

Zanthoxylum avicennae* extracts inhibit cell proliferation through protein phosphatase 2A activation in HA22T human hepatocellular carcinoma cells *in vitro* and *in vivo

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Abstract. Hepatocellular carcinoma is a common type of cancer that is usually associated with poor prognosis. In this study, we examined the *in vitro* and *in vivo* mechanisms of the traditional Vietnamese herb *Zanthoxylum avicennae* on the inhibition of HA22T human hepatocellular carcinoma cell proliferation. HA22T cells were treated with different concentrations of *Zanthoxylum avicennae* extracts (YBBEs) and analyzed with the MTT assay, western blot analysis, flow cytometry, siRNA transfection assays and co-immunoprecipitation assay. Additionally, the HA22T-implanted xenograft nude mouse model was applied to confirm the cellular effects. YBBEs showed a strong inhibition of HA22T cell viability in a dose-dependent manner and significantly reduced cell proliferation-related proteins as well as induced cell cycle arrest in the G2/M phase. Protein phosphatase 2A (PP2A) siRNA or okadaic acid totally blocked YBBE-mediated cell proliferation inhibition. In addition, an HA22T-implanted nude mouse model further confirmed that YBBEs inhibit HA22T tumor cell growth and downregulate the survival and cell cycle regulating proteins, as well as activate the PP2A protein. Our findings indicate that the inhibition of HA22T cell proliferation by YBBEs is mediated through PP2A activation.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the most frequent type of liver cancer, accounting for almost 90% of primary malignant hepatic tumors in adults (1), particularly in parts of the developing world (2). According to the World Health Organization, the burden of HCC is expected to continue to increase. Most HCC cases occur in Asia, with a particularly higher incidence in East Asia, accounting for over 20 cases/100,000 population (3). In spite of the great developments in diagnosis and therapy, the prognosis for patients with HCC remains dismal due to its high rate of metastasis and recurrence. For patients in the advanced stages, the median survival rate is less than 6 months, regardless of the type of the treatment modality used (4).

Alternative treatment options for HCC, along with other types of cancers, are in great demand. Traditional medicinal herbs stand out as promising candidates for cancer therapy due to their low toxicity and side effects. *Zanthoxylum avicennae* [Ying Bu Bo (YBB)] is a traditional Vietnamese herb with known medicinal applications. It removes rheumatic symptoms, invigorates blood circulation, promotes diuresis and disperses swelling (5-7). The root is used to treat hepatitis and edema due to nephritis as well as rheumatoid arthritis (5-8). In addition, YBB is also used to treat hepatitis B, hepatocirrhosis, colitis and stomatitis in Vietnamese folk medicine (9).

Our previous studies showed that extracts of *Zanthoxylum avicennae* (YBBEs) induced cell apoptosis through protein phosphatase 2A activation in HA22T human hepatocellular carcinoma cells and blocked tumor growth in xenografted nude mice (9). We further demonstrated the effects of YBBEs on the proliferation of human hepatocellular carcinoma HA22T cells, and evaluated the mechanisms behind its inductive effect on cell proliferation inhibition *in vitro* and *in vivo*. Results show that YBBEs can remarkably activate the PP2A.

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Materials and methods

Materials. Antibodies against PCNA, cyclin A, cyclin D1, cyclin E, α -tubulin, p21, p27, p-p53, p53, c-Fos, c-Myc, p-PI3K, PP2A-C α , p-Cdc25C, Cdc25C, p-Cdk1, donkey anti-goat IgG, goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against Cdk1 were purchased from GeneTex, Inc. (Irvine, CA, USA). Antibodies against p-Akt and p-MDM2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Dulbecco's modified Eagle's medium (DMEM), phenol red-free DMEM, Basal Medium Eagle (BME), sodium bicarbonate NaHCO₃, thiazolyl blue tetrazolium bromide MTT, propidium iodide and ribonuclease A (RNase A) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (PBS) was purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Thermo Scientific HyClone, Logan, UT, USA). Fetal bovine serum (FBS) was purchased from HyClone. Okadaic acid (OA) (PP2A inhibitor, cat.no. 495604) was purchased from Alexis Biochemicals (San Diego, CA, USA). The root bark wood of YBB was collected from Nam Dan, Nghe An, Vietnam.

Ying Bu Bo extraction. YBB extraction was performed according to the methods described in our previous study (9). According to the experimental design, the HA22T cell line was incubated with 0, 50, 100, 150, 200 or 250 μ g/ml of the extract for 24 h. The dose for the animal experiments was 20 or 40 mg/kg YBBEs.

Cell culture and treatments. HA22T cells (BCRC no. 60168) were obtained from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were grown as described in our previous study (9). Cells were cultured in serum-free medium for the invasive and metastatic experiments.

Animals. About 20 male NU/NU nude mice 20–22 g in weight, 5 weeks of age were obtained from the Biolasco Taiwan Co., Ltd. (Taipei, Taiwan). The mice were given food and water *ad libitum*. The detailed procedure has been described in a previous study (9). All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] assay. The YBBEs effect on HA22T cell proliferation was estimated using the MTT assay. HA22T cells were cultured in a 24-well plates (2x10⁵ cells/well) and treated with different concentrations of YBBEs (0, 50, 100, 150, 200 or 250 μ g/ml) and then incubated at 37°C for 24 h to determine cell viability. Cell viability was determined using the MTT assay. In the MTT assay, after dissolving formazan crystals, light absorbance was measured at 570 nm using a spectrophotometer (U-2001, Hitachi Instruments, Inc., Tokyo, Japan). The quantity of the formazan product is directly proportional to the number of viable cells in the culture medium.

Flow cytometric analysis. The YBBEs effect on the HA22T cell cycle was estimated using flow cytometric analysis. Cells were seeded in 6-well plates (5x10⁵ cells/well) and incubated at different YBBEs concentrations (0, 50, 100, 150, 200 or 250 μ g/ml) at 37°C for 24 h. After YBBEs treatment, cells were washed with PBS and treated with trypsin and harvested after trypsin removal. Cells were fixed in 70% ethanol, washed with ice-cold PBS and stained with propidium iodide (PI) buffer, which consisted of 400 μ g/ml PI, 1% Triton X-100 and 0.5 mg/ml RNase A in PBS. Cellular PI content was measured on a BD FACSCanto™ flow cytometer (BD Biosciences, USA) equipped with an argon ion laser at a wavelength of 488 nm. Data were analyzed using the ModFit LT software.

Western blot analysis. HA22T cells were scraped and washed once with PBS. Cell pellets were lysed for 30 min in lysis buffer and spun down at 12,000 x g for 10 min. Tissue samples were homogenized with ice-cold PBS and then subjected to lysis in a solution containing 20 mM Tris, 2 mM EDTA, and 1% glycerol. Supernatants were obtained after centrifugation at 12,000 x g for 40 min. Western blot analyses were performed according to the methods described in our previous study (9).

Gene knockdown using siRNA. HA22T cells were seeded into 6-well plates and grown to 80% confluence. siRNA transfection was carried out with DharmaFECT Duo transfection reagent (Dharmacon, Inc., Lafayette, CO, USA). The 100 μ l PP2A-C α siRNA (Santa Cruz Biotechnology, Inc.) and 100 μ l negative control transfect Non-Targeting Pool (NT) (Dharmacon, Inc.) were mixed with 100 μ l serum-free medium. The detailed procedure has been described in our previous study (9).

Co-immunoprecipitation assay (Co-IP). HA22T cells were transfected with PP2A-C α siRNA first and then treated with YBBEs. Two hundred milliliters Co-IP cell lysis buffer (1.5 mM MgCl₂, 1% Triton X-100, 50 mM HEPES, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM NaVO₃, 10 mM NaF, 10 mM β -glycerolphosphate, and 5 mg/ml protease inhibitor) was added to each plate to lyse cells for 30 min. The cells were then scraped and centrifuged for 10 min at 12,000 x g at 4°C. For each group, 100 μ g of the total protein sample was added into each microcentrifuge tube, with a suitable amount of lysis buffer without protease inhibitor added so that the mixture in each of the microcentrifuge tubes had a final volume of 500 μ l. Seven milliliters of protein G PLUS-agarose (Santa Cruz Biotechnology, Inc., cat. no. sc-2002) was then added followed by vortexing at 4°C for 1 h with a vortex mixer. Centrifugation at 4°C and 1,300 x g was performed for 30 sec. The supernatant was then removed. For each group, 2.5 μ l of each antibody listed as follows was added into each of the microcentrifuge tubes: PP2A-C α and Akt. Afterward, vortexing at 4°C overnight with a vortex mixer was conducted. Twenty milliliters of protein G PLUS-agarose was added, followed by vortexing at 4°C for 2 h with the vortex mixer. Centrifugation at 4°C and 1,300 x g was conducted for 30 sec. After the supernatant was removed, 1 ml of lysis buffer without protease inhibitor was added to wash the pellet, followed by centrifugation at 4°C and 1,300 x g for 30 sec. Western blotting was performed to detect the PP2A-C α , p-Akt and Akt proteins.

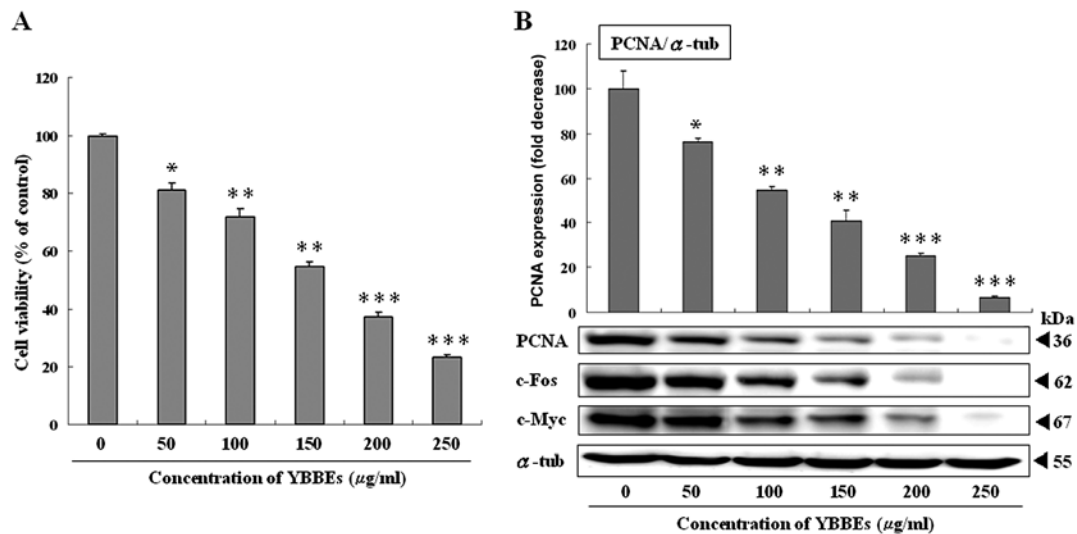


Figure 1. The suppressive effects of YBBEs on HA22T cell proliferation. HA22T cells were incubated with 0, 50, 100, 150, 200 or 250 $\mu\text{g/ml}$ of YBBEs for 24 h. (A) Cell viability as measured using MTT assay. (B) Downregulation of PCNA, c-Fos, and c-Myc proteins expression as revealed by western blot analysis; α -tubulin was used as a loading control. Data are shown as the means \pm SE of three independent experiments and denote significant differences from control values with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, respectively.

Liver cancer mice model. The detailed procedure has been described in a previous study (9). Tumors were excised and then examined by western blot analysis.

Statistical analysis. Each sample was analysed based on results that were repeated at least three times using SigmaPlot 10.0 software. The detailed procedure has been described in previous study (9).

Results

The anti-proliferative effect of YBBEs on HA22T cells. No known study has been carried out to investigate the effect of YBBEs on human cancer cell lines. We therefore tested the antiproliferative effect of YBBEs on HA22T human hepatocellular carcinoma cells. We first determined whether YBBEs significantly attenuated cell viability. The MTT assay was used to determine cell viability in cell proliferation and cytotoxicity assays. Our experiments found dose-dependent inhibition of cell viability in the YBBEs-treated groups compared to the control group (Fig. 1A), accompanied with a significant decrease in PCNA, c-Fos and c-Myc markers in a dose-dependent manner (Fig. 1B). These findings provide evidence that YBBEs significantly inhibited the viability and protected against HA22T cell proliferation.

YBBEs inhibit cell cycle progression in HA22T cells. We evaluated the inhibitory effect of YBBEs on the p-MDM2-p53 pathway that regulates cell-cycle related protein expression. YBBEs treatment reduced phosphorylated MDM2 protein levels, accompanied with a significant increase in p53 and p-p53 proteins (Fig. 2A). Therefore, there was no change in the total MDM2 expression levels. We also observed p21 and p27 enhancement at the 24 h time point (Fig. 2B). The cell cycle progression is tightly regulated by a complex network of cell cycle regulatory molecules, such as cyclins. To elucidate how YBBEs modulate the cell cycle in HA22T cells,

we measured the cell cycle modulator proteins cyclin D1, cyclin E, and cyclin A expression using western blot analysis. The results showed decreased expression of cyclin D1, cyclin E, and cyclin A proteins in a dose-dependent manner following YBBEs treatment (Fig. 2C). In addition, we treated cells with or without YBBEs and subjected them to flow cytometric analysis to test whether YBBEs induced G2/M phase cell cycle arrest in HA22T cells. We found that YBBEs (0-250 μM) increased the G2 phase population from 15.12 to 45.05% and reduced the G1 phase population from 67.08 to 43.62%, suggesting an accumulation at the G2/M phase in the cell cycle (Fig. 2D and E). To further explore the underlying mechanisms of G2/M phase arrest induced by YBBEs, we examined the regulatory effects of YBBEs on p-Cd25C, Cd25C, p-Cdk1 and Cdk1 expression. As shown in Fig. 2F, cell exposure to YBBEs also resulted in a dose-dependent increase in p-Cd25C and p-Cdk1 protein levels, accompanied with a significant reduction in Cd25C and Cdk1 proteins. According to our results, YBBEs were able to arrest HA22T cells at the G2/M phase.

Okadaic acid (OA) inhibits YBBEs reduced cell cycle progression through the downregulation of PP2A-Ca in HA22T cells. We further determined the role of PP2A on the YBBEs inhibition of HA22T cell proliferation. HA22T cells were pretreated with OA, a pharmacological inhibitor of PP2A, followed by treatment with YBBEs at a concentration of 200 $\mu\text{g/ml}$ for 24 h. OA significantly reversed the YBBEs-induced inhibition of cell proliferation in a dose-dependent manner, as revealed by the MTT assay (Fig. 3A). YBBEs (200 $\mu\text{g/ml}$) treatment significantly inhibited p-PI3k, p-Akt and p-MDM2 expression and was accompanied with an increase in PP2A-Ca and p53 expression. However, this situation was totally reversed after treatment with OA in the presence of YBBEs (Fig. 3B).

PP2A-Ca siRNA blocks the antiproliferative effect of YBBEs in HA22T cells. To further confirm that YBBEs induce cell prolifer-

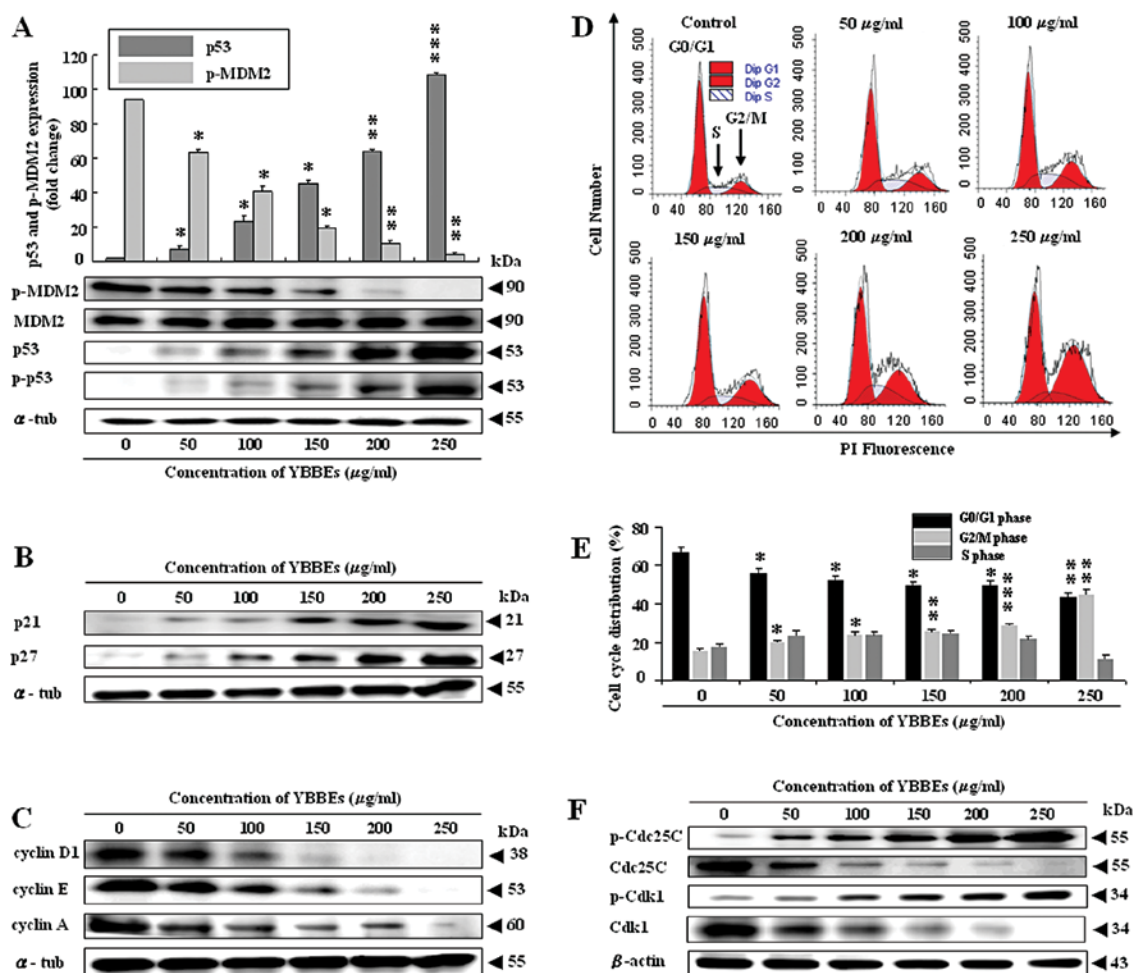


Figure 2. Cell cycle progression inhibition by YBBEs in hepatocellular carcinoma HA22T cells. HA22T cells were cultured and incubated with 0, 50, 100, 150, 200 or 250 $\mu\text{g/ml}$ of YBBEs for 24 h. (A) Western blot analysis showing decreased p-MDM2 and increased p53 and p-p53 protein expression. (B and C) Cell cycle controlling protein expression was measured using western blot analysis with antibodies against the proteins indicated. Equal loading was assessed with an anti- α -tubulin antibody. (D) The cell cycle was determined using flow cytometric analysis. (E) Representative histograms clearly showing a significant YBBEs effect on inducing G2 phase cell cycle arrest in HA22T cells. * $P < 0.05$, ** $P < 0.01$ vs. the control group. (F) Western blot analysis showing decreased Cdc25C, Cdk1, and increased p-Cdc25C and p-Cdk1 protein expression. β -actin was used as a loading control.

eration inhibition in HA22T cells through PP2A, we transfected HA22T cells with PP2A-C α siRNA. Western blot analysis showed a significant reduction in PP2A-C α and p53 proteins, accompanied with increased protein levels of p-PI3K, p-Akt, p-MDM2, PCNA, c-Fos and c-Myc (Fig. 3E). Our results suggested that PP2A is an important mediator in the YBBEs-induced antiproliferative inhibition effect in HA22T cells.

YBBEs-induced Akt dephosphorylation is regulated by PP2A. PP2A regulates the protein kinase signaling pathway activities, including the PI3/Akt pathways. We further assessed the physical interaction between PP2A and Akt. Surprisingly, our results showed that YBBEs induced PP2A-C interaction with Akt (Fig. 4). The present study provides evidence that YBBEs activated PP2A and induced a physical interaction between PP2A and Akt proteins, which may directly dephosphorylate Akt.

YBBEs inhibits cell proliferation and tumor growth in an in vivo model. To assess the YBBEs antiproliferative effect on tumor growth in the *in vivo* model, western blot analysis

was conducted to observe the cell proliferation and cell cycle modulator protein expression levels in the tumor tissues of mice treated with or without different doses of YBBEs. In our experiments, the p53, p21, p27, p-Cdc25C, and p-Cdk1 expression levels in the tumor tissues of mice in the positive control group were lower than those in the negative control group (mice without injection of HA22T cells and without YBBEs treatment). However, the expression levels of p53, p21, p27, p-Cdc25C and p-Cdk1 significantly increased in YBBEs groups treated with YBBEs (20 or 40 mg/kg) in a dose-dependent manner (Fig. 5A, C and D). The expression levels of p-PI3k, p-Akt, p-MDM2, PCNA, c-Fos, c-Myc, cyclin D1, cyclin E, cyclin A, Cdc25C and Cdk1 in the tumor tissues of mice in the positive control group were higher than those in the negative control group. The expression levels of these proteins gradually decreased as the YBBEs treatment concentration increased (Fig. 5A-D). The *in vivo* and *in vitro* experimental results are totally consistent. Thus, YBBEs are able to suppress tumor cell proliferation *in vitro*. Based on the *in vivo* tests we also strongly believe that YBBEs effectively inhibit liver cancer cell growth.

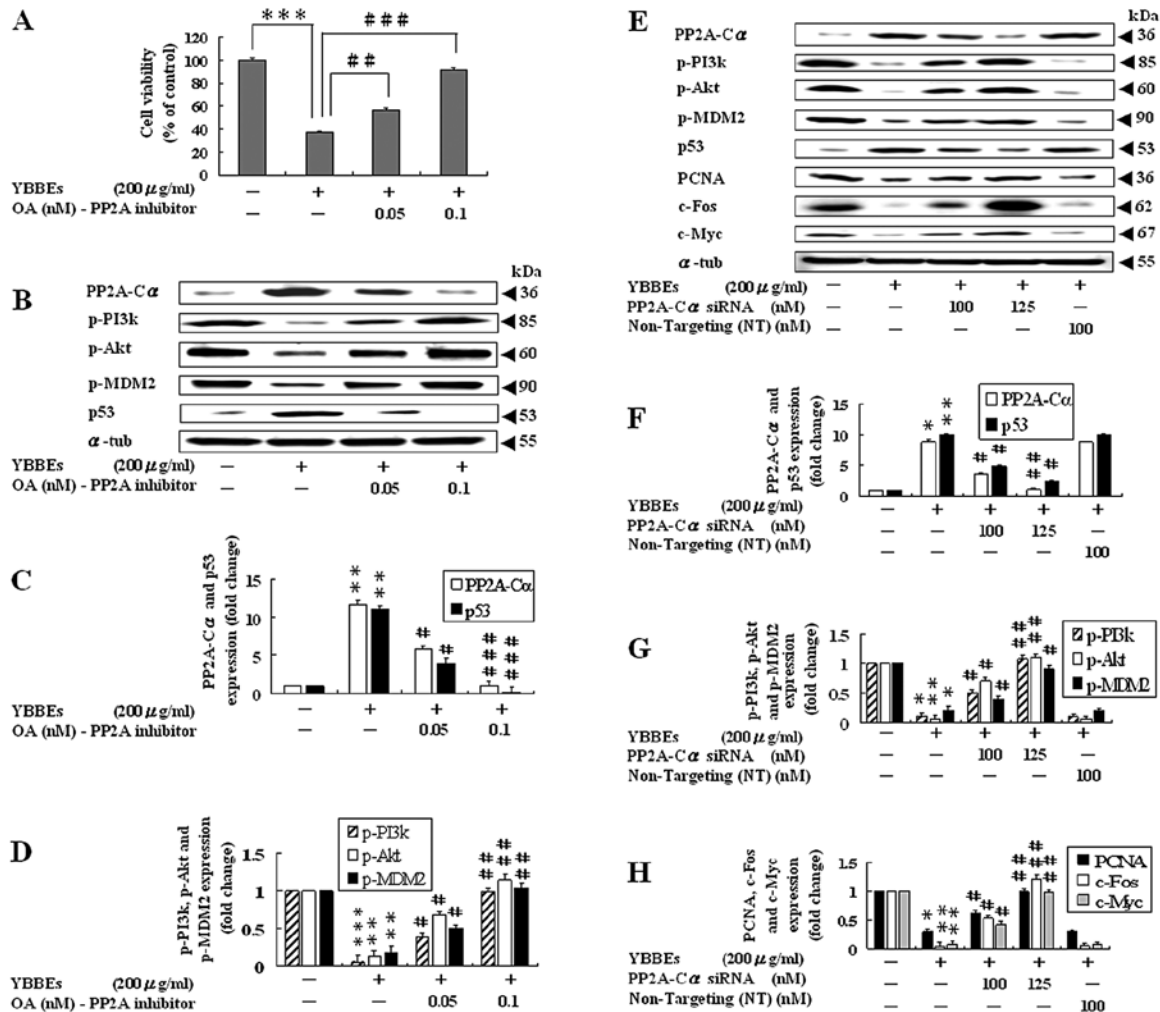


Figure 3. YBBEs inhibit HA22T cell viability through PP2A activation. HA22T cells were treated with okadaic acid (OA), a PP2A inhibitor (0.05 and 0.1 nM for 1 h) or 100 or 125 nM PP2A-Cα siRNA, a PP2A gene silencer and 100 nM control Non-Targeting (NT) siRNA and then subsequently treated with YBBEs (200 µg/ml) for another 24 h. (A) The cell viability was examined using the MTT assay. (B) OA inhibits the YBBEs-induced inhibition of cell cycle progression by modulating the expression of PP2A-Cα, p-PI3K, p-Akt, p-MDM2 and p53 proteins in HA22T cells. (E) siRNA knockdown of PP2A-Cα to inhibit the YBBEs-induced inhibition of HA22T cell proliferation as determined by western blot analysis. α-tubulin was used as a loading control. (C and F) Bars represent the relative quantification of PP2A-Cα and p53 relative to the control levels. (D and G) Bars represent the relative quantification of p-PI3K, p-Akt and p-MDM2 relative to the control levels. (H) Bars represent the relative quantification of PCNA, c-Fos and c-Myc relative to the control levels. The quantitative results are expressed as the mean value ± SE (n=3). *P<0.05, **P<0.01 and ***P<0.001 denote significant differences from control values with YBBEs treatment. #P<0.05, ##P<0.01 and ###P<0.001, respectively denote significant differences from YBBEs treatment of OA or siRNA.

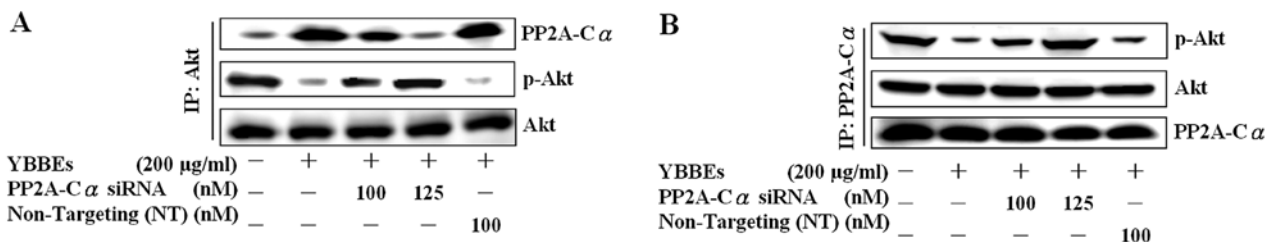


Figure 4. YBBEs-activated PP2A dephosphorylates phospho-Akt. HA22T cells were treated with 100 or 125 nM PP2A-Cα siRNA, a PP2A gene silencer and 100 nM control Non-Targeting (NT) siRNA and then subsequently treated with YBBEs (200 µg/ml) for another 24 h. (A) Co-immunoprecipitation result regarding the degree of association of Akt with PP2A-Cα, or p-Akt. (B) Co-immunoprecipitation result regarding the degree of association of PP2A-Cα with p-Akt or Akt.

Discussion

Uncontrolled tumor cell proliferation plays the most crucial role in hepatocellular carcinoma growth. Therefore, the

prevention of cancer proliferation is an important target for improving patient prognosis. There is still no available information addressing the antiproliferative and inhibitory effects of YBBEs in human cancer cells. This study investigated whether

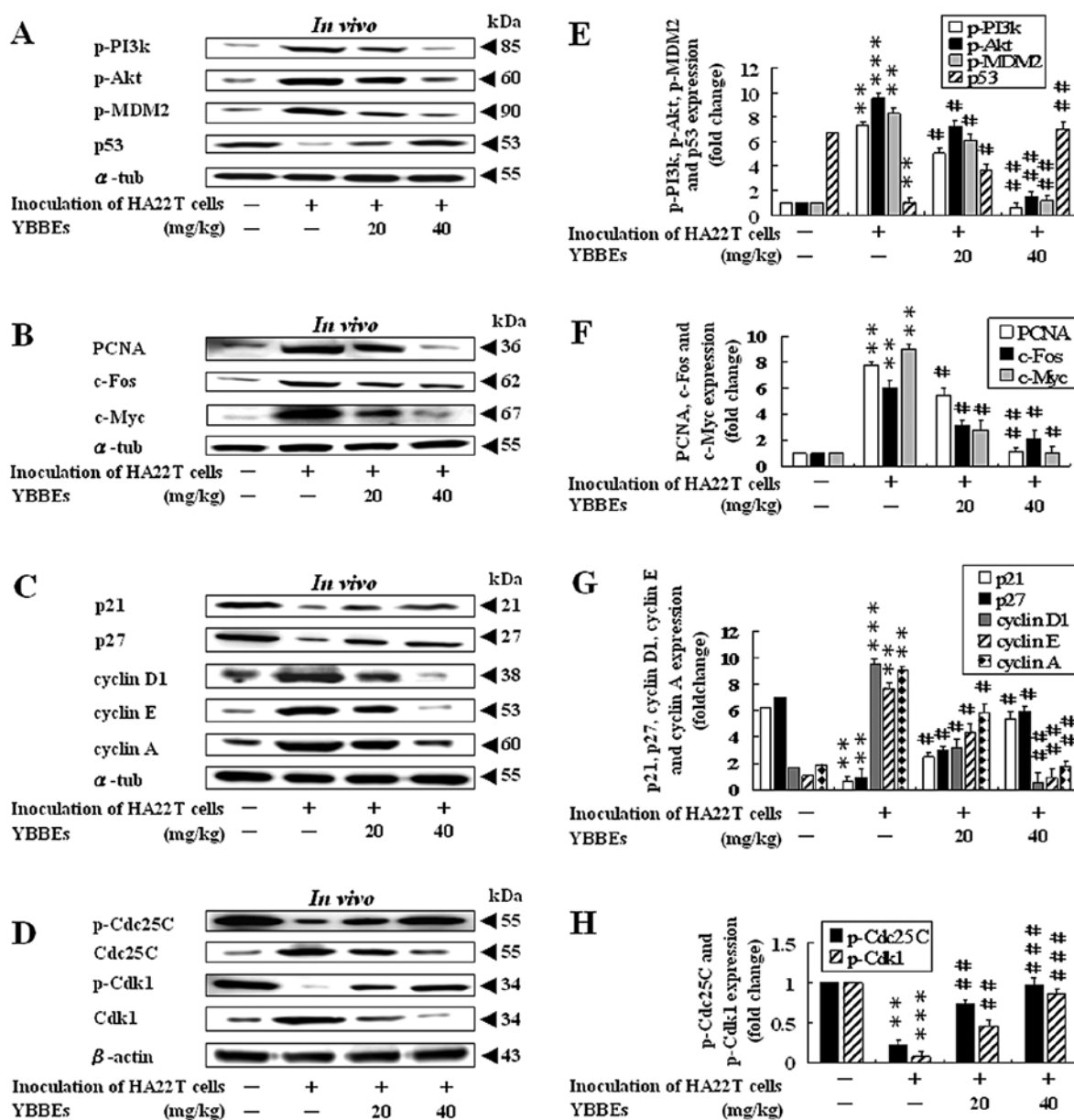


Figure 5. Western blot analysis in different tumor tissue groups in the HA22T xenograft nude mouse model treated or not treated with YBBEs. HA22T cells (1×10^6 in $100 \mu\text{l}$ DMEM) were subcutaneously injected into the left flank of NU/NU mice. Starting at 4 days after inoculation, when the tumors had reached a volume around 600 mm^3 , mice were orally treated with YBBEs (20 or 40 mg/kg) every day. (A-D) Western blot analysis of the expression levels of different proteins compared to the relative control values. (E) Bars representing the relative quantification of p-PI3k, p-Akt, p-MDM2 and p53 relative to the control levels. (F) Bars representing the relative quantification of PCNA, c-Fos and c-Myc relative to the control levels. (G) Bars representing the relative quantification of p21, p27, cyclin D1, cyclin E and cyclin A relative to the control levels. (H) Bars representing the relative quantification of p-Cdc25C and p-Cdk1 relative to the control levels. Experiments were repeated five times with similar results. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ denote significant differences of the groups treated with YBBEs (20 or 40 mg/kg) from the positive control group inoculated with HA22T cells; ** $P < 0.01$ and *** $P < 0.001$ denote significant differences of the positive control group from the negative control group, (mice without injection of HA22T cells and without YBBEs treatments).

YBBEs can inhibit cell proliferation in human hepatocellular carcinoma HA22T cells. We found a significant inhibitory effect by YBBEs on HA22T cell viability as revealed by the MTT assay.

PCNA, c-Fos, and c-Myc are associated with tumor cell proliferation. PCNA is a useful marker for proliferative activity, which functions as a cofactor of DNA polymerase and as an important marker for evaluating the proliferation of several cancers, including hepatocellular carcinoma (10). In normal cells, c-Myc is induced upon growth factor stimulation and is constitutively high in transformed cells. c-Myc overexpression

is estimated to occur in 70% of human tumors (11). Our results showed that YBBEs significantly inhibited PCNA, c-Fos, and c-Myc levels in HA22T cells.

Chronic cyclin D1 overexpression in transgenic mice is reported to be associated with the rapid development of hepatocellular adenomas and carcinomas (12). Cyclin D1 is a major regulator of cell cycle progression, serving as a rate limiting agent for the G1/S cell-cycle checkpoint (13). In this study, YBBEs administration significantly decreased PCNA, cyclin D1, cyclin E, cyclin A, c-Fos, and c-Myc protein levels. Thus, it can be concluded that YBBEs efficiently suppressed

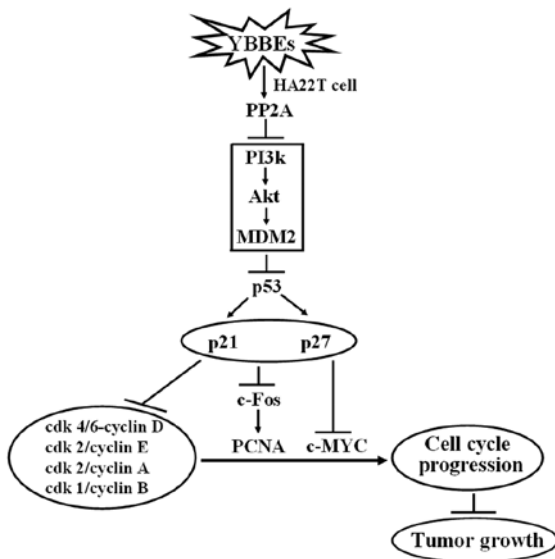


Figure 6. Schematic representation of the mechanism of YBBEs-induced inhibition of cell proliferation in human HA22T hepatocellular carcinoma cells and inhibition of the xenografted HA22T tumor growth in the nude mouse model.

the proliferation of human hepatocellular carcinoma HA22T cells by inhibiting PCNA, c-Fos, and c-Myc accumulation and cyclin D1, cyclin E, cyclin A protein expression.

The p53 protein inhibits tumor growth by arresting cell proliferation and inducing apoptosis. The p53 gene alteration is the most frequently identified mutation in human cancers. The loss of p53 function allows cells with damaged DNA to continue to proliferate and, therefore, it is associated with tumor progression (14). There is increasing evidence supporting p53 as an attractive target in cancer therapy (15). Our results revealed that YBBEs treatment resulted in a dose-dependent increase in p53 level. It has been shown that p53 may directly facilitate cytochrome c release. p53 has been associated with various biological functions, such as cell cycle arrest (16), metastasis (17).

MDM2 is an oncoprotein that is overexpressed in a range of human cancers (18). Under non-stress conditions, p53 levels are tightly controlled by MDM2 through a well-established auto-regulatory feedback loop (19). It induces MDM2 gene expression, which in turn leads to p53 inactivation and degradation (20). Since MDM2 is reported to be one of the main factors causing p53 degradation via the proteasome-related pathway (21), we therefore examined its involvement in YBBEs-mediated p53 regulation. Western blot analysis assay showed that YBBEs inhibited MDM2 expression in a dose-dependent manner. We also found that p53, p-p53, p21 and p27 were significantly upregulated in the presence of YBBEs. Our research suggested that p53 protein upregulation is involved in MDM2 inhibition by YBBEs in HA22T cells. p53/MDM2 modulation may be one of the important mechanisms behind the YBBEs-mediated anticancer activity.

According to previous studies, to prove that asparanin A causes G2/M arrest in HepG2 cells, it was shown that cyclin A and Cdk1 were downregulated to varying degrees following asparanin A treatment. Exposure to asparanin A also resulted in an increase in p-Cdk1 protein levels (22). In a recent study,

HKH40A was used to treat Hep3B cells and was found to increase p-Cd25C and p-Cdk1 expression in a dose-dependent manner (23). Treating HA22T hepatocarcinoma cells with different doses of YBBEs also had similar results, leading to cell cycle arrest at the G2/M phase.

It has previously been reported that sorafenib plus bortezomib significantly suppressed PLC5 hepatocellular carcinoma cell xenograft tumor growth, downregulated p-Akt expression, and upregulated PP2A activity (24). Use of ceramide to treat PC-3 human prostate cancer cells revealed that ceramide-activated PP2A dephosphorylates phospho-Akt (25). In this study, we found that OA reversed YBBEs-mediated downregulation of p-PI3K, p-Akt and p-MDM2, suggesting that targeting PP2A may be a feasible way to affect the pivotal stage. Silencing PP2A using RNA-interference suppressed the YBBEs mediated antiproliferative effect, confirming that PP2A is indispensable in mediating the effects of YBBEs. These results showed that PP2A has different roles in different cancer cell types in various cellular contexts, which should be further investigated in future studies.

We also found that YBBEs dramatically suppressed HA22T cell proliferation and tumor growth in the nude mouse model. YBBEs showed the strongest effect at the 40 mg/kg concentration.

Based on these findings, YBBEs upregulated PP2A, leading to decreased p-PI3K, p-Akt, and p-MDM2, and further upregulated cell cycle checkpoint proteins, such as p53, p21, p27, p-Cdc25C and p-Cdk1; and decreased expression of c-Fos, c-Myc, cyclin A, cyclin D, cyclin E, Cdc25C, Cdk1 and PCNA. These results present the molecular mechanisms behind human hepatocellular carcinoma HA22T cell proliferation and tumor growth inhibition by YBBEs. This was confirmed using an *in vitro* and *in vivo* model system. The signaling pathways involved are shown in Fig. 6.

In conclusion, our study provides strong evidence that YBBEs significantly inhibit HA22T cell proliferation and tumor growth through PP2A activation. These results are promising enough to consider further investigation of the YBBEs anticancer effect using preclinical studies and clinical trials. Efforts aimed at enhancing the YBBEs function and/or activity may provide an alternative therapy against liver cancer.

Acknowledgements

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References

1. El-Serag HB and Rudolph KL: Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 132: 2557-2576, 2007.
2. Gomaa AI, Khan SA, Leen EL, Waked I and Taylor-Robinson SD: Diagnosis of hepatocellular carcinoma. *World J Gastroenterol* 15: 1301-1314, 2009.
3. Gomaa AI, Khan SA, Toledano MB, Waked I and Taylor-Robinson SD: Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. *World J Gastroenterol* 14: 4300-4308, 2008.

4. Ziparo V, Balducci G, Lucandri G, Mercantini P, Di Giacomo G and Fernandes E: Indications and results of resection for hepatocellular carcinoma. *Eur J Surg Oncol* 28: 723-728, 2002.
5. Xiang-Ping GPZ and Shen T: Dictionary of Chinese Herbs. Vol. 2. Shanghai Science and Technology Press, pp3825-3826, 2006.
6. Zhang LC: Dictionary of Chinese Herbs. Vol. 5. Chao Ren Press, pp5513-5514, 1981.
7. Zu BX: Whole China herb conglomeration edition. *J People's Public Health* 1: 928-929, 1975.
8. Hsu HY, Chen YP, Shen SJ, Hsu CS, Chen CC and Chang HC: Oriental Materia Medica. Oriental Healing Arts Institute, Long Beach, CA, pp365-366, 1986.
9. Dung TD, Chang HC, Chen CY, Peng WH, Tsai CH, Tsai FJ, Kuo WW, Chen LM and Huang CY: *Zanthoxylum avicennae* extracts induce cell apoptosis through protein phosphatase 2A activation in HA22T human hepatocellular carcinoma cells and block tumor growth in xenografted nude mice. *Int J Mol Med* 28: 927-936, 2011.
10. Wu WY, Xu Q, Shi LC and Zhang WB: Inhibitory effects of *Curcuma aromatica* oil on proliferation of hepatoma in mice. *World J Gastroenterol* 6: 216-219, 2000.
11. Gordan JD, Thompson CB and Simon MC: HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* 12: 108-113, 2007.
12. Deane NG, Parker MA, Aramandla R, Diehl L, Lee WJ, Washington MK, Nanney LB, Shyr Y and Beauchamp RD: Hepatocellular carcinoma results from chronic cyclin D1 overexpression in transgenic mice. *Cancer Res* 61: 5389-5395, 2001.
13. Sherr CJ: Cancer cell cycles. *Science* 274: 1672-1677, 1996.
14. Greenblatt MS, Bennett WP, Hollstein M and Harris CC: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 54: 4855-4878, 1994.
15. Bell HS and Ryan KM: Targeting the p53 family for cancer therapy - 'big brother' joins the fight. *Cell Cycle* 6: 1995-2000, 2007.
16. Giono LE and Manfredi JJ: The p53 tumor suppressor participates in multiple cell cycle checkpoints. *J Cell Physiol* 209: 13-20, 2006.
17. Roger L, Gadea G and Roux P: Control of cell migration: a tumour suppressor function for p53. *Biol Cell* 98: 141-152, 2006.
18. Brooks CL and Gu W: p53 ubiquitination: Mdm2 and beyond. *Mol Cell* 21: 307-315, 2006.
19. Erster S, Mihara M, Kim RH, Petrenko O and Moll UM: In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol* 24: 6728-6741, 2004.
20. Li M, Brooks CL, Wu-Baer F, Chen D, Baer R and Gu W: Mono-versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* 302: 1972-1975, 2003.
21. Inoue T, Geyer RK, Yu ZK and Maki CG: Downregulation of MDM2 stabilizes p53 by inhibiting p53 ubiquitination in response to specific alkylating agents. *FEBS Lett* 490: 196-201, 2001.
22. Liu W, Huang XF, Qi Q, Dai QS, Yang L, Nie FF, Lu N, Gong DD, Kong LY and Guo QL: Asparagin A induces G(2)/M cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells. *Biochem Biophys Res Commun* 381: 700-705, 2009.
23. Wang Z, Wang M, Kar S and Carr BI: Involvement of ATM-mediated Chk1/2 and JNK kinase signaling activation in HKH40A-induced cell growth inhibition. *J Cell Physiol* 221: 213-220, 2009.
24. Chen KF, Yu HC, Liu TH, Lee SS, Chen PJ and Cheng AL: Synergistic interactions between sorafenib and bortezomib in hepatocellular carcinoma involve PP2A-dependent Akt inactivation. *J Hepatol* 52: 88-95, 2010.
25. Kim SW, Kim HJ, Chun YJ and Kim MY: Ceramide produces apoptosis through induction of p27(kip1) by protein phosphatase 2A-dependent Akt dephosphorylation in PC-3 prostate cancer cells. *J Toxicol Environ Health A* 73: 1465-1476, 2010.