expression.

5 Conclusions

In conclusion, TT-ACFM can not only inhibit the proliferation of human hepatocellular carcinoma HepG2 cells and promote their apoptosis, but also inhibit their invasion and invasion. The mechanism may be related to its increase of ROS and down-regulation of MMP-2, MMP-9 and VEGF expression. This study has provided a basis for preparation of TT-ACFM and their application to treatment of hepatocellular carcinoma. However, there may be other action mechanisms of TT-ACFM on HepG2 cells which have not been explored in this study. These issues should be solved in further research.

Disclosure

The authors declare that there is no conflict of interest.

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