Mechanism of the growth inhibitory effects of *Zizyphus jujuba* and green tea extracts in human hepatoma cells

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Mechanism of the growth inhibitory effects of Zizyphus jujuba and green tea extracts in human hepatoma cells

（なつめ抽出物と緑茶抽出物のヒト肝ガン細胞増殖抑制作用とその作用メカニズムについて）

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黄 雪丹
INTRODUCTION

CHAPTER I  Mechanism of the anticancer activity of *Zizyphus jujuba* in HepG2 cells

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Hepatocellular carcinoma accounts for 80% to 90% of primary liver cancer. Hepatocellular carcinoma is a major health problem worldwide, with an estimated incidence ranging between 500,000 and 1,000,000 new cases annually. It is the fifth most common cancer in the world, and the third most common cause of cancer-related death \(^1\). The rates of hepatocellular carcinoma in men are 2 to 4 times higher than in women. It usually develops between 35 and 65 years of age, when people are most productive and have most family responsibilities \(^2, 3, 4, 5\). Hepatocellular carcinoma shows great geographical variation, with a very high incidence in Asia and sub-Saharan Africa \(^6\). Although its incidence is far lower in the United States and Europe, those rates have been increasing in recent years.

There are many treatment options for hepatocellular carcinoma, such as liver resection (partial hepatectomy and orthotopic liver transplantation), local ablative therapy (injection of cytotoxic agent and application of an energy source), hepatic artery transcatheter
treatment (transarterial chemoembolisation and transarterial radioembolisation), systemic therapy (chemotherapy, immunotherapy, chemo-immunotherapy, hormonal therapy and somatostation analogue) and other treatments (gene therapy and supportive therapy). The most effective and potentially curative therapy is liver transplantation, because it eradicates the hepatocellular carcinoma, however, this option is available to a very small fraction of hepatocellular carcinoma patients whose tumors are discovered at a very early stage before there is multifocal or vascular involvement. The other options used in clinic also depend on the status of the tumors. Combination chemotherapy appeared promising in two phase II studies of cisplatin, interferon, doxorubicin, and 5-fluorouracil and was notable for a small, but significant, number of pathologic complete remissions discovered after tumor downstaging (patients were all deemed unresectable as a criterion for entering the study) allowed surgical resection. Therefore, it is important to find a new drug for chemotherapy in the future.

For thousands of years, natural products have played an
important role throughout the world in treating and preventing human diseases. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates\textsuperscript{15}). The importance of natural products in modern medicine has been discussed in recent reviews and reports\textsuperscript{15, 16, 17, 18}). The value of natural products in this regard can be assessed using 3 criteria: (1) the rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semisynthetic and total synthetic modification, (2) the number of diseases treated or prevented by these substances, and (3) their frequency of use in the treatment of disease. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87\% of all categorized human diseases, including as antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressant agents, among others. Moreover, there are more than 60\% of drugs of natural origin used in the treatment of cancer\textsuperscript{15}). Recently, the low side effects of natural products in anticancer treatment have been noticed. Hence,
finding a new natural source with anticancer activities would aid in finding new tools for cancer therapy.

In this study, we aimed to investigate the anticancer activity and mechanism of action of *Zizyphus jujuba* Mill (*Z. jujuba*), a natural product, in human liver cancer cells (HepG2 cells). To achieve this aim, the following studies were conducted.

In Chapter I, we described that the chloroform fractions from *Z. jujuba* extract (CHCl$_3$-F) induced a concentration dependent effect on apoptosis and a differential cell cycle arrest in HepG2 cells.

In Chapter II, we described that combination of CHCl$_3$-F and green tea extracts (GTE) produced an enhanced cell growth inhibition effect, and the resultant G1 arrest was caused via different mechanism as that of CHCl$_3$-F treatment alone in HepG2 cells.

In Chapter III, we described that CHCl$_3$-F and GTE enhanced anti-cancer activity was via reducing the expression of APRIL, moreover, the anti-cancer activity of CHCl$_3$-F and GTE mixture was stronger than that of the anti-cancer drug cisplatin in HepG2 cells.
CHAPTER I

Mechanism of the anticancer activity of *Zizyphus jujuba* in HepG2 cells

1. Introduction

Chinese date is scientifically known as *Zizyphus jujuba* Mill (*Z. jujuba*). It is also “Hongzao” or “Dazao” in China and “Natume” in Japan. It has been mentioned in the famous Chinese ancient medical book - *Sheng Nong Ben Cao Jing*, and has traditionally been used in oriental medicines. For example, in Chinese traditional medicine, the dried fruits are prescribed as anodyne, anti-tumor, pectoral, refrigerant, sedative, stomachic, styptic and tonic. In Japan, the extracts of *Z. jujuba* are used to treat chronic hepatitis or distress and fullness in the chest and ribs. Some studies reported that there are 11 major components of 2 saponins and 9 fatty acids, namely jujuboside A, jujuboside B, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid and docosanoic acid in *Z. jujuba*. 
Many physiological activities have been found but there are no report about the anticancer activity of these components\textsuperscript{19, 20, 21}. A study reported the ethyl acetate-soluble fraction of \textit{Z. jujuba} showed high cytotoxic activity against some tumor cell lines\textsuperscript{22}. However, in these studies the mechanism of action of \textit{Z. jujuba} has not so far been investigated. More importantly, the effect of \textit{Z. jujuba} in human hepatoma cells (HepG2 cells), which come from hepatocellular carcinoma, has not yet been reported.

In this Chapter, we investigated the anticancer activity and mechanism of action of \textit{Z. jujuba} in HepG2 cells.

2. Materials and Methods

2.1. Materials

\textit{Z. jujuba} extract was kindly donated from Sea Load Co. Ltd. (Fukui, Japan). 2’, 7’- Dichlorodihydrofluorescein diacetate (DCFH-DA) and rhodamine 123 were purchased from Sigma–Aldrich Fine Chemical (Tokyo, Japan). Rb (retinoblastoma protein) and p27\textsuperscript{Kip1} antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa
Cruz, CA, USA). Fetal bovine serum (FBS) was purchased from Equitech-Bio Inc. (Kerrville, Texas, USA). Other chemicals used in this chapter were special grade commercial products.

2.2. Cell culture

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS in humidified incubator containing 5% CO₂ in air at 37°C. The cells were washed and cultured again at a concentration of 2×10⁵/ml in fresh medium. Z. jujuba extract was diluted in the culture medium immediately before use. In all the experiments, control cultures were made up of medium, water and the cells only.

2.3. Cell viability

Cell viability was determined with the neutral red uptake assay, based on the lysosomal uptake of neutral red. Following specified incubations with test agents, neutral red solution (0.25 mg/ml) was added to the cell cultures at a final concentration of 50 μg/ml. After
incubation at 37°C for 2 hrs, cells were rinsed twice with a mixture of 1% (v/v) formaldehyde, 1% (v/v) calcium chloride, and 98% (v/v) distilled water. Subsequently, 1 ml of destaining buffer consisting of 1% (v/v) acetic acid, 50% (v/v) ethanol, and 49% (v/v) distilled water was added to the cells, and the culture plates were kept for 30 mins. Lysosomal uptake of neutral red was determined spectrophotometrically at 540 nm \(^{24,25,26}\). Viability was expressed as \( (A_{540}^{\text{treated cells}}/A_{540}^{\text{appropriate control}}) \times 100\% \) after correction for background absorbance (100% cytotoxicity).

2.4. Fractionation of *Z. jujuba* extract

*Z. jujuba* extract was diluted at 1/20 with water. These liquids were extracted with chloroform, followed by ethyl acetate, butanol and water. Each of the fractions was evaporated to dryness *in vacuo* (Fig. 2).

2.5. Cell cycle assay

The cell cycle distribution was analyzed by laser scanning
cytometer (Olympus LSC 101) using PI staining. Briefly, after designated treatment, cells were washed twice in PBS and incubated with freshly prepared PI-stained buffer (0.1% Triton X-100 in PBS, 20 µg/ml PI, 200 µg/ml RNase) for 1 hr at 37°C in the dark \(^{27}\).

2.6. Reactive oxygen species (ROS) assay

The generation of ROS was determined with the DCFH-DA reagent as described by Ablise, M. \(^{28}\). Briefly, DCFH-DA (8 µM) was added at the last 15 mins of Z. jujuba extract treatment. Cells were washed with PBS twice and resuspended in Hanks solution. Fluorescence intensity was measured using a micro-plate reading fluoroscan plate-reader (Wallac 1420 ArVOsx, Amarsham Pharmacia Biotech) with the excitation wavelength at 485 nm and the emission wavelength at 535 nm \(^{29,30}\). The amount of intracellular ROS was calculated from a standard curve derived from 2’, 7’–dichlorofluoresceina (DCF). Protein concentration was measured by the Bradford method \(^{31}\).
2.7. Mitochondrial membrane potential assay

The HepG2 cells were incubated with *Z. jujuba* extract for 30, 60, 120 and 240 mins and rhodamine 123 (10 µg/ml) was added at the last 30 mins of *Z. jujuba* treatment. Cells were washed twice with PBS and fixed with 4% *p*-formaldehyde. After washing with PBS, the fluorescence intensity of cells was measured by a micro-plate reading fluoroscan plate-reader with the excitation wavelength at 505 nm and the emission wavelength at 525 nm.32

2.8. Western blot analysis of Rb and p27^Kip1^

Cells were harvested at the indicated time points and were washed twice in PBS. Then the cells were dissolved for 30 mins with lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.2), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM NaF, 20 µg/mL aprotinin, 50 µg/mL leupeptin, 10 µg/mL Pepstatin A and 100 µg/mL phenylmethylsulfonyl fluoride). Finally, the solution was centrifuged at 2000 ×g for 20 mins at 4°C. The supernatant was collected and protein concentrations were determined
by Bradford method. Equal amounts of protein were fractionated on 10% SDS-PAGE gels and transferred to 0.45 µm PVDF (Hybond; Amersham Pharmacia Biotech). After blocking overnight in 0.1% Tween-20 and 5% non-fat dry milk, in PBS, blots were incubated with anti-Rb antibody or anti-p27^Kip1 antibody for 1 hr at room temperature. After washing, the membrane was reincubated with 1:750 diluted biotinulated mouse immunoglobulin G (IgG) or rabbit IgG for 1 hr at room temperature. The membrane was washed several times, and incubated with 1:750 diluted horse radish peroxidase-coupled streptavidin for 1 hr at room temperature. After several washing steps, the color reaction was developed with DAB. Densitometry analysis of the protein bands was performed with the software Scion Image (Scion Corporation).

2.9. Statistical analysis

Data are represented as means ± S.D. (standard deviation from the mean). The significance of difference in assay values was evaluated with ANOVA followed by Tukey multiple tests. p <0.05 was
used to indicate a statistically significant difference.

3. Results

3.1. Effect of Z. jujuba extract on cell viability

The effect of Z. jujuba on cell viability was examined in HepG2 cells by neutral red method. As shown in Fig. 1, Z. jujuba extract decreased cell viability in a dose-dependent manner. The Z. jujuba extract contains many substances such as protein, carbohydrate and minerals (Data are not shown), and it has been reported that the ethyl acetate-soluble fraction of Z. jujuba has high cytotoxic activity \(^{22}\). Therefore, we extracted the Z. jujuba extract again by organic solvent (Fig. 2), and examined the cell viability with these fractions from the original Z. jujuba extract in HepG2 cells. As shown in Fig. 3, the chloroform fraction of Z. jujuba extract (CHCl\(_3\)-F) decreased cell viability the most as compared with the other fractions and non-treated control cells, in a dose-dependent manner (Fig. 4). Hence, this fraction was used in subsequent experiments.
3.2. Cell cycle changes with CHCl$_3$-F

We evaluated cell cycle dynamics with PI staining to examine the effect of CHCl$_3$-F induced decrease of the cell viability in HepG2 cells. As shown in Fig. 5, cells incubated with 100 µg/ml of CHCl$_3$-F resulted in an accumulation of G1 cell cycle region and a decrease in S cell cycle region. However, the cell number of G2/M phase was increased by the addition of 200 µg/ml of the CHCl$_3$-F, suggesting that the cell cycle was arrested at G2/M phase. Furthermore, CHCl$_3$-F also increased the cell number of Sub-G1 phase, suggesting that the CHCl$_3$-F also induced apoptosis in HepG2 cells.

3.3. Effect of CHCl$_3$-F on intracellular ROS levels

In recent years many studies on apoptosis have been associated with the excessive production of reactive oxygen species (ROS). We examined the effect of CHCl$_3$-F on intracellular peroxides levels by the DCFH-DA method. As shown in Fig. 6, the intracellular ROS level increased rapidly when cells were incubated with 100 µg/ml of CHCl$_3$-F at 15 mins, but not with 200 µg/ml of the CHCl$_3$-F. These
results suggest that the cause of apoptosis may be associated with increase of ROS under the 100 µg/ml treatment of CHCl₃-F but not under the 200 µg/ml treatment.

The effect of catalase on cell viability was examined by the neutral red method. As shown in Fig. 7, cell viability decrease induced by CHCl₃-F (100 µg/ml) was improved with catalase.

3.4. Effect of CHCl₃-F on mitochondrial membrane potential

The increase of intracellular ROS is well known to impair a variety of intra- and extra-mitochondrial membrane transport system such as distribution of mitochondrial ion-transport system and decrease of mitochondrial membrane potential, which may contribute to apoptosis. As shown in Fig. 8, the mitochondrial membrane potential was declined by CHCl₃-F (100-200 µg/ml) after 60 mins of incubation.

The increase in ROS is also considered the cause of changes in mitochondrial membrane potential. Most likely, catalase contributes to the formation of water and molecular oxygen from hydrogen peroxide.
To examine whether the decline of the mitochondrial membrane potential is associated with ROS, we used catalase to scavenge the increased ROS. We then examined the relationship between the decline of the mitochondrial membrane potential and the increase of ROS. As shown in Fig. 9, the decline of mitochondrial membrane potential induce by CHCl\textsubscript{3}-F was not recovered by the addition of catalase. This result indirectly explains that the increase in ROS was not the cause of a decline in mitochondrial membrane potential.

3.5. Effect of CHCl\textsubscript{3}-F on Rb protein

Cell cycle analysis showed a cell cycle arrest at G1 phase by addition of 100 µg/ml of CHCl\textsubscript{3}-F and at the G2/M phase by the addition of 200 µg/ml of CHCl\textsubscript{3}-F. Rb protein is a tumor suppressor protein found to be dysfunctional in a number of cancers. The normal function of Rb is to prevent the cell from dividing or progressing through the cell cycle. The product of the Rb gene prevents S-phase entry during the cell cycle, and inactivation of this growth-suppressive function is presumed to result from phosphorylated retinoblastoma protein (ppRb)
during the late G1 phase and S phase, with dephosphorylation during the G2/M phase. Western blot analysis showed that treatment of CHCl$_3$-F increased Rb levels compared with control at 8 hrs (Fig. 10).

To determine whether the CHCl$_3$-F induced increase in intracellular peroxide was associated with Rb protein, we examined the effect of catalase on Rb protein. As shown in Fig. 11, treatment of CHCl$_3$-F increased Rb levels, however, these effects were not reversed by the addition of 200 units/ml catalase at 5 mins before the treatment of 100 µg/ml of CHCl$_3$-F. This result suggests that the increase in ROS was not associated with Rb levels in CHCl$_3$-F of *Z. jujuba*.

3.6. Effect of CHCl$_3$-F on p27$^{\text{Kip1}}$

The p27$^{\text{Kip1}}$ is a member of the Cip/Kip family of cyclin-dependent kinase inhibitor. The overexpression of p27$^{\text{Kip1}}$ protein in mammalian cells induces G$_1$ arrest of the cell cycle. As shown in Fig. 12, the p27$^{\text{Kip1}}$ levels increased when the cells were treated with 100 µg/ml of CHCl$_3$-F, but phosphorylation of p27$^{\text{Kip1}}$ was observed under the treatment of 200 µg/ml of CHCl$_3$-F.
To determine whether the CHCl₃-F of *Z. jujuba* extract-induced increase in intracellular peroxide is associated with p27⁰ᴷⁱᵖ¹ protein, we examined the effect of catalase on p27⁰ᴷⁱᵖ¹ protein. As shown in Fig. 13, the increase of p27⁰ᴷⁱᵖ¹ induced by CHCl₃-F could not be reversed when catalase was added at 5 mins before the treatment 100 µg/ml of CHCl₃-F. This result suggests that the increase in ROS is not associated with p27⁰ᴷⁱᵖ¹ levels in CHCl₃-F of -treated cells.
Fig. 1. Effect of *Z. jujuba* extract on cell viability in HepG2 cells

Cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS for 3 days, diluted and incubated again in fresh medium with or without *Z. jujuba* extract. Cell viability was measured 24 hrs later by neutral red method. Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments. *p<0.05, **p<0.01
Fig. 2. Method of fractionation of *Z. jujuba* extract
Fig. 3. Effect of various fractions of *Z. jujuba* extract on cell viability in HepG2 cells

Cells were treated with or without various fractions of *Z. jujuba* extract. Cell viability was measured 24 hrs later by neutral red method. Each bar is the mean (± S.D.) of three experiments. *p<0.05, **p<0.01
Fig. 4. Effect of CHCl₃-F of *Z. jujuba* extract on cell viability in HepG2 cells

Cells were treated with or without CHCl₃-F. Cell viability was measured 24 hrs later by neutral red method. Each bar is the mean (± S.D.) of three experiments. *p<0.05, **p<0.01
Fig. 5. Effect of CHCl$_3$-F on cell cycle in HepG2 cells

Cells were incubated with CHCl$_3$-F of Z. jujuba extract for 24 hrs, washed twice in PBS, and incubated with fleshly prepared PI-stained buffer for 1 hr at 37°C in dark. The staining was analyzed by laser scanning cytometer. Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments. *p<0.05, **p<0.01
Fig. 6. Effect of CHCl₃-F on intracellular ROS levels in HepG2 cells

The intracellular ROS levels were measured by the DCFH-DA method. DCF fluorescence intensity of cells was measured at 15, 30 and 60 mins after treatment with the 100 µg/ml or 200 µg/ml of CHCl₃-F. Data are presented as means ± S.D. *p<0.05, **p<0.01 compared to the control group at the same time points.
Fig. 7. Effect of catalase on cell viability in HepG2 cells

The cell viability was examined by the neutral red method. After 3-4 days culture in Dulbecco's modified Eagle's medium supplemented with 10% FBS, the cells were washed and cultured again in flesh medium with or without the CHCl₃-F. Catalase (200 units/ml) was added at 5 mins before incubation of CHCl₃-F and cell viability was measured at 24 hrs later. Each bar is the mean (± S.D.) of three experiments. p<0.01
Fig. 8. Effect of CHCl₃-F on mitochondrial membrane potential in HepG2 cells

The mitochondrial membrane potential in the cells were measured by the rhodamine 123 method at 30, 60, 120 and 240 mins after treatment with the 100 µg/ml or 200 µg/ml of CHCl₃-F. Data are presented as means ± S.D. **p<0.01 compared to the control group at the same time points.
Fig. 9. Effect of catalase on mitochondrial membrane potential in HepG2 cells

The mitochondrial membrane potential in the cells in the presence of catalase were measured by the rhodamine 123 method at 2 hrs after treatment with the 100 µg/ml or 200 µg/ml of CHCl₃-F. Catalase (200 units/ml) was added 5 mins before CHCl₃-F treatment. Data are presented as means ± S.D. **p<0.01 compared to the control group at the same time points.
### Fig. 10. Effect of CHCl$_3$-F on Rb protein in HepG2 cells

HepG2 cells were treated with 100 µg/ml or 200 µg/ml CHCl$_3$-F for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.

<table>
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<td>8</td>
<td>200</td>
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**Fig. 11. Effect of catalase on Rb protein in HepG2 cells**

Catalase (200 units/ml) was added 5 mins before treatment of 8 hrs-incubated HepG2 cells with 100 µg/ml CHCl_3-F. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 12. Effect of CHCl₃-F on p27^{Kip1} protein in HepG2 cells

HepG2 cells were treated with 100 µg/ml or 200 µg/ml CHCl₃-F for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 13. Effect of catalase on p27<sup>Kip1</sup> protein in HepG2 cells

Catalase (200 units/ml) was added 5 mins before treatment of HepG2 cells with 100 µg/ml CHCl<sub>3</sub>-F. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
4. Discussion

In this chapter, we showed that the *Z. jujuba* extract has cytotoxic activities in HepG2 cells. Furthermore, the chloroform fraction of *Z. jujuba* extract (CHCl₃-F) was the most effective component. It was not only cytotoxic but it had cytostatic activity as well. Also, different concentrations of CHCl₃-F showed different growth inhibition effects in HepG2 cells. The results obtained from cell cycle analysis also showed that in HepG2 cells, there was a concentration dependent effect of CHCl₃-F induced apoptosis and arrest of cell cycle in different phases.

These results differ from a previously reported study that the EtOAc-F of *Z. jujuba* had cytotoxic activities in some tumor cell lines. This may be due to the fact that various fractions of *Z. jujuba* have different effects in various tumor cell lines.

An imbalance between cell proliferation, apoptosis, and differentiation leads to the development of malignant cells clones. Based on the understanding of tumor biology in respect of the kinetics of cell populations, two new strategies, induction of apoptosis and
anti-proliferation, have recently emerged in the fields of cancer chemoprevention and chemotherapy by phytochemicals. Apoptosis is a subtype of cell death that is involved in diverse physiological and pathological processes. Many studies have shown that apoptosis is closely associated with excessive production of reactive oxygen species (ROS)\textsuperscript{33, 34, 35}. ROS include free radicals such as superoxide anion (O\textsubscript{2} \textsuperscript{-}), hydroxyl radicals (\cdot OH) and nonradical hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). The excessive production of ROS may damage various intracellular macromolecules, leading to oxidative stress accompanied by loss of cell function and possibly apoptosis and/or necrosis\textsuperscript{36, 37, 38}. Our results showed that at low concentration of CHCl\textsubscript{3}-F there is a rapid elevation of the intracellular ROS level (Fig. 6). Furthermore, cell viability was significantly improved with the addition of catalase (Fig. 7). These results suggest that the CHCl\textsubscript{3}-F of \textit{Z. jujuba} extract induced apoptosis in HepG2 cells was related to the rapid increase of intracellular ROS, i.e., the increase in hydrogen peroxide when cells were treated at low CHCl\textsubscript{3}-F concentrations. Furthermore, recent studies have suggested that mitochondria play an important role in
apoptosis triggered by many stimuli such as oxidative stress and radial ray\textsuperscript{39, 40}. The increase of ROS was probably due to the affected mitochondria cycling dioxygen through the electron transport assembly, and by generating ROS by one-electron-transfer mitochondria could be a main target of nonspecific damage because of oxidative stress at the level of the outer and inner membrane\textsuperscript{41}. As a consequence of oxidative membrane damage, membrane potential and permeability-barrier function is impaired which leads to apoptosis. On the other hand, other studies suggested that the apoptosis of various cell types, such as human leukemia HL-60 cells and human leukemia K562 cells\textsuperscript{42, 43, 44}, were induced by ROS-independent mitochondrial dysfunction pathway. In this chapter, we also found the CHCl\textsubscript{3}-F decreased mitochondrial membrane potential at both low and high concentration in another pathway unrelated to ROS (Fig.8 and 9). Taken together, these results suggest that the CHCl\textsubscript{3}-F-induced apoptosis was, at low concentration, related to both a ROS-increase pathway and a ROS-independent mitochondrial dysfunction pathway, but at the higher concentration, apoptosis was only induced in ROS-independent
mitochondrial dysfunction pathway.

Another effect of CHCl$_2$-F observed in this chapter in HepG2 cells was anti-proliferation. In general, anti-proliferation is usually expressed in cell cycle arrest. The cell cycle transitions are controlled by cyclin-dependent kinase (CDKs). The p27$^{\text{Kip1}}$ is a member of Cip/Kip family of cyclin-dependent kinase inhibitor (CDKI). It plays a critical role in negative regulation of cell division \textit{in vivo}. Its ability to enforce G1 restriction point is derived from its inhibitory binding to cyclin E-cdk2 and other cyclin E-cdk2 complexes\textsuperscript{45, 46, 47).} p27$^{\text{Kip1}}$ binds to a wide variety of cyclin/CDK complexes including CDK2 and CDK4\textsuperscript{46, 48, 49)} inhibiting kinase activity\textsuperscript{50, 51) and blocking cell cycle\textsuperscript{52, 53, 54).} The over expression of p27$^{\text{Kip1}}$ protein in mammalian cells induces G$_1$ arrest in cell cycle. Furthermore, in the case of progression from G$_1$ to S, another apparent target is Rb protein. Rb is bound to the transcription factor E2F during G$_1$ but upon phosphorylation, E2F is released and cell cycle progresses to S phase\textsuperscript{55).} Many studies have reported that the accumulation of p27$^{\text{Kip1}}$ protein inhibits CDK2 activity followed by an increase in
hypophosphorylated levels of Rb protein. In this chapter, low concentration CHCl$_3$-F of Z. jujuba extract induced the hypophosphorylation of Rb protein and an accumulation of p27$^{\text{Kip1}}$ protein when compared with the control at 8 hrs (Fig. 10 and 12). These results were consistent with the G1 phase arrest in cell cycle analysis under the treatment with low concentrations of CHCl$_3$-F. On the other hand, some studies have shown that phosphorylation of p27$^{\text{Kip1}}$ results in elimination of p27$^{\text{Kip1}}$ from the cell, allowing cells to transit from G1 to S phase. The Rb protein was phosphorylated following cell cycle progress to S phase and finally dephosphorylated to hypophosphorylated Rb at G2/M phase. In this chapter, the high concentration of CHCl$_3$-F decreased the p27$^{\text{Kip1}}$ levels and generated the phosphorylation of p27$^{\text{Kip1}}$ in HepG2 cells (Fig. 12), and the hypophosphorylation of Rb protein (Fig. 10) was remained. This result suggests that the cell cycle progressed from G1 to S phase and was finally arrested at G2/M phase. However, the precise mechanism of the G2/M phase arrest at high concentration is still unclear. Additional studies are needed to clarify this mechanism in HepG2.
cells. Furthermore, other studies have reported that the increase of ROS levels, such as H$_2$O$_2$, may trigger signal transduction mechanisms that regulate cell growth, transformation, aging, and apoptosis $^{56, 57}$. These data suggested that apoptosis and ROS responses may be tied to p53-dependent regulation of cell cycle control and stress-activated pathways $^{57}$. In contrast, our results showed that the increase of ROS levels were not tied to the p27$^{Kip1}$ protein levels and the hypophosphorylation Rb protein levels (Fig. 11 and 13).

On the components in Z. jujuba, Lee et al.$^{22}$ identified eleven triterpenoic acids (colubrinic acid, alphitolic acid, 3-0-cis-p-coumaroylalphaltolic acid, 3-O-trans-p-coumaroylalphaltolic acid, 3-O-cis-p-coumaroylmaslinic acid, 3-O-trans-p-coumaroylmaslinic acid, betulinic acid, oleanolic acid, betulonic acid, oleanonic acid and zizyberenalic acid) using repeated column chromatography of EtOAc-soluble fraction of the methanol extract of Z. jujuba on silica gel followed by gel filtration on Sep-Pak C18 cartridge and preparative HPLC. Furthermore, they showed that
the lupine-type triterpenes, such as 3-O-cis p-coumaroylalphitolic acid, 3-O-trans-p-coumaroylalphitolic acid, betulinic acid and betulonic acid, had high cytotoxic activities against K562, B16, SK-MEL-2, LOX-IMVI and A549 tumor cell lines. The main and effective component(s) of the CHCl₃-F extract in this chapter is/are not clear but currently under investigation.

An interesting finding in this chapter was the differential effects induced in HepG2 cells by different concentrations of *Z. jujuba* extract. Some studies have shown such phenomenon, including Liu et al. (2006), who demonstrated that differences of cell cycle arrest in human breast cancer cells were induced by different drug concentrations. However, the reasons for these effects are still not clear.¹⁵⁸

Our results showed that *Z. jujuba* extract induced apoptosis and different cell cycle arrests in HepG2 cells, and therefore suggest that *Z. jujuba* may play an effective contribution in the search for anticancer treatment for hepatoma. However, the molecular basis of such effects needs further investigation.
5. Summary

The extract of *Z. jujuba* decreased the viability of the cells. Further extraction of the initial *Z. jujuba* extract with organic solvents revealed that the chloroform fraction (CHCl$_3$-F) was the most effective. Interestingly, the CHCl$_3$-F induced not only apoptosis but also G1 arrest at a low concentration (100 μg/ml) and G2/M arrest at a higher concentration (200 μg/ml) by cell cycle assay. Apoptosis, an increase in intracellular ROS level, a decline of mitochondrial membrane potential at low *Z. jujuba* concentrations, and a ROS-independent mitochondrial dysfunction pathway at high concentrations were all observed. CHCl$_3$-F-induced G1 arrest in HepG2 cells was associated with an increase in hypophosphorylation of Rb and \( p27^{Kip1} \), and a decrease of phosphorylated Rb. However, CHCl$_3$-F-induced G2/M arrest in HepG2 cells correlated with a decrease of the \( p27^{Kip1} \) levels and generation of the phosphorylation of \( p27^{Kip1} \), however the hypophosphorylation of Rb protein remained. Collectively, our findings suggest that the CHCl$_3$-F extract of *Z. jujuba* extract induced a concentration dependent effect on apoptosis.
and a differential cell cycle arrest in HepG2 cells.
CHAPTER II

Green tea extracts enhances the selective cytotoxic activity of *Zizyphus jujuba* extracts in HepG2 cells

1. Introduction

In Chapter I, we investigated the anticancer activity of the chloroform fractions from *Z. jujuba* extract (CHCl$_3$-F) and its underlining mechanisms of action in HepG2 cells, and found that the CHCl$_3$-F decreased the viability of HepG2 cells. Interestingly, the CHCl$_3$-F induced not only apoptosis but also G1 arrest at a low concentration (100 µg/ml) and G2/M arrest at a high concentration (200 µg/ml). We also showed that CHCl$_3$-F-induced G1 arrest in HepG2 cells was associated with an increase in hypophosphorylation of Rb and p27$^{Kip1}$, an inhibitor of cyclin-dependent kinase, and a decrease in phosphorylated Rb. However, CHCl$_3$-F-induced G2/M arrest in HepG2
cells correlated with a decrease in the p27\textsuperscript{Kip1} levels and the phosphorylation of p27\textsuperscript{Kip1}, but the hypophosphorylation of Rb protein still remained. Our findings suggested that the CHCl\textsubscript{3}-F of Z. jujuba might contribute to the antineoplastic activity of HepG2 cells, and might become a new plant component that could prevent or be used to treat cancer in the future.

\textit{Z. jujuba} has various biological activities and is traditionally used in oriental medicines. It is also used as jujuba tea which contains \textit{Z. jujuba} and green tea. Many people in China drink jujuba tea as opposed to tea alone and believe that the combination of \textit{Z. jujuba} with green tea (GTE) have synergistic effects that enhance immune function. GTE alone has been shown to prevent or inhibit cancer growth \textit{in vitro} and \textit{in vivo}\textsuperscript{59, 60}. However, the additive or synergistic effect of combining \textit{Z. jujuba} with the extracts of GTE on anticancer activity \textit{in vitro} or \textit{in vivo} has not been reported.

In this Chapter, we hypothesized that the combination of \textit{Z.
*jujuba* with GTE might influence HepG2 cells, and their effects would be apparent in the apoptotic and cell cycle inhibition pathway. Therefore, we used low concentrations (100 µg/ml) of *Z. jujuba* extract in combination with GTE, and investigated their interaction in HepG2 cells.

2. Materials and Methods

2.1. Materials

*Z. jujuba* extract and GTE were kindly donated by Sea Load Co. Ltd. (Fukui, Japan) and Taiyo Kagaku (Japan), respectively. 2’, 7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma–Aldrich Fine Chemical (Tokyo, Japan). Rb, p21\(^{\text{Waf1/Cip1}}\), p27\(^{\text{Kip1}}\), p53 and cyclin E antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). FBS was purchased from Equitech-Bio Inc. (Kerrville, Texas, USA). Other chemicals used in this study were special grade commercial products.
2.2. Cell culture

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS in a humidified incubator containing 5% CO₂ in air at 37°C for 3-4 days, then washed and cultured again in fresh medium at a concentration of 2.5×10⁵/ml in 35 mm plastic dishes overnight.

Rat hepatocytes were isolated from 10 week-old male Wistar rats anesthetized with diethyl ether by collagenase perfusion. The isolated hepatocytes were plated in 35 mm plastic dishes at a density of 2.5×10⁵ cells/ml in 2 ml Williams’ Medium E supplemented with 10% FBS, and were cultured in humidified atmosphere of 5% CO₂ and 95% air at 37°C overnight.

CHCl₃-F and GTE were dissolved in dimethyl sulfoxide (DMSO) and water respectively, then CHCl₃-F and GTE were diluted in the culture medium immediately before use (final DMSO concentration < 0.125%). In all the experiments, control cultures
comprised the medium, DMSO and the cells.

2.3. Fractionation of Z. jujuba extract

*Z. jujuba* extract was diluted at 1/20 with water, and the mixtures were extracted with chloroform. The fractions were evaporated to dryness *in vacuo*. Finally, the powder was dissolved in DMSO at a concentration of 200 µg/2.5 µl.

2.4. Cell viability

Cell viability was determined with the neutral red uptake assay, based on the lysosomal uptake of neutral red\(^{23}\). Following specified incubations with test agents, neutral red solution (0.25 mg/ml) was added to the cell cultures at a final concentration of 50 µg/ml. After incubation at 37°C for 2 hrs, cells were rinsed twice with a mixture of 1% (v/v) formaldehyde, 1% (v/v) calcium chloride, and 98% (v/v) distilled water. Subsequently, 1 ml of destaining buffer consisting of 1%
(v/v) acetic acid, 50% (v/v) ethanol, and 49% (v/v) distilled water was added to the cells, and the culture dishes were stood for 30 mins. Lysosomal uptake of neutral red was determined spectrophotometrically at 540 nm. Viability was expressed as \( \frac{A_{540 \text{-treated cells}}}{A_{540 \text{ of appropriate control}}} \times 100\% \) after correction for background absorbance (100% cytotoxicity)\(^{61}\).

2.5. cell cycle assay

Cell cycle distribution was analyzed by laser scanning cytometer (Olympus LSC 101) using PI staining. Briefly, after designated treatments, cells were washed twice in PBS and incubated with freshly prepared PI-stained buffer (0.1% Triton X-100 in PBS, 20 µg/ml PI, 200 µg/ml RNase) for 1 hr at 37°C in the dark\(^{27}\).

2.6. DNA synthesis assay

HepG2 cells were cultured in the presence of CHCl\(_3\)-F or GTE
as well as bromodeoxyuridine (BrdU) (0.1 mM) for 24 hr. Cells were washed twice with PBS and added to a mixture of 95% ethanol and 5% acetic acid at 4°C for 30 mins, then washed with PBS. Subsequently, 1 ml of formamide was added to the cells, and the culture dishes were cultured for 1 hr at 70°C in the dark, and then washed with PBS. After washing with PBS 3 times for 2 mins, the culture dishes were incubated with 1:50 dilute anti-BrdU antibody at room temperature for 1 hr, and then washed with PBS for 2 mins, and incubated with 1:200 diluted biotinylated mouse IgG for 1 hr at room temperature. The culture dishes were washed with PBS once more for 2 mins and incubated with 1:400 dilute horse radish peroxidase-coupled streptavidin for 1 hr at room temperature. After washing with PBS, the color reaction was developed with 3,3'-Diaminobenzidine (DAB). The number of BrdU positive nuclei was counted in three microscopic fields in each specimen.

2.7. ROS assay
DCFH-DA (8 μM) was added at the last 15 mins of CHCl₃-F or (and) GTE treatment. Cells were washed with PBS twice and resuspended in Hanks solution. Fluorescence intensity was measured using a micro-plate reading fluoroscan plate-reader (Wallac 1420 ArVOsx, Amersham Pharmacia Biotech) with the excitation wavelength at 485 nm and the emission wavelength at 535 nm. The amount of intracellular ROS was calculated from a standard curve derived from 2’, 7’ –dichlorofluorescein (DCF). Protein concentration was measured by the Bradford method ³¹).

2.8. Western blot analysis of Rb, p27Kip¹, p21Waf1/Cip1, p53 and cyclin E

Cells were harvested after 8 hrs and were washed twice in PBS. Then, the cells were dissolved for 30 mins with lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.2), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM NaF, 20 µg/mL aprotinin, 50 µg/ml leupeptin, 10 µg/ml Pepstatin A and 100 µg/ml
phenylmethylsulfonyl fluoride). Finally, the solution was centrifuged at 2000 ×g for 20 mins at 4°C. The supernatant was collected and protein concentrations were determined by Bradford method. Equal amounts of protein were fractionated on 10% SDS-PAGE gels and transferred to 0.45 µm PVDF (Hybond; Amersham Pharmacia Biotech). Efficiency of transfer and equal loading of protein were confirmed by staining membranes with Coomassie Brilliant blue (0.1%) in 5% acetic acid. After overnight blocking in 0.1% Tween-20 and 5% non-fat dry milk in PBS, the blots were incubated with the respective antibodies for 1 hr at room temperature. The antibodies included anti-Rb, anti- p21\textsuperscript{Waf1/Cip1}, anti- p27\textsuperscript{Kip1}, anti-p53 and anti-cyclin E. After washing, the membrane was reincubated with 1:750 diluted biotinylated mouse IgG or rabbit IgG for 1 hr at room temperature. The membrane was washed several times, and incubated with 1:750 diluted horse radish peroxidase-coupled streptavidin for 1 hr at room temperature. After several washing steps, the color reaction
was developed with DAB. Densitometry analysis of the protein bands was performed with the software Scion Image.

2.9. Statistical analysis

Data are represented as means ± S.D (standard deviation from the mean). The significance of difference in assay values was evaluated with ANOVA followed by Dunnett’s multiple comparison post hoc tests. A $p$ value of less than 0.05 was considered significant. The observed interaction was confirmed by isobolographic analysis of data derived from the neutral red uptake assay analysis at 24 h.

3. Results

3.1. Effect of GTE on cell viability in HepG2 cells

To determine the adequate concentration of GTE, we examined the effect of GTE with different concentrations in HepG2 cells on cell viability. As shown in Fig. 1, cell viability decreased with 60 µg/ml,
but not with 30 µg/ml of GTE. Therefore, we used 30 µg/ml of GTE for subsequent experiments as this concentration of GTE that did not affect cell viability.

3.2. Effect of CHCl₃-F and GTE on cell viability in HepG2 cells

To investigate whether the combination of CHCl₃-F with GTE induces cytotoxicity in HepG2 cells, we examined the cell viability by neutral red method. As shown in Fig. 2, cell viability was decreased in the presence of CHCl₃-F and GTE.

3.3. Effect of CHCl₃-F and GTE on cell viability in rat hepatocytes

To investigate the effect of CHCl₃-F and GTE in normal cell lines, we examined the effect of CHCl₃-F and GTE on cell viability in rat hepatocytes. As shown in Fig. 3, no effect on cell viability was observed in rat hepatocytes after treatment of CHCl₃-F and/or GTE.
3.4. Effect of CHCl₃-F and GTE on intracellular ROS levels

We have shown that the intracellular ROS level increased rapidly when cells were incubated with 100 µg/ml of CHCl₃-F, and that the increase in ROS was associated with apoptosis. Therefore, we examined the effects of CHCl₃-F and GTE on intracellular ROS levels to see whether ROS is related to the enhanced cytotoxicity with CHCl₃-F and GTE. There was no enhanced effect on ROS levels when cells were treated with both CHCl₃-F and GTE (Fig. 4).

3.5. Effect of CHCl₃-F and GTE on cell cycle in HepG2 cells

We examined the effect of CHCl₃-F and GTE on cell cycle in HepG2 cells. As shown in Fig. 5, significant increase in G1, but not in sub-G1 cell cycle region, was observed when cells were treated with the combined CHCl₃-F and GTE.

3.6. Effect of CHCl₃-F and GTE on DNA synthesis in HepG2 cells
To confirm the G1 arrest by the combination of CHCl₃-F and GTE, we investigated the effect on the DNA synthesis with the treatment of CHCl₃-F and GTE in HepG2 cells. As shown in Fig. 6, an enhanced effect on DNA synthesis was observed.

3.7. Effect of CHCl₃-F and GTE on Rb protein in HepG2 cells

To investigate the mechanism of G1 arrest in CHCl₃-F and GTE-treated in HepG2 cells, we examined Rb protein, which acts as a tumor suppressor by providing a cell cycle checkpoint between the G1 and S phase. As shown in Fig. 7, the treatment with CHCl₃-F and GTE decreased hypophosphorylation Rb protein levels and increased the phosphorylation of Rb protein levels after 8 hrs of culture. This result suggests that the enhanced inhibition of cell cycle arrest is caused by the suppression of phosphorylation of Rb protein.

3.8. Effect of CHCl₃-F and GTE on p27Kip1 in HepG2 cells
In an earlier study we showed that p27\textsuperscript{Kip1} protein increased in cells incubated with 100 µg/ml of CHCl\textsubscript{3}-F. Hypophosphorylated Rb was induced, followed by an arrest at the G1 cell cycle phase. Therefore, we examined the effect of the combination of CHCl\textsubscript{3}-F and GTE on the status of the phosphorylation of p27\textsuperscript{Kip1}. As shown in Fig. 8, the p27\textsuperscript{Kip1} levels decreased when the cells were treated with CHCl\textsubscript{3}-F and GTE for 8 hrs.

3.9. Effect of CHCl\textsubscript{3}-F and GTE on p21\textsuperscript{Waf1/Cip1} in HepG2 cells

To investigate the cause of cell cycle arrest by CHCl\textsubscript{3}-F and GTE in HepG2 cells, we measured the p21\textsuperscript{Waf1/Cip1} protein, another protein associated with the phosphorylation of Rb in G1 phase. As shown in Fig. 9, the p21\textsuperscript{Waf1/Cip1} protein levels increased when the cells were treated with CHCl\textsubscript{3}-F and GTE for 8 hrs.

3.10. Effect of CHCl\textsubscript{3}-F and GTE on p53 in HepG2 cells
To investigate the up-stream regulation of $p21^{\text{Waf1/Cip1}}$ protein, we measured p53 protein, a tumor suppressor protein associated with the expression of $p21^{\text{Waf1/Cip1}}$. As shown in Fig. 10, p53 protein levels increased after the cells were treated with CHCl$_3$-F and GTE for 8 hrs.

3.11. Effect of CHCl$_3$-F and GTE on cyclin E in HepG2 cells

Cyclin E is another checkpoint protein in G1 cell cycle phase arrest. To investigate the changes in cyclin E, cells were treated with CHCl$_3$-F and GTE in HepG2 cells for 8 hrs. As shown in Fig. 11, the cyclin E levels decreased with the treatment of GTE alone or the combination of CHCl$_3$-F and GTE.
Fig. 1. Effect of GTE on cell viability in HepG2 cells

Cells were cultured in Dulbecco's modified Eagle's medium with or without various concentration of GTE. Cell viability was measured 24 hrs later by neutral red method as described in Materials and Methods. Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments. *p<0.05.
Fig. 2. Effect of CHCl₃-F and GTE on cell viability in HepG2 cells

Cells were cultured in Dulbecco's modified Eagle's medium with or without CHCl₃-F and GTE (30 µg/ml). Cell viability was measured 24 hrs later by neutral red method. Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments. Data not sharing common alphabet are significantly different (p<0.01).
Fig. 3. Effect of CHCl₃-F and GTE on viability of rat hepatocytes

The isolated hepatocytes were cultured in Williams’ Medium E with or without CHCl₃-F and GTE (30 µg/ml). Cell viability was measured 24 hrs later by neutral red method as described in Materials and Methods. Results are representative of three separate determinations. Each bar is the mean (± S.D.) from three experiments.
Fig. 4. Effect of CHCl$_3$-F and GTE on intracellular ROS levels in HepG2 cells

The intracellular ROS levels were measured by the DCFH-DA method. DCF fluorescence intensity of cells was measured at 15, 30 and 60 mins after treatment with GTE, CHCl$_3$-F, or CHCl$_3$-F and GTE. Data are presented as means ± S.D. **p<0.01 compared to the control group at the same time points.
Fig. 5. Effect of CHCl$_3$-F and GTE on cell cycle in HepG2 cells

Cells were incubated with GTE, CHCl$_3$-F, or CHCl$_3$-F and GTE for 24 hrs, washed twice in PBS, and incubated with prepared PI-stained buffer for 1 hr at 37°C in dark. The stained cells were analyzed by laser scanning cytometer.

Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments. *$p<0.05$, **$p<0.01$
### Fig. 6. Effect of CHCl₃-F and GTE on DNA synthesis in HepG2 cells

Cells were incubated with GTE, CHCl₃-F, or CHCl₃-F and GTE for 24 hrs. DNA synthesis was measured by the percentage of BrdU positive cells. Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments. Data not sharing common alphabet are significantly different (p<0.01).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA Synthesis (% of BrdU positive cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
</tr>
<tr>
<td>GTE (30 µg/ml)</td>
<td>30</td>
</tr>
<tr>
<td>100 CHCl₃-F (µg/ml)</td>
<td>b</td>
</tr>
<tr>
<td>100+GTE CHCl₃-F (µg/ml)</td>
<td>c</td>
</tr>
</tbody>
</table>

Data not sharing common alphabets are significantly different (p<0.01).
Fig. 7. Effect of CHCl₃-F and GTE on Rb protein in HepG2 cells

Cells were incubated with GTE, CHCl₃-F, or CHCl₃-F and GTE for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 8. Effect of CHCl₃-F and GTE on p27^Kip₁ protein in HepG2 cells

Cells were incubated with GTE, CHCl₃-F, or CHCl₃-F and GTE for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 9. Effect of CHCl₃-F and GTE on p21<sub>Waf1/Cip1</sub> protein in HepG2 cells

Cells were incubated with GTE, CHCl₃-F, or CHCl₃-F and GTE for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 10. Effect of CHCl₃-F and GTE on p53 protein in HepG2 cells

Cells were incubated with GTE, CHCl₃-F, or CHCl₃-F and GTE for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 11. Effect of CHCl₃-F and GTE on cyclin E in HepG2 cells

Cells were incubated with GTE, CHCl₃-F, or CHCl₃-F and GTE for 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Data shown are representative of at least three independent experiments.
Fig. 12. Proposed mechanism of action of CHCl₃-F alone and CHCl₃-F with GTE on G1 arrest in HepG2 cells.
4. Discussion

The results obtained in this chapter demonstrate for the first time, to the best of our knowledge, that GTE enhances the cytotoxic activity of CHCl₃-F in HepG2 cells. The cytotoxic activity of CHCl₃-F was enhanced after combining with low concentrations of GTE which initially had no affect on HepG2 cell viability. Furthermore, in rat hepatocytes used as a normal cell line, there was no effect on cell viability when the cells were treated with CHCl₃-F, GTE or the combination of CHCl₃-F and GTE. Our findings show that combining CHCl₃-F and GTE can enhance their individual neoplastic toxicity, providing a new approach to the design of chemotherapy strategies.

In chapter I, we have shown that the CHCl₃-F of Z. jujuba extract produced ROS resulting in apoptosis in HepG2 cells. Therefore, we hypothesized that a possible synergistic decrease in cell viability, by combining CHCl₃-F of Z. jujuba extract with GTE, could be the result of the production of ROS levels. However, we showed that
combining CHCl$_3$-F and GTE did not affect ROS levels (Fig. 3), nor increase apoptosis in HepG2 cells. We also have shown that CHCl$_3$-F induced not only apoptosis but also cell cycle arrest at the G1 phase in HepG2 cells. The present results show an enhanced effect in G1 arrest and a decrease in DNA synthesis when cells were treated with CHCl$_3$-F and GTE (Fig. 5 and 6), suggesting that the cytotoxic activities with the combined CHCl$_3$-F and GTE were related to growth inhibition but not to apoptosis.

Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have acquired damage to genes that directly regulate their cell cycles. In the cell cycle, the period from late G1 to the S phase is the most important for cell proliferation. The cell cycle progression from G1 to S-phase requires phosphorylation of the retinoblastoma tumor suppressor protein, Rb, a member of the pocket protein family and one of the checkpoint of G1 arrest, by the cyclin D1-cdk4/6 and cyclin E-cdk2 complexes $^{62}$. Phosphorylation of Rb in early G1 by
cyclin D1/cdk4/6 triggers a cascade of events that begins with the dissociation of E2F from Rb and the activation of transcription of cyclin E by E2F, and culminates with the stimulation by E2F of its own transcription and assembly of cyclin E with its catalytic partner Cdk2. The cyclin E-cdk2 complexes promote further phosphorylation of Rb and the release of E2F, thus establishing a positive feedback loop that accelerates the irreversible progression through late G1\(^{63}\).

To prevent unrestrained proliferation, there are a number of cyclin-dependent kinase inhibitors (CDKIs) which bind with specific CdkS, preventing the CdkS from binding to cyclin and hence blocking a cascade of events which ultimately lead to cell proliferation. p21\(^{\text{Waf1/Cip1}}\) and p27\(^{\text{Kip1}}\) belong to the family of CDKIs\(^ {64}\), whose expression regulates the G1 phase CdkS\(^ {63}\). We have shown that CHCl\(_3\)-F- induced G1 arrest in HepG2 cells was associated with an increase in hypophosphorylation of Rb and a decrease of phosphorylated Rb, and these changes depended on the up-regulation
of $p27^{Kip1}$ protein. Therefore, we hypothesized that the effect of combining CHCl$_3$-F of $Z$. jujuba extract with GTE was also associated with an up-regulation of $p27^{Kip1}$ protein and induced hypophosphorylation of Rb. However, our results showed that the hypophosphorylation of Rb did not cause any up-regulation of $p27^{Kip1}$ protein but up-regulation of $p21^{Waf1/Cip1}$ protein instead in the presence of CHCl$_3$-F and GTE (Fig. 8 and 9).

The tumor suppressor protein p53 is also the major mediator of the checkpoint-induced arrest in the G1 phase of the cell cycle$^{65}$. A variety of cellular stresses including DNA damage, hypoxia, nucleotide depletion, viral infection, and cytokine-activated signaling pathways transiently stabilize the p53 protein, causing it to accumulate in the nucleus, and activating it as a transcription factor$^{66}$. p53 induces cell cycle arrest, through the Cdk inhibitor $p21^{Waf1/Cip1}$, preventing the replication of damaged DNA. $p21^{Waf1/Cip1}$ interacts with different cyclin/Cdk complexes and other regulators of transcription
and signal transduction, exerting broad effects on cell survival, gene expression and morphology \(^{67}\). \(p21^{\text{Waf1/Cip1}}\) effects are partially mediated by Rb, which is inactivated in proliferating cells through phosphorylation by Cdk2 and Cdk4/6, both of which are inhibited by \(p21^{\text{Waf1/Cip1}}\). As a result, \(p21^{\text{Waf1/Cip1}}\) induction leads to Rb dephosphorylation and activation, with ensuing G1 arrest \(^{68}\). Our results suggested that in the presence of the CHCl\(_3\)-F and GTE, p53 protein levels were increased and induced the Cdk inhibitor \(p21^{\text{Waf1/Cip1}}\) (Fig. 9 and 10), Rb dephosphorylation and activation. Finally, as a result, there was a G1 phase cell cycle arrest (Fig. 8). However, for CHCl\(_3\)-F alone, only the \(p27^{\text{Kip1}}\) protein levels were increased, but not p53 protein or the \(p21^{\text{Waf1/Cip1}}\) protein.

Cyclin E, whose catalytic partner is Cdk2, is another rate-limiting regulator of the G1 phase of the cell cycle, and increased expression of cyclin E has been found in several types of tumors \(^{69}\). Some studies suggested that decreasing cyclin E level induced the cell
cycle arrest at G1 phase\textsuperscript{70, 71}. In this chapter, the treatment of CHCl\textsubscript{3}-F alone did not decrease the expression of cyclin E levels. However, treatment with GTE alone or combination of CHCl\textsubscript{3}-F and GTE decreased the expression of cyclin E levels (Fig. 11), but the concentration of 30 µg/ml of GTE had no effect on the p53 level and the cell viability in HepG2 cells. These results suggested that both the increase in p53 and p21\textsuperscript{Waf1/Cip1} levels and the decrease in cyclin E levels were related to the effect of combination of CHCl\textsubscript{3}-F and GTE on G1 arrest.

GTE contains a variety of polyphenols, the most important class of polyphenols called catechins. The major catechins found in GTE are called epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). Of these four catechins, EGCG is the most abundant and has been shown to be the most important cancer inhibiting chemical found in GTE\textsuperscript{60, 72}. It has been reported that the combination of green tea polyphenols
and grape polyphenols had synergistic anticancer effects in HeLa cells, and that the polyphenolic mixture was more effective than those from the individual sources\textsuperscript{73).} Suganuma \textit{et al.} reported that whole green tea is a more reasonable mixture of tea polyphenols for cancer prevention in humans than EGCG alone and that it is even more effective when it is used in combination with other cancer preventives\textsuperscript{74).} We examined the effects of these four catechins alone or the interaction between these four catechins in combination with \textit{Z. jujuba.} However, there was no effective interaction in HepG2 (data not shown). Hence, we suggest that the active components in GTE are not the individual catechins.

Collectively, we have found that GTE enhances the cytotoxic effect of CHCl\textsubscript{3}-F in HepG2 cells causing cell growth inhibition, and the involved mechanisms may be via two pathways. One pathway involved increased p53 and p21\textsuperscript{Waf1/Cip1} proteins. The increased p21\textsuperscript{Waf1/Cip1} bound with Cdk2 and prevented the Cdk2 from binding to
cyclinE, resulting in a G1 phase arrest. Also, decreasing the cyclin E levels directly might have led to a decrease in the cyclin E-cdk2 complex levels and caused G1 arrest (Fig. 12). Based on these findings, we suggest that the combination of CHCl₃-F and GTE is more effective than the CHCl₃-F alone in the growth inhibition of HepG2 cells and the mechanisms of G1 arrest by CHCl₃-F and GTE are different from that with the treatment of CHCl₃-F alone.
5. Summary

GTE enhanced the effect of CHCl₃-F on cell viability in HepG2 cells, without cytotoxicity in rat hepatocytes, which was used as a normal cell model. Furthermore, combination of CHCl₃-F and GTE caused an effect on G1 phase arrest but not on apoptosis. Interestingly, the mechanism of the G1 arrest was associated, not with an increase in p27^Kip1 levels and the hypophosphorylation of Rb, which are pathways by CHCl₃-F on G1 arrest in HepG2 cells, but with increases in p53 and p21^{Waf1/Cip1} levels, and a decrease in cyclin E levels. Our findings suggest that combining CHCl₃-F and GTE produces an enhanced cell growth inhibition effect, and that the resultant G1 arrest was caused via a different mechanism as that of CHCl₃-F treatment alone.
Combination of *Zizyphus jujuba* and green tea extracts exert excellent cytotoxic activity in HepG2 cells via reducing the expression of APRIL

1. Introduction

Cytokines regulate cellular proliferation and differentiation by binding to their specific receptors on target cells. Tumor necrosis factor (TNF) is the prototypic member of a family of cytokines playing an important role in immune regulation and cancer. A proliferation-inducing ligand (APRIL), a novel member of the TNF family, is reported to stimulate tumor cell growth via signaling in an autocrine and/or paracrine mode\(^ {75}\). The expressions of APRIL mRNA and protein were high in various tumor cell lines and tissues, but almost undetectable in various normal tissues\(^ {76}\). It is possible that APRIL may play a role in tumorigenesis. Furthermore, it has been demonstrated that the expression of APRIL protein was detected in
human hepatocellular carcinoma cell, where it can promote neovascularization, as estimated by the human umbilical vein endothelial cell tube formation \(^{77}\). We have shown that combining CHCl\(_3\)-F and GTE caused an effect on G1 phase arrest but not on apoptosis. Interestingly, the mechanism of the G1 arrest is associated, not with an increase in p27\(^{\text{Kip1}}\) levels and the hypophosphorylation of Rb, which are pathways of CHCl\(_3\)-F on G1 arrest in HepG2 cells, but with increases in p53 and p21\(^{\text{Waf1/Cip1}}\) levels, and a decrease in cyclin E levels. However, how these factors are induced in HepG2 cells is still unclear.

Knowledge of the molecular mechanisms governing malignant transformation brings new opportunities for therapeutic intervention against cancer using novel approaches. Therefore, in order to demonstrate the particular molecular mechanisms of the growth inhibitory effects of CHCl\(_3\)-F and GTE mixture in HepG2 cells, especially elucidate the relationship between APRIL and growth related factors such as p27\(^{\text{Kip1}}\), p53 and p21\(^{\text{Waf1/Cip1}}\), we examined the time-dependent expression of APRIL, p27\(^{\text{Kip1}}\), p53 and p21\(^{\text{Waf1/Cip1}}\) by
combination CHCl₃-F with GTE in HepG2 cells. The chemotherapeutic potential of treatment of hepatocellular carcinoma with combining CHCl₃-F and GTE is discussed below.

2. Materials and Methods

2.1. Materials

APRIL antibody was purchased from ψProSci. Inc. (Poway, CA, USA). p27^Kip1, p53 and p21^Waf1/Cip1 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Other chemicals used in this study were special grade commercial products.

2.2. Cell culture

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS in a humidified incubator containing 5% CO₂ in air at 37°C for 3-4 days, then washed and cultured again in fresh medium at a concentration of 2.5×10⁵/ml in 35 mm plastic dishes overnight.

Rat hepatocytes were isolated from 10 week-old male Wistar
rats anesthetized with diethyl ether by collagenase perfusion. The isolated hepatocytes were plated in 35 mm plastic dishes at a density of 2.5×10^5 cells/ml in 2 ml Williams’ Medium E supplemented with 10% FBS, and were cultured in humidified atmosphere of 5% CO₂ and 95% air at 37°C overnight.

CHCl₃-F and GTE were dissolved in DMSO and water respectively. These were then diluted in the culture medium immediately before use (final DMSO concentration < 0.125%). Final concentration of CHCl₃-F and GTE were 100 µg/ml and 30 µg/ml, respectively. In all the experiments, control cultures comprised the medium, DMSO and the cells.

2.3. Fractionation of Z. jujuba extract

Z. jujuba extract was diluted at 1/20 with water, and the mixtures were extracted with chloroform. The fractions were evaporated to dryness in vacuo. Finally, the powder was dissolved in DMSO at a concentration of 200 µg/2.5 µl.
2.4. Western blot analysis of APRIL, $p27^{\text{Kip1}}$, p53 and $p21^{\text{Waf1/Cip1}}$

Cells were harvested after 1, 2, 4 and 8 hrs and were washed twice in PBS. Then, the cells were dissolved for 30 mins with lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.2), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM NaF, 20 µg/mL aprotinin, 50 µg/ml leupeptin, 10 µg/ml Pepstatin A and 100 µg/ml phenylmethylsulfonyl fluoride). Finally, the solution was centrifuged at 2000 ×g for 20 mins at 4°C. The supernatant was collected and protein concentrations were determined by Bradford method. Equal amounts of protein were fractionated on 10% SDS-PAGE gels and transferred to 0.45 µm PVDF (Hybond; Amersham Pharmacia Biotech). Efficiency of transfer and equal loading of protein were confirmed by staining membranes with Coomassie Brilliant blue (0.1%) in 5% acetic acid. After overnight blocking in 0.1% Tween-20 and 5% non-fat dry milk in PBS, the blots were incubated with the respective antibodies for 1 hour at room temperature. The antibodies included anti-APRIL, anti-$p27^{\text{Kip1}}$, anti-p53 and anti-$p21^{\text{Waf1/Cip1}}$. After washing, the membrane was
reincubated with 1:750 diluted biotinylated mouse IgG or rabbit IgG for 1 hour at room temperature. The membrane was washed several times, and incubated with 1:750 diluted horse radish peroxidase-coupled streptavidin for 1 hour at room temperature. After several washing steps, the color reaction was developed with DAB. Densitometry analysis of the protein bands was performed with the software Scion Image (Scion Corporation).

2.5. Cell viability

Cell viability was determined with the neutral red uptake assay, based on the lysosomal uptake of neutral red \(^ {23} \). Following specified incubations with test agents, neutral red solution (0.25 mg/ml) was added to the cell cultures at a final concentration of 50 µg/ml. After incubation at 37°C for 2 hrs, cells were rinsed twice with a mixture of 1% (v/v) formaldehyde, 1% (v/v) calcium chloride, and 98% (v/v) distilled water. Subsequently, 1 ml of destaining buffer consisting of 1% (v/v) acetic acid, 50% (v/v) ethanol, and 49% (v/v) distilled water was added to the cells, and the culture dishes were left for 30 mins.
Lysosomal uptake of neutral red was determined spectrophotometrically at 540 nm. Viability was expressed as \( \frac{A_{540\text{-treated cells}}}{A_{540\text{ of appropriate control}}} \times 100\% \) after correction for background absorbance (100% cytotoxicity)\(^{61}\).

2.6. Cell cycle assay

Cell cycle distribution was analyzed by laser scanning cytometer (Olympus LSC 101) using PI staining. Briefly, after designated treatments, cells were washed twice in PBS and incubated with freshly prepared PI-stained buffer (0.1% Triton X-100 in PBS, 20 µg/ml PI, 200 µg/ml RNase) for 1 hour at 37°C in the dark.

2.7. Statistical analysis

Data are represented as means ± S.D (standard deviation from the mean). The significance of difference in assay values was evaluated with ANOVA followed by Dunnett’s multiple comparison post hoc tests. A \( p \) value of less than 0.05 was considered significant.
3. Results

3.1. Effect of CHCl₃-F and GTE on the expression of APRIL in HepG2 cells

To investigate whether the combination of CHCl₃-F with GTE induce APRIL in HepG2 cells, we examined the time-dependent expression of APRIL. HepG2 cells were cultured with or without GTE, CHCl₃-F or both together, for 1, 2, 4 and 8 hrs and the expression levels of APRIL were examined by Western blot analysis. As shown in Fig. 1, APRIL was expressed in HepG2 cells after 4 hrs of the incubation, and the addition of the mixture of CHCl₃-F and GTE, but not the addition of CHCl₃-F alone, reduced the expression levels of APRIL from 4 hrs of the incubation.

3.2. Effect of CHCl₃-F and GTE on cell viability after incubation for various times in HepG2 cells

APRIL was expressed in HepG2 cells after incubation for 4 hrs. To investigate whether APRIL is involved in cell proliferation in HepG2 cells, and whether the effect of anticancer activity of
combining CHCl$_3$-F with GTE or CHCl$_3$-F alone is involved in the expression of APRIL, CHCl$_3$-F or CHCl$_3$-F with GTE were added to HepG2 cell culture after 0, 1, 2, 4 and 8 hrs of the incubation, and cell viability was examined after incubation for 24 hrs. As shown in Fig. 2, CHCl$_3$-F decreased cell viability at all the indicated times. However, the addition of the CHCl$_3$-F and GTE mixture did not decrease cell viability after 8 hrs of the incubation. It shows that the addition of CHCl$_3$-F and GTE mixture to the APRIL expressed cells did not decrease cell viability.

3.3. Effect of CHCl$_3$-F and GTE on cell cycle after various hours of incubation in HepG2 cells

To further investigate whether APRIL is involved in cell proliferation in HepG2 cells, and whether the effect of G1 arrest by CHCl$_3$-F and GTE mixture or CHCl$_3$-F alone is involved in the expression of APRIL, we examined the changes in cell cycle dynamics. GTE, CHCl$_3$-F or CHCl$_3$-F with GTE was added to HepG2 cell cultures after 0 and 8 hrs of incubation. At the end of 24-hrs incubation, the cell
cycle distribution was analyzed. As shown in Fig. 3, the addition of CHCl$_3$-F alone increased the cell numbers in the G1 cell cycle region, and the addition of CHCl$_3$-F and GTE mixture caused a further increase. However, G1 arrest was not induced in HepG2 cells incubated 8 hrs before the addition of combined CHCl$_3$-F and GTE.

3.4. Effect of CHCl$_3$-F and GTE on p27$^{\text{Kip1}}$, p53 and p21$^{\text{Waf1/Cip1}}$ in HepG2 cells

We showed in a previous study that the mechanism of G1 arrest was associated with an increase in p27$^{\text{Kip1}}$ levels at 8 hrs of incubation with CHCl$_3$-F alone, and with an increase in p53 and p21$^{\text{Waf1/Cip1}}$ levels at 8 hrs of incubation with combination CHCl$_3$-F with GTE. To investigate whether APRIL is associated with these factors which control cell cycle progression in G1 phases, we examined the time-dependent expression of p27$^{\text{Kip1}}$, p53 and p21$^{\text{Waf1/Cip1}}$ by Western blotting. As shown in Fig. 4, the p27$^{\text{Kip1}}$ levels were increased only by CHCl$_3$-F alone after 2 hrs of the incubation. Only the CHCl$_3$-F and GTE mixture increased p53 levels significantly at 4 hrs of incubation,
and p21<sup>Waf1/Cip1</sup> levels at 8 hrs of incubation.

3.5. Selective Inhibition of CHCl<sub>3</sub>-F and GTE Mixture

To demonstrate the selective inhibition of the CHCl<sub>3</sub>-F and GTE mixture, we examined the effect of the mixture in rat hepatocytes, as a normal cell model, and in HepG2 cells. As shown in Table 1, the combination of CHCl<sub>3</sub>-F with GTE did not affect cell viability in rat hepatocytes, but in HepG2 cells.
Fig. 1. Effect of CHCl₃-F and GTE on the expression of APRIL in HepG2 cells

Cells were incubated with or without GTE, CHCl₃-F, or CHCl₃-F and GTE mixture for 0, 1, 2, 4 and 8 hrs. Cell lysis and Western blotting were performed as described in Materials and Methods. Inner graphs represent the densitometric values quantified by densitometer. Data shown are representative of at least three independent experiments.
Fig. 2. Effect of CHCl₃-F and GTE on cell viability after incubation for various times in HepG2 cells

GTE, CHCl₃-F or CHCl₃-F and GTE mixture were added to HepG2 cells culture after 0, 1, 2, 4 and 8 hrs of the incubation, and cell viability examined at the end of incubation for 24 hrs by neutral red method. Results are representative of three separate determinations. Each bar is the mean (± S.D.) of three experiments compared with control (**p<0.01).
**Fig. 3.** Effect of CHCl₃-F and GTE on cell cycle after incubation for various times in HepG2 cells

GTE, CHCl₃-F or CHCl₃-F and GTE mixture were added to HepG2 cells cultured for 0 and 8 hrs. After 24 hrs of the incubation, the cells were collected as described in Materials and Methods. Cell cycle distribution was analyzed by laser scanning cytometer. Results are representative of three separate determinations. Data not sharing common alphabet are significantly different (p<0.05).
Fig. 4. Effect of CHCl₃-F and GTE on p27^Kip1 and p53 in HepG2 cells

The cells were treated with or without GTE, CHCl₃-F or CHCl₃-F and GTE mixture for 1, 2, 4 and 8 hrs, and cell lysis and Western blotting were performed as described in Materials and Methods. Inner graphs represent the densitometric values quantified by densitometer. Data shown are representative of at least three independent experiments.
Table 1. Selective inhibition of combined CHCl₃-F and GTE on cell viability

<table>
<thead>
<tr>
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<th>Rat hepatocytes (% of control)</th>
<th>HepG2 cells (% of control)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0±2.01</td>
<td>100.0±0.32</td>
</tr>
<tr>
<td>CHCl₃-F+GTE</td>
<td>97.8±3.08</td>
<td>60.3±1.27**</td>
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Values are means ± S. D. (n = 4). **P < 0.01

CHCl₃-F and GTE mixture were added to rat hepatocytes and HepG2 cells.

Cell viability was measured 24 hrs later by neutral red method as described in Materials and Methods.
4. Discussion

We showed that the APRIL was expressed in HepG2 cells after 4 hrs of incubation in vitro. Furthermore, addition of a mixture of CHCl_3-F and GTE, but not CHCl_3-F alone, reduced the expression of APRIL in HepG2 cells (Fig. 1). Our results also showed that HepG2 cell viability was decreased in the presence of the mixture of CHCl_3-F and GTE or CHCl_3-F alone. However, CHCl_3-F and GTE mixture did not decrease cell viability after 8 hrs of incubation (Fig. 2). Furthermore, the CHCl_3-F and GTE mixture or CHCl_3-F alone caused a G1 arrest, with CHCl_3-F and GTE mixture having a stronger effect than that of CHCl_3-F alone. Addition of CHCl_3-F and GTE mixture to the 8 hrs incubation HepG2 cells did not induce an arrest of G1 phase (Fig. 3).

A previous study showed that *Blumea balsamifera*, a medicinal plant, decreased cell viability via reducing the expression of APRIL in HepG2 cells. When CHCl_3-F was added at 8 hrs, the decrease in cell viability was maintained. Theses results suggest that APRIL may be playing an important role in cell proliferation but there may be
other factors also involved in cell proliferation in HepG2 cells.

APRIL is practically undetectable in normal tissues. However, it is strongly up-regulated in many tumor cells in vivo and in vitro and stimulates tumor cell growth. APRIL can be produced by tumor cells and thus can act in an autocrine manner, or by accessory cells to act in a paracrine manner. APRIL can induce both cell proliferation and death of malignant tumors. Some studies have shown that the mechanism of cell death could be caused by activated NF-κB and c-jun N-terminal kinase (JNK), or down-regulated Bcl-2 and Bcl-xL and up-regulated Bax in lymphoid malignancies. APRIL was also found to up-regulate c-Myc (an inducer of cell proliferation), to down-regulate p53 and to increase Bcl-6, an inhibitor of B-cell differentiation. Although APRIL is an important ligand of cell proliferation in lymphoid malignancies, not many studies have shown its mechanism in solid tumor cells, especially in HepG2 cells. Okano et al. suggested that APRIL induced proliferation in hepatocellular carcinoma, and functional expression of APRIL might contribute to neovascularization via upregulation of Vascular endothelial growth
factor (VEGF) in hepatocellular carcinoma \cite{77}. However, the mechanism of inducing cell proliferation is still unclear. The relationship between APRIL and downstream signaling of cell cycle regulating factors have not been described thus far.

\( \text{p21}^{\text{Waf1/Cip1}} \) and \( \text{p27}^{\text{Kip1}} \) belong to the family of CDKIs (cyclin-dependent kinase inhibitors) \cite{64}, whose expression regulates the G1 phase Cdks (cyclin-dependent kinase) in the cell cycle \cite{63}. Furthermore, the tumor suppressor protein p53 is also the major mediator of the checkpoint-induced arrest in the G1 phase of the cell cycle \cite{65}. p53 induces cell cycle arrest, through the Cdk inhibitor \( \text{p21}^{\text{Waf1/Cip1}} \) \cite{21}, preventing the replication of damaged DNA. \( \text{p21}^{\text{Waf1/Cip1}} \) interacts with different cyclin/Cdk complexes and other regulators of transcription and signal transduction, exerting broad effects on cell survival, gene expression and morphology \cite{67}. We have shown that in HepG2 cells, the induction G1 phase arrest was via \( \text{p27}^{\text{Kip1}} \) pathway with the addition of CHCl\textsubscript{3}-F alone, but via p53 and \( \text{p21}^{\text{Waf1/Cip1}} \) pathway with the addition of the CHCl\textsubscript{3}-F and GTE mixture. In this chapter, we hypothesized that the combined CHCl\textsubscript{3}-F and GTE
induces expression of APRIL and involves the regulation of these three factors, and therefore the time-dependent expression of these factors in HepG2 cells were examined. Our results showed that $p27^{\text{Kip1}}$ was increased after 2 hrs of the incubation by CHCl$_3$-F only, suggesting that the effect of $p27^{\text{Kip1}}$ by CHCl$_3$-F appeared before the expression of APRIL in HepG2 cells. However, p53 and p21$^{\text{Waf1/Cip1}}$ were increased in the presence of the CHCl$_3$-F and GTE mixture, and this effect was expressed after 8 hrs of the incubation (Fig. 4). Theses results suggest that the combination CHCl$_3$-F with GTE-induced APRIL is not associated with $p27^{\text{Kip1}}$ protein, but most likely with p53 and p21$^{\text{Waf1/Cip1}}$ proteins.

We have previously demonstrated that extracts of green tea$^{27}$, evening primrose$^{29, 30}$, Cape aloe$^{83, 84}$, Zingiberaceae plant such as Languas galanga and Alpinia galangal$^{85}$ and Blumea balsamifera$^{78}$ have anti-cancer activities in various tumor cell lines. We have also demonstrated synergistic anti-cancer activity of natural substances in various tumor cell lines$^{84, 86}$. In the present chapter, we have shown the different mechanisms of anti-cancer activity with treatment with
CHCl$_3$-F alone and with a CHCl$_3$-F and GTE mixture in HepG2 cells. Our study suggests that these natural substances have different anticancer activities via independent pathways *in vitro*, and these could be new tools for cancer therapy. In addition, in order to demonstrate whether the CHCl$_3$-F and GTE mixture has selective inhibitory effects in tumor cells, its effect on hepatocytes, as a normal cell line, and in HepG2 cells were examined via cell viability dynamics. Our results showed that the CHCl$_3$-F and GTE mixture exerted no cytotoxicity in rat hepatocytes, unlike in HepG2 cells.
5. Summary

Our results showed that APRIL was expressed in HepG2 cells after 4 hrs of incubation in vitro and played an important role in cell proliferation. Furthermore, the anticancer activity of combination CHCl$_3$-F with GTE was stronger than the CHCl$_3$-F alone in HepG2 cells. The mechanism of the anti-cancer activity is via reduction of the expression of APRIL, and involves an up-regulation of p53 and p21$^{\text{Waf1/Cip1}}$ proteins in HepG2 cells. We speculate from our results that the CHCl$_3$-F and GTE mixture might provide ways to new drug design to treat hepatocellular carcinoma in the future.
Natural products have played an important role throughout the world in treating and preventing human diseases. In this study, we showed that the Z. jujuba, a famous fruit in China, have anticancer activity in HepG2 cells. Furthermore, the chloroform fraction of Z. jujuba extract (CHCl₃-F) was the most effective component, and was not only cytotoxic but had cytostatic activity as well. Also, different concentrations of CHCl₃-F showed different growth inhibition effects in HepG2 cells. Apoptosis, an increase in intracellular ROS level, a decline of mitochondrial membrane potential at low Z. jujuba concentrations (100 µg/ml), and a ROS-independent mitochondrial dysfunction pathway at high concentrations (200 µg/ml) were all observed. The CHCl₃-F induced not only apoptosis but also G1 arrest at a low concentration and G2/M arrest at a high concentration by cell cycle assay. CHCl₃-F-induced G1 arrest in HepG2 cells was associated with an increase in hypophosphorylation of Rb and p27KiP1, and a decrease of phosphorylated Rb. However, CHCl₃-F-induced
G2/M arrest in HepG2 cells was correlated with the decrease of the $p27^{\text{Kip1}}$ levels and generation of the phosphorylation of $p27^{\text{Kip1}}$, but the hypophosphorylation of Rb protein remained. Moreover, GTE enhanced selective cytotoxic activity of CHCl$_3$-F in HepG2 cells. Combining CHCl$_3$-F and GTE caused an effect on G1 phase arrest but not on apoptosis. Interestingly, the mechanism of the G1 arrest was associated, not with an increase in $p27^{\text{Kip1}}$ levels and the hypophosphorylation of Rb, which are pathways of CHCl$_3$-F on G1 arrest in HepG2 cells, but with increases in p53 and p21$^{\text{Waf1/Cip1}}$ levels, and a decrease in cyclin E levels. We also found that APRIL was expressed in HepG2 cells from 4 hrs of incubation *in vitro* and play an important role in cell proliferation. The mechanism of the anti-cancer activity of CHCl$_3$-F and GTE mixture was via reducing the expression of APRIL. Decreasing APRIL involved an up-regulation of p53 and p21$^{\text{Waf1/Cip1}}$ proteins in HepG2 cells.

Our study is the first to demonstrate that CHCl$_3$-F possesses anticancer activity in HepG2 cells. Furthermore, the anticancer activity of CHCl$_3$-F and GTE mixture was stronger than the CHCl$_3$-F
alone in HepG2 cells. We speculate from our results that the CHCl$_3$-F alone and the CHCl$_3$-F and GTE mixture might provide a lead to new drug design to treat hepatocellular carcinoma in the future.
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Finally, I should mention my son, Xingan, who always stands by me, gives me the power and inspires me to go on…
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APRIL</td>
<td>a proliferation-inducing ligand</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<td>BuOH-F</td>
<td>butanol fraction of <em>Z. jujuba</em> extract</td>
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<tr>
<td>CDKs</td>
<td>cyclin-dependent kinase</td>
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<td>EGCG</td>
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<tr>
<td>Water-F</td>
<td>water fraction of <em>Z. jujuba</em> extract</td>
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*Z. jujuba*  
*Zizyphus jujuba* Mill
LIST OF PUBLICATIONS RELATED TO THIS THESIS

Mechanism of the anticancer activity of *Zizyphus jujuba* in HepG2 cells

**Xuedan Huang, Akiko Kojima-Yuasa, Toshio Norikura, David Opare Kennedy, Tadayoshi Hasuma and Isao Matsui-Yuasa**

*The American Journal of Chinese Medicine 35: 3, 517-532 2007*

Green tea extract enhances the selective cytotoxic activity of *Zizyphus jujuba* extracts in HepG2 cells

**Xuedan Huang, Akiko Kojima-Yuasa, Shenghui Xu, Toshio Norikura, David Opare Kennedy, Tadayoshi Hasuma and Isao Matsui-Yuasa**


Combination of *Zizyphus jujuba* and green tea extracts exert excellent cytotoxic activity in HepG2 cells via reducing the expression of APRIL

**Xuedan Huang, Akiko Kojima-Yuasa, Shenghui Xu, David Opare Kennedy, Tadayoshi Hasuma and Isao Matsui-Yuasa**

*The American Journal of Chinese Medicine 37:1, 169-179 2009*