Chung Shan Medical Journal 2024; 35:57-64

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Original Article

The anti-hepatoma activity of *Juniperus indica Bertol extract in vitro*

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Purpose: *Juniperus indica Bertol* is belong to *Juniperus* genus and *Juniperus* used to treat infections and diabetes in usage of herbal medicine, and scientific studies have proven that it has anti-inflammatory and anti-cancer effects. However, it is still not clear whether *J. indica Bertol* extract (JIB extract) has anti-hepatoma activity. Therefore, this study investigated the mechanisms underlying the anti-cancer activity of JIB extract in hepatocellular carcinoma cells. Hepatocellular carcinoma (HCC) is one of the most malignant cancers and its prevalence has increased over the past few years. Patients with HCC often do not respond effectively to chemotherapy; therefore, other therapeutic approaches are required to treat the disease.
Methods: Using *Juniperus indica Bertol* extract (JIB extract) treated hepatoma cell lines and detected anticancer effects with MTT assay, cell cycle analysis, TUNEL assay, Western blot and statistical analysis to verify JIB extract suppressed hepatoma cell survival, anti-proliferation activities, cell cycle arrest and tumor apoptosis induction pathways.
Results: Our results indicated that the anti-cancer activity of JIB extract in HCC involved blocking the cell cycle progression by reducing phosphorylated Rb and increasing p21 levels. Moreover, JIB extract triggered HCC cell death by inducing caspase dependent apoptotic pathways. Furthermore, JIB extract reduced VEGF/VEGFR1/VEGFR2 levels, which are associated with tumor angiogenesis and proliferation, and suppressed MMP2 levels to exert an antimetastatic capacity.
Conclusion: JIB extract might be developed as an anti-cancer agent for clinical treatment or daily prevention.

Keywords: *Juniperus indica Bertol*, hepatocellular carcinoma, cell cycle, apoptosis

1. Introduction

Juniperus is one of the most important genera belonging to the Cuppressaceae family. *Juniperus indica Bertol* is an evergreen coniferous dioecious shrub that is widely distributed throughout the mountains in the cool temperate regions of North America, Europe, India, and Asia. *Juniperus* is traditionally used as food appetizer, flavoring agent, and medicine for treating diuresis due to terpinenol-4, which has a marked diuretic effect in juniper oil (1); it is also used as a remedy for

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diabetes in streptozotocin-diabetic rat model(2), and arthritis in freund's adjuvant induced rat arthritis model (3). Previous scientific studies have reported that *Juniperus* possesses anti-inflammatory in acute-dextran induced rat model(4), antihyperlipidemic effect in alloxan-induced diabetic rat model(5), antioxidant effect by DPPH, TBA and FRAP assays(6), and antimicrobial activities (7-9). To date, various kinds of *Juniperus* have been reported to possess diverse potential anti-cancer effects in vitro (10, 11). Among these, *Juniperus indica Bertol* has found the cytotoxic activity against brine shrimp eggs (12); however, it remains to be studied whether *J. indica Bertol* has anti-hepatoma activity.

Traditional Chinese medicine has been used to treat liver disease and prevent liver cancer development. Liver cancer is the 6th most prevalent cancer type and has a high mortality rate; its incidence rate has increased worldwide in recent years, especially for hepatocellular carcinoma (HCC) (13). Moreover, HCC patients with poor prognosis are usually less responsive to clinical drugs. Many studies regarding hepaprotective activity have been done using *J. indica Bertol*. extract as a natural product to overcome these problems (14-16); however, its anti-hepatoma effects and mechanisms are still unknown.

The anti-tumor strategy is commonly preferred for inducing cell apoptosis. Apoptosis is a biological adaptation that maintains cell homeostasis, and is divided into the extrinsic and intrinsic apoptotic pathways (17). The extrinsic apoptotic pathway is induced by death receptors related to caspase-8 activation. While the intrinsic pathway involves the mitochondria related to caspase-9 activation. Both pathways activate caspase-3 cleavage. Current chemotherapeutic drugs usually stimulate cancer cell death via cell apoptosis, but the toxicity and resistance in HCC chemotherapy caused failure; further increasing the potential of natural products as a reliable alternative treatment (18). The cell cycle is known to be associated with the proliferation and survival of cancer cells. The biological role for normal cells regulates cellular division and replication; in cancer cells the regulation of the cell cycle fails, causing uncontrolled cell proliferation and survival. The cell cycle is controlled by cyclin dependent kinase, cyclin, and cell cycle regulators. Moreover, cell cycle checkpoints play an important role in regulating the cell cycle to repair cellular damage and cell cycle-related genes (19). Consequently, cell cycle-related and cell apoptotic therapies are promising strategies for cancer treatment. In this study, *J. indica Bertol*. extracts (JIB extract) obtained via steam distillation demonstrated that JIB extract has potential anti-hepatoma activity in $HepG_2$ cell, and could be used as a treatment to induce apoptosis and cell cycle arrest.

2. Materials and Methods

2.1. Extraction of *Juniperus indica Bertol* extract (JIB extract)

The leaves and fruits of *J. indica Bertol*. were obtained from Nepal. The dried leaves and fruits of *J. indica Bertol* were extracted with steam distillation and obtained the oil layer as *J. indica Bertol* extract which was purchased from PHOENIX (New Jersey, USA). Fresh JIB extract was dissolved in dimethyl sulfoxide (DMSO) diluted with growth medium. The drug concentration was calculated into formula: JIB extract (μg)/volume (ml). The final concentration of DMSO was adjusted to 0.1% (v/v) in the culture media. JIB extract was preserved at 4°C in a brown glass bottle, and sealed with paraffin to prevent extract being subjected to moisture and light.

2.2. Cell culture and reagent

The human liver cancer $HepG_2$, Mahlavu, canine kidney epithelial cell (MDCK), and mouse vascular endothelial cell (SVEC) were purchased from BCRC (Bioresource Collection and Research Center, Hsinchu, Taiwan). Cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% HEPES, and 1% Penicillin/Streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The dimethyl sulfoxide (DMSO), MTT and *in situ* cell death detect kit, fluorescein, were purchased from Sigma-Aldrich Chemicals Co. (Missouri, MO, USA). Protease inhibitor was purchased from Bio Basic Inc. (Toronto, Canada). BCA Protein Assay Reagent was purchased from Thermo Fisher Scientific (Waltham, MA USA). T-Pro LumiFast plus Chemiluminescence Detection Kit was purchased from T-Pro Biotechnology (New Taipei County, Taiwan).

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2.3. MTT assay

Cells were plated in a 96-well culture plate (5×10^3 cells/well) and treated with various JIB extract concentrations. The medium was removed after the indicated time interval, MTT was added to each well, and formazan crystals were dissolved in DMSO. The absorbance value was read at 550 nm with SpectraMax M5 Molecular Devices (San Jose, California, USA).

2.4. Cell cycle analysis

Cell cycle analysis was performed by treating the $HepG_2$ cell (2×10^6/dish) with JIB extract (0, 30, 50 and 70 μg/ml) for 24 h, and staining the harvested cell with propidium iodide (PI) and RNase overnight (in the dark) at 4°C. Afterwards, cells were analyzed via flow cytometer (BD Biosciences, USA) and presented as percentages (%).

2.5. TUNEL assay

Cells were stained via TUNEL assay kit after JIB extract treatment, according to the manufacturer's procedure. Cells were spread on slides after treatment, fixed with 10% formalin, and then stained with TUNEL reaction solution for 2 hours. After washing with phosphate-buffered saline (PBS), the cells were stained with 40 μg/ml of propidium iodide (PI) for 10 minutes. The morphology of apoptotic cells was then observed under microscopy (×400).

2.6. Western blot analysis

Cell lysate were collected, and the protein concentration was quantified using the BCA Protein Assay Reagent. Whole cell lysates were fractionated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated at 4°C overnight with each primary antibody to visualize the target protein. The membranes were then washed and incubated with the appropriate secondary antibody and horseradish peroxidase. Detection was carried out using a T-Pro LumiFast plus Chemiluminescence Detection Kit. Antibodies against Rb, pRb, p21, cdk2, cdk4, cyclin D1, cyclin B1, cyclin A, bax, bcl2, Fas, Fas-L, cleaved caspase 3, procaspase 8, procaspase 9, VEGF, VEGFR1, VEGFR2, MMP2, and actin (1:200) were purchased from Santa Cruz Biotechnology (California, USA). The densitometry analysis was performed using Image J software (NIH, Betlesda, MD, USA).

2.7. Statistical analysis

All data are expressed as mean ± SD of duplicated independent replicates for each group. Comparisons were made by Student's t -test; where $p < 0:05$ was considered statistically significant.

3. Results

3.1. JIB extract inhibited HCC cell growth and was less cytotoxic to normal vascular endothelial and kidney epithelial cells

Initially, MTT assay was used to determine the cytotoxic effects of JIB extract. The cell viability

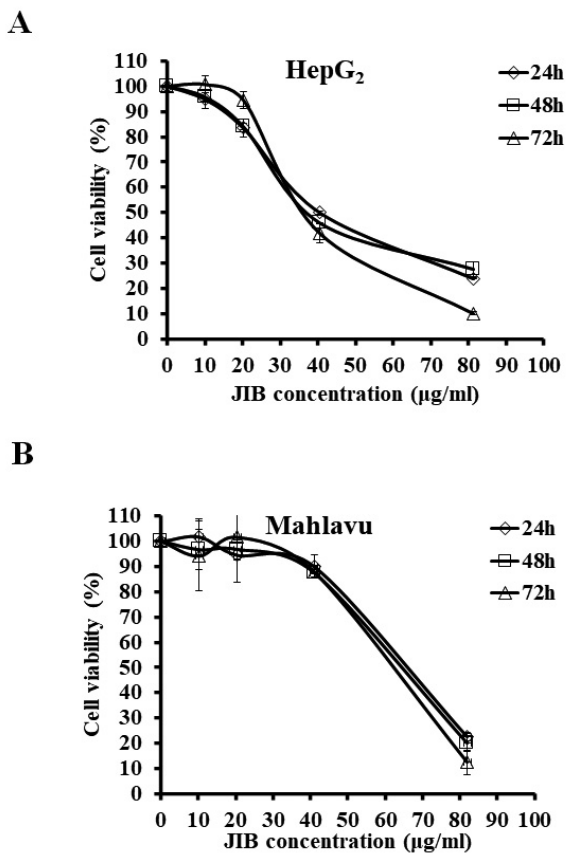


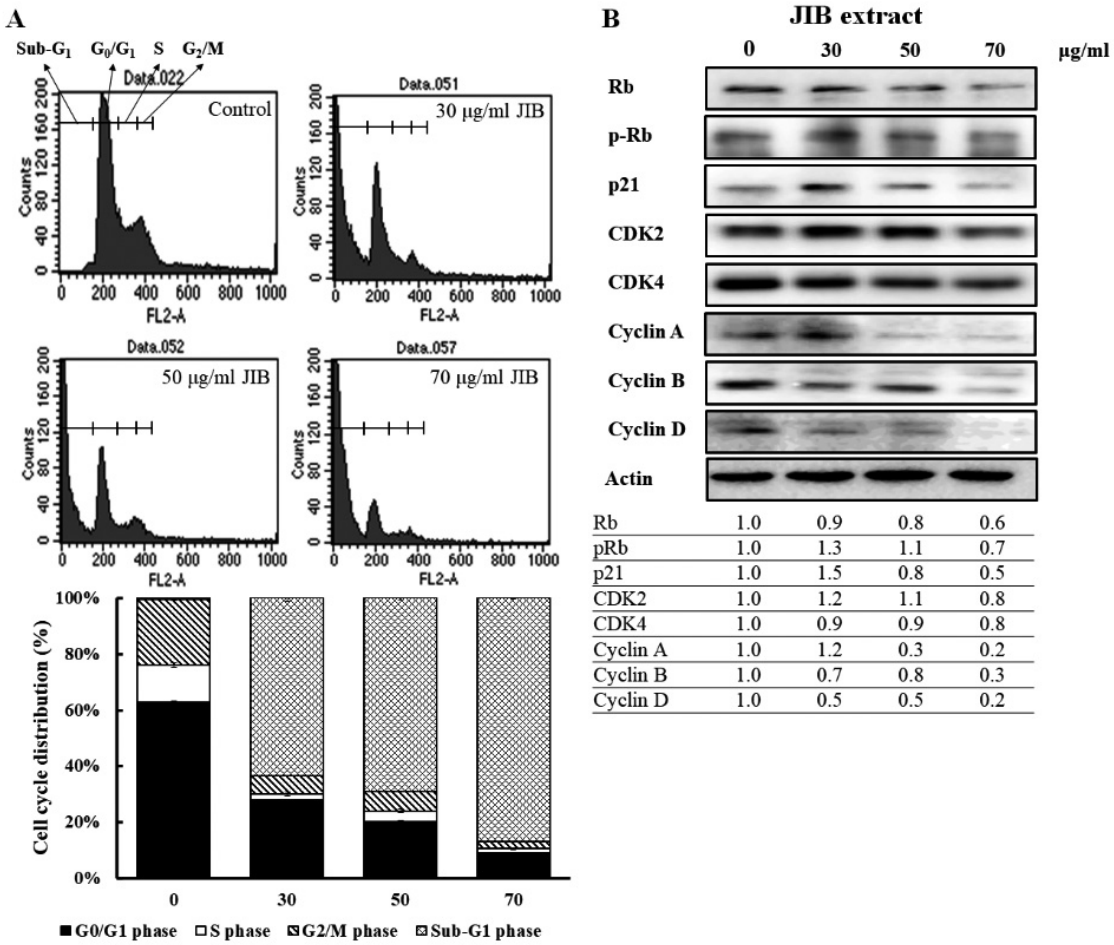
Figure 1. Effects of JIB extract on HCC cells
HCC cells were treated with JIB extract (< 90 μg/ml) or growth medium as control for 24, 48, and 72 h. Cell viability was measured using the MTT assay.

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Table 1. The inhibition potential of JIB extract and VP-16 on HCC and normal cells

Cell line	Tumor type	JIB extract (μg/ml)			VP-16 (μg/ml)		
Hepatocellular carcinoma cell		**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
$HepG_2$	HCC cells	40.59±0.67	34.42±0.29	37.45±3.59	> 200	6.57±2.46	< 6.25
Mahlavu	HCC cells	65.33±2.84	63.82±1.82	61.54±2.94	45.35±1.13	< 6.25	< 6.25
Normal cell							
MDCK	Canine kidney epithelial cell	75.8 ±2.71	74.17±2.73	77.89±0.6	> 50	25.01±0.04	18.2±3.3
SVEC	Mouse vascular endothelial cell	91.13±5.99	82.04 ±0.43	77.84±0.44	21.7±4.9	1.9±0.06	1.8±0.01

Note: All the values showed by mean ± standard deviation (SD).



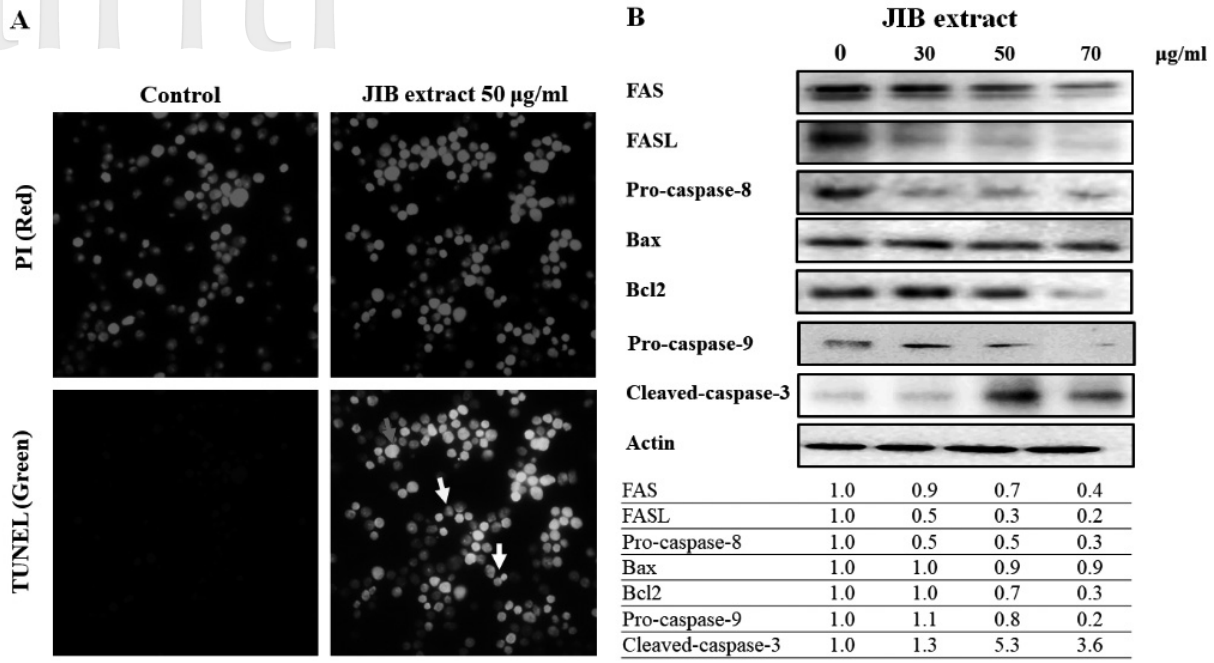
Rb	1.0	0.9	0.8	0.6
pRb	1.0	1.3	1.1	0.7
p21	1.0	1.5	0.8	0.5
CDK2	1.0	1.2	1.1	0.8
CDK4	1.0	0.9	0.9	0.8
Cyclin A	1.0	1.2	0.3	0.2
Cyclin B	1.0	0.7	0.8	0.3
Cyclin D	1.0	0.5	0.5	0.2

Phase/JIB (μg/ml)	0	30	50	70
$G_0/G_{1\ phase}$	62.72±0.64	27.81±0.14*	20.27±0.45*	9.16±0.14*
S phase	13.25±0.73	2.24±0.87*	3.74±0.51*	1.37±0.22*
G_2/M phase	23.40±0.45	6.53±0.17*	6.99±0.22*	2.56±0.03*
Sub-G_1 phase	0.62±0.03	63.42±1.18#	68.99±0.53#	86.91±0.38#

Figure 2. JIB extract induced cell cycle arrest

(A) After cell treatment with or without JIB extract (30, 50 and 70 μg/ml), the cell cycle population was analyzed via flow cytometer and quantified as percentages (%). (B) Cells analyzed by immunoblotting with antibodies for Rb, p-Rb, p21, CDK2, CDK4, Cyclin A, cyclin B and cyclin D1 after 24 h of cell treatment with various JIB extract concentrations (0, 30, 50 and 70 μg/ml). *: significant difference decreasing between the untreated and treated groups. $p < 0.05$. #: significant difference increasing between the untreated and treated groups. $p < 0.05$.

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FAS	1.0	0.9	0.7	0.4
FASL	1.0	0.5	0.3	0.2
Pro-caspase-8	1.0	0.5	0.5	0.3
Bax	1.0	1.0	0.9	0.9
Bcl2	1.0	1.0	0.7	0.3
Pro-caspase-9	1.0	1.1	0.8	0.2
Cleaved-caspase-3	1.0	1.3	5.3	3.6

Figure 3. JIB extract induced apoptosis via extrinsic and intrinsic pathways in $HepG_2$ cell
(A) The apoptotic cells were detected by TUNEL assay after cell was treated with JIB extract (50 μg/ml) for 24 h; red: PI; green: positive TUNEL; red arrow: DNA fragments; yellow arrow: anoikis; white arrow: apoptotic bodies. (B) Cells analyzed by immunoblotting with antibodies for FAS, FASL, pro-caspase-8, bax, bcl2, pro-caspase-9, and cleaved-caspase-3 after 24 h of JIB extract treatment at various concentrations.

of the JIB extract treatments reduced in a dose dependent manner; suggesting that JIB extract inhibited the $HepG_2$ and Mahlavu cell growth in vitro (Figure 1). The inhibitory concentration (IC_{50}) is presented in Table 1. Both JIB extract and etoposide (VP-16) presented inhibitory effects to those cells. The results revealed that the IC_{50} of $HepG_2$ and Mahlavu cells ranged from 37.45 ± 3.59 to 65.33 ± 2.84 μg/ml, at the indicated time interval after the JIB extract treatment. Furthermore, the IC_{50} of normal cells with the JIB extract treatment ranged from 74.17 ± 2.73 to 92.13 ± 5.99 μg/ml. These results indicated that JIB inhibited HCC cell growth, and that JIB extract may be less cytotoxic to kidney epithelial cells (MDCK) and vascular endothelial cells (SVEC) with IC_{50} ranging from 1.3- to 2.3-fold higher than tumor cells.

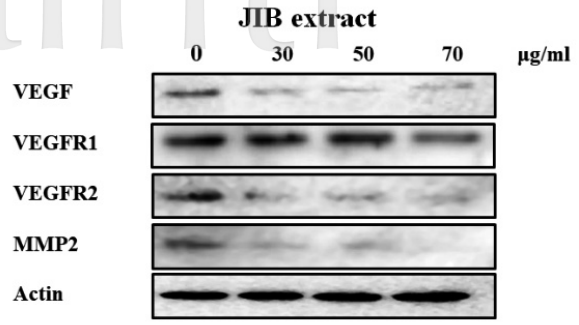
3.2. JIB extract induced cell cycle arrest by down-regulating phosphorylated Rb and p21 protein expressions

The results revealed that doses of JIB extract significantly induced the cell cycle arrest at the G_0/G_1 phase with decreasing of G_2/M and S phase in living cell population (Data not shown). The sub-G_1 phase significantly increased after JIB extract treatment (Figure 2A). Western blot analysis was performed on the key signaling proteins associated with the cell cycle to better understand the mechanisms of the JIB extract on cell cycle regulations. JIB extract reduced the protein levels of total Rb and phosphorylated Rb, and also significantly reduced the protein levels of CDK2, CDK4, cyclin A, cyclin B, and cyclin D, but increased the levels of p21 protein compared to the drug free treatment (Figure 2B). This outcome suggested that the JIB extract induced cell cycle arrest at G_0/G_1 phase and contributed to inhibiting the growth of $HepG_2$ cells.

3.3. Typical morphological changes and mediated apoptosis illustrated that the JIB extract induced caspase dependent apoptosis in $HepG_2$ cells

TUNEL staining revealed that JIB extract caused anoikis, DNA fragmentation, chromatin condensation, and apoptotic bodies formation (Figure 3A). Cellular apoptosis of $HepG_2$ caused by JIB extract

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VEGF	1.0	0.3	0.2	0.2
VEGFR1	1.0	1.0	1.0	0.7
VEGFR2	1.0	0.2	0.2	0.1
MMP2	1.0	0.2	0.2	0.1

Figure 4. JIB extract inhibited angiogenesis and expression of metastasis related proteins. VEGF, VEGFR1, VEGFR2 and MMP2 were detected by immunoblotting analysis at various concentrations of JIB extract (30, 50 and 70 μg/ml) after 24 h.

was demonstrated by increased levels of caspase-3 cleavage. Proteins related to extrinsic apoptosis such as FAS, FASL, and pro-caspase-8 were reduced. Moreover, proteins related to intrinsic apoptosis such as bcl2 and pro-caspase-9 decreased, while the bax/bcl2 ratio increased (Figure 3B); indicating that JIB extract induced $HepG_2$ cell apoptosis via activation of caspase cascade.

3.4. JIB extract inhibited VEGF/VEGFR-1, -2 proteins and MMP2 protein expressions

HCC tumors with an overexpressed vascular endothelial growth factor (VEGF); that generally have high proliferative capacity via autocrine proliferation, were investigated to determine the inhibitory effects of JIB extract on $HepG_2$ cells. The results showed that JIB extract inhibited the levels of VEGF, VEGFR1, and VEGFR2 protein expression, and reduced the level of MMP2 protein expression. These findings indicated that JIB extract might exert the inhibitory effects on $HepG_2$ cells through repression of VEGF, VEGFR1, and VEGFR2 protein expression (Figure 4).

4. Discussion

The hepatoprotective effects of *Juniperus* has been studied, however, its anti-cancer activity on hepatocellular carcinoma is still unclear. As a result, our study first demonstrated the anti-hepatoma effects of the JIB extract. Chemotherapy is the main treatment used for patients with late stage of hepatocellular carcinoma, however, the toxicity of chemodrugs often induced strong side effects such as liver or bone marrow toxicity. JIB extract is a natural plant extract that may be easily metabolized in the liver compared to chemodrugs. The results revealed that JIB extract treatment inhibited HCC cell growth and had less cytotoxic potential towards normal cells than the VP-16 treatment.

The cell cycle and apoptotic pathway was further investigated to address the inhibitory effects induced by the JIB extract. The cell cycle is the progression of cell proliferation, and is commonly overprocessed in cancer cells. The retinoblastoma protein (Rb), which is a tumor suppressor protein that inhibits the cell cycle progression and excessive cell growth, is usually dysfunctional in cancers (19). While Rb is phosphorylated, the cell cycle is activated and improves cell growth. The results found that JIB extract induced cell cycle arrest at the G_0/G_1 phase by downregulating phosphorylated Rb and total Rb, reducing cell cycle regulator levels, and increasing the p21 protein, which is also known as the cyclin-dependent kinase inhibitor.

On the other hand, after JIB extract treatment, HCC cells had been inhibited and triggered cell death. However, there was no precise evidence to determine whether JIB extract induced HCC cell apoptosis. Therefore, we performed the TUNEL assay to detect the cell apoptosis morphology, and the results exhibited a cell apoptotic cell morphology with anoikis, DNA fragmentation, chromatin condensation, and apoptotic body formation. Afterwards, the caspase associated pathway was investigated. The data showed that the JIB extract induced increasing of cleaved caspase-3 expression contributing to caspase cascade activation. Furthermore, the JIB extract suppressed the VEGR/VEGFR induced autocrine in hepatocellular carcinoma, and also inhibited the metastatic related protein levels (MMP2), which may prevent the possibility of cancer metastasis. In previous study, Lohani et al. has descripted the components of *J. indica Bertol* essential oil by GC/MS and the results showed that the major components characterized in the leaves and berries of *J. indica*

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Bertol were sabinene (27.75% and 23.17%), terpinen-4 (16.11% and 23.61%), α-pinene (6.34% and 8.82%) and γ-terpinene (6.05% and 6.58%) (20, 21). In addition, we had analyzed the bioactive compounds in JIB extract by GC/MS. The five major components in JIB extract involves α-pinene (34.87%), citronellyl acetate (14.26%), limonene (10.72%), terpinolene (10.65%) and cymene (6.24%). Among these components, α-pinene and terpinen-4 has found the anticancer ability on hepatoma and gastrointestinal cancer in vitro and in vivo (22, 23). As a result, α-pinene as the one of the major components in JIB extract that might contribute to exerted the antihepatoma activity in our study.

Taken together, the JIB extract might be a potential anti-cancer extract that inhibits hepatocellular carcinoma growth by inhibiting the progression of the cell cycle and inducing cell apoptosis via caspase cascade.

Acknowledgments

This study was funded by the research grant CSH-2015-A-011 from the Chung Shan Medical University Hospital, Taiwan and acknowledged the Instrument Center of Chung Shan Medical University for the usage of facility.

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