International Journal of Applied Science and Engineering

Vernonia amygdalina extract induces apoptosis and inhibits epithelial-mesenchymal transition in Hep 3B cells through the inhibition of PI3k/Akt signaling pathway

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ABSTRACT

Hepatocellular carcinoma (HCC) is more common around the world and due to multiple hepatocarcinogenic causes, it is imperative to find drugs that can alleviate the worsening of liver disease or inhibit the occurrence of liver cancer. Recent studies found that *Vernonia amygdalina* (VA) extracts exhibited the anti-cancer ability, including breast, prostate and nasopharynx cancer. Moreover, recent experiments showed that VA extract has the potential to be hepatoprotective, antioxidant, and antifibrotic to the liver. In this study, the anti-cancer effects and its possible mechanisms of VA extracts in human hepatoma cancer Hep 3B cells were investigated. Western blot, flow cytometry, and cell migration and invasion assays were used to explore the anticancer effects of VA extracts against HCC cells. The MTT assay revealed that the chloroform-leave extract of VA (VA-6) exhibited the most potent ability to inhibit the proliferation of Hep 3B cells than other extracts. Flow cytometric analysis found that VA-6 induced apoptosis in Hep 3B cells. Western blotting demonstrated VA-6 induced apoptosis through the inhibition of PI3K/Akt signaling pathway. The results showed that cancer migration and invasion could be repressed by VA-6 through the inhibition of epithelial-mesenchymal transition (EMT). Interestingly, we found that VA-6 enhanced the sensitivity of paclitaxel and doxorubicin to Hep 3B cell growth.

Keywords: Hepatocellular carcinoma, Vernonia amygdalina, Epithelial-mesenchymal transition, Apoptosis.

OPEN ACCESS

Received: March 17, 2021
Revised: November 2, 2021
Accepted: November 3, 2021

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Publisher:
Chaoyang University of Technology
ISSN: 1727-2394 (Print)
ISSN: 1727-7841 (Online)

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and the fifth lethal cancer in the world. HCC mortality rates have increased in recent decades around the world, particularly in underdeveloped countries (Sanaei et al., 2018; Bertuccio et al., 2017; Schlachterman et al., 2015). Although research has confirmed that risk factors such as chronic HBV or HCV infection, diabetes, cirrhosis, and excessive alcohol consumption can increase the risk of primary liver cancer, most people do not have symptoms and signs in the early stages of HCC (Corey et al., 2017; Gallagher and LeRoith, 2015; Kudo et al., 2014; Morgan et al., 2013). Furthermore, hepatocarcinogenic causes are multiple and intricate, HCC still does not have clear curative treatment (Schlachterman et al., 2015). The major treatment methods for HCC in clinical settings are surgical treatment, immunotherapy, chemotherapy, and radiotherapy. Unfortunately, most patients did not experience long-term benefit and the overall survival rate of HCC patients remained unchanged (Allemani et al., 2018). Above all, it has become crucial to develop novel effective drugs that can inhibit the occurrence of HCC.

Recent studies have demonstrated that epithelial-mesenchymal transition (EMT) is closely related to HCC development and promote invasion, metastasis, recurrence, and poor prognosis (Gurzu et al., 2019). Through the EMT process, HCC cells develop powerful invasive and metastatic abilities and allow tumor cells to migrate through the extracellular circulatory system to different organs. Therefore, EMT regulators may possibly become a novel biomarker and attractive therapeutic target in HCC (Giannelli et al., 2016). The most important molecular features of EMT could be deduced in terms of the epithelial markers (such as E-cadherin) decreased and mesenchymal markers (such as N-cadherin, vimentin, and fibronectin) increased (Mikesh et al., 2010). However, the specific regulatory factors and molecular networks that initiate EMT are not yet fully understood.

HCC can easily metastasize and traditional therapeutic agents including doxorubicin, cisplatin and paclitaxel are limited by therapeutic resistance, which results in poor efficacy of chemotherapeutic agents (Jin et al., 2010; Burroughs et al., 2004; Ganne-Carrie and Trinchet, 2004). Consequently, the development of some novel therapeutic agents is urgently required to treat HCC patients clinically. In an effort to improve response rates to chemotherapy, systemic combination chemotherapy is usually used. Therefore, new strategies in which the discovery of a new combination of drugs can increase the efficacy and safety are important to improve the therapeutic efficacy of HCC.

Vernonia amygdalina (VA), also called 'bitter leaf' due to its taste, is a kind of the wood shrub of Asteraceae family (Farombi and Owoeye, 2011). VA has been shown to have various therapeutic effects such as anti-hepatitis (Yineger et al., 2007), anti-malaria (Abosi and Raseroka, 2003), anti-diabetes (Wu et al., 2018), and anti-cancer. Previous studies have provided some useful evidence on the anticancer activities of VA against human nasopharynx cancer (Kupchan et al., 1969), prostate cancer (Johnson et al., 2017), and leukemia cells (Jisaka et al., 1993). Recent experiments showed that VA have the potential effects of hepatoprotective, antioxidant and anti-fibrotic of liver (Imafidon et al., 2018; Iwalokun et al., 2006).

All of these studies indicated that VA may be a potential drug to inhibit the occurrence of HCC. However, there is no relevant research on whether VA extracts could inhibit HCC cell growth. The objective of this study is to examined the anti-cancer effects of VA extracts and possible mechanisms of action in HCC Hepa 3B cells.

2. MATERIALS AND METHODS

2.1 Materials

Doxorubicin, paclitaxel, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), anti-β-actin antibody, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for poly (ADP-ribose) polymerase (PARP), caspase-3, caspase-9, Bcl-2, Bcl-xL, Survivin, Akt and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). The antibodies for Bax, X-linked inhibitor of apoptosis protein (XIAP), E-cadherin, N-cadherin, Vimentin, Snail, Slug, and Twist were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Preparation of Plant Extract

The VA used in this study was provided by our group. The process is depicted in Fig. 1. Briefly, fresh harvested plants are divided into two groups, stems and leaves. First, both groups are sun dried before being soaked in 85% denatured ethanol, separately obtaining ethanol-stem VA extract (VA-5) and ethanol-leave VA extract (VA-9). Secondly, part of VA-5 and VA-9 were added to hexane for liquid- liquid extracting, the hexane layer was concentrated by rotary evaporation to achieve the hexane-stem VA extract (VA-4) and the hexane-leave VA extract (VA-8). At the same time, the aquifer material was added to chloroform for the chloroform-stem leave extract (VA-2) and chloroform-leave extract (VA-6). Finally, the water layer will be added to *n*-butanol to separate into *n*-butanol stem extract (VA-3), *n*-butanol leave extract (VA-7), and water stem extract (VA-1).

2.3 Cell Lines

The human HCC cell line (Hepa 3B cells), normal liver cells and hepatocytes were purchased from the American Type Culture Collection (ATCC) (Manassa, VA). Cells were cultured as monolayers at 37°C in an incubator of 95% air

and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin – streptomycin (PS).

2.4 Cell Viability Assay

The MTT assay was used to evaluate cell viability. Cells were cultured in a 96-well plate at a specific density. When 70% to 80% confluence was achieved, the compounds tested were added. The MTT (500 μg/mL) was then added into each well. The culture medium was removed after incubation for 2 h and the formed crystals were dissolved in 80 μL dimethyl sulfoxide (DMSO). Subsequently, the absorbance at 570 nm was determined using an ELISA reader.

2.5 Western Blotting

Equal amounts (50 μg/lane) of total proteins were fractionated on SDS-PAGE gels and transferred onto PVDF membrane (polyvinylidene difluoride). After blocking with PBS containing BSA (3%) for 2 h at room temperature, cells were washed twice with the washing buffer and then incubated with the indicated primary antibodies for 2 h at room temperature. After washing with PBS, the membranes were incubated with secondary antibodies for 1 h. Finally, the western blot bands were developed using the ECL Western blotting kit.

2.6 Annexin V-FITC (Fluorescein Isothiocyanate)/ PI Assay

An evaluation of necrotic and apoptotic cells was performed using the Annexin V-FITC kit according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were cultured in six-well plates (5×10^4 cells/well) and incubated for 24 h prior to treatment with VA extracts. The cells were then incubated for another 48 h with VA extracts. Cells were harvested in a 0.25% trypsin-EDTA solution and washed twice with PBS. After centrifugation, 250 μL Annexin V binding buffer was mixed with the cell, resuspended in 250 μL Annexin V-FITC added binding buffer for 5 min in darkness at room temperature, then an equal volume of Annexin V-FITC and PI solutions was added. Samples are immediately analyzed by flow cytometry. For each experiment, 10,000 events per sample were recorded.

2.7 Cell Migration and Invasion Assay

Migration and invasion assays were performed in a transwell system (8 μm pore size; Millipore). The cells (3×10^4) in serum-free medium were cultured in the upper compartment of the transwell chambers, and 10% FBS was employed to the lower compartment. For invasion assays, the transwell filter was additionally coated on the upper side with Matrigel (Collaborative Research, Inc., Boston, MA). Cells were scraped from the upper side of the filter at the end of the incubation period, while cells adhered to the underside of the membrane were fixed with 4% formaldehyde for 20 min and stained with crystal violet for 30 to 45 min. To evaluate the number of cells that had invaded the membrane, the cells were counted using a light microscope.

2.8 Statistical Analysis

All experiments were performed in triplicate and all data were presented as mean ±SD. The comparison between means was determined using the Student's *t*-test. The mean data were determined to be statistically significant if the values were 0.05 or less.

3. RESULTS

3.1 The Chloroform Extract of VA Inhibits Cell Proliferation in Hep 3B Cells

In this study, the MTT assay was used to determine the effect of VA-1 to VA-9 on Hep 3B cell proliferation. According to the extract process shown in Fig. 1, nine VA extracts were obtained, VA-1 to VA-9. As shown in Fig. 2A, Hep 3B cells were treated with different concentrations of each VA extract fraction for 48 h. The ethanol-stem VA extract (VA-5) and chloroform-leave extract (VA-6) exhibited the most potent ability to inhibit the proliferation of Hep 3B. However, VA-6 was selected for the subsequent experiments because it has a more effective ability to induce Hep 3B at higher concentration. After treatment, Hep 3B cells with various concentrations (0-100 μg/mL) of VA-6 for 24, and 72 h. The cell survival rate decreased significantly with the increase of VA-6 concentration (Fig. 2B). Morphological features were characterized with a microscope, and our result found that VA-6 altered the cellular morphology and the number of cells (Fig. 2C). To determine whether VA-6 has no cytotoxicity to normal liver cells, hepatocytes were treated with the indicated VA-6 for 48 h (Fig. 2D). After treatment with VA-6 for 48 h, hepatocytes still have more than 80% cell viability of the control, which demonstrated that VA-6 is not cytotoxic to normal liver cells under this condition.

3.2 VA-6 Induces Apoptosis in Hep 3B Cells

To investigate the reduction in total viable cells whether or not through VA-6 induction of cell apoptosis, Hep 3B cells were treated with 30, 60 and 90 μg/mL VA-6 for 48 h and stained with Annexin V/PI. The flow cytometric assays indicated that VA-6 induced apoptotic cell death in a dose-dependent manner (Fig. 3A). For further confirming that VA-6 could induced an apoptotic cell death-associated protein expression, collected cells were subjected for Western blotting analysis and the results are shown in Fig. 3B. After VA-6 treatment at different concentrations, Hep 3B cells exhibited increased PARP activity. The activities of caspase-3 and -9 in Hep 3B cells also increased after VA-6 treatment at different concentrations. To further clarify the circumstances of VA-6-related apoptosis-related proteins, protein expression levels of various established apoptotic

markers were examined. As demonstrated in Fig. 3C, 3D, VA-6 treatment resulted in a decrease in the protein expression levels of Bcl-2, Bcl-xL, Survivin, XIAP and an increase in Bax expression.

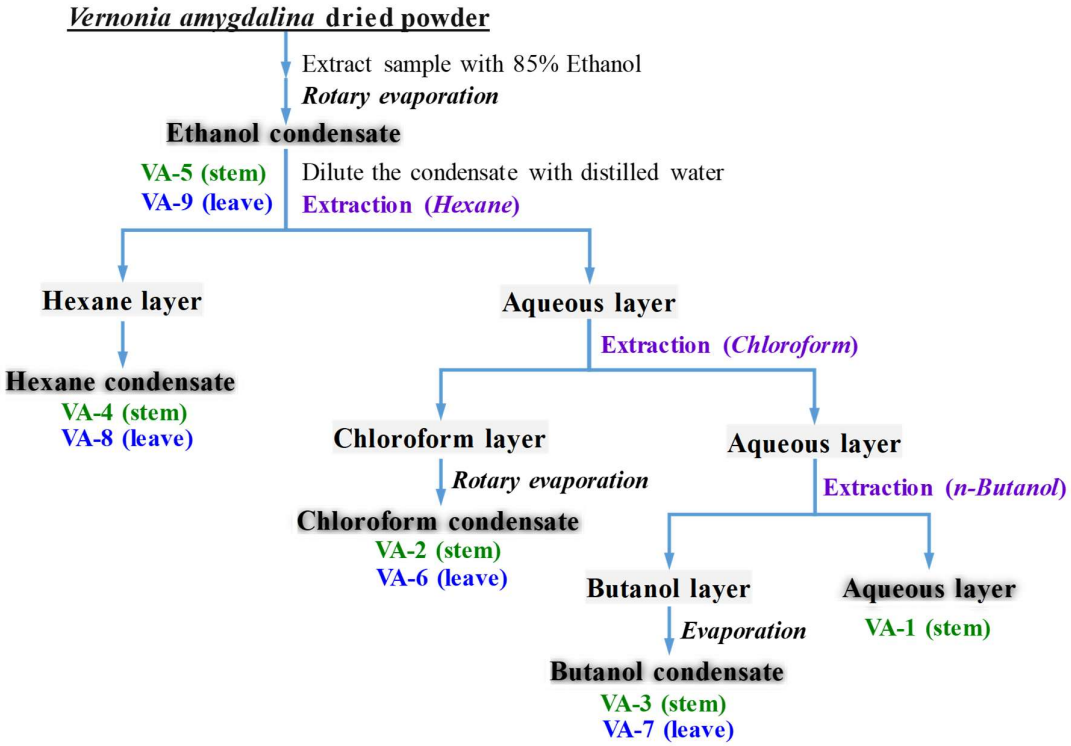
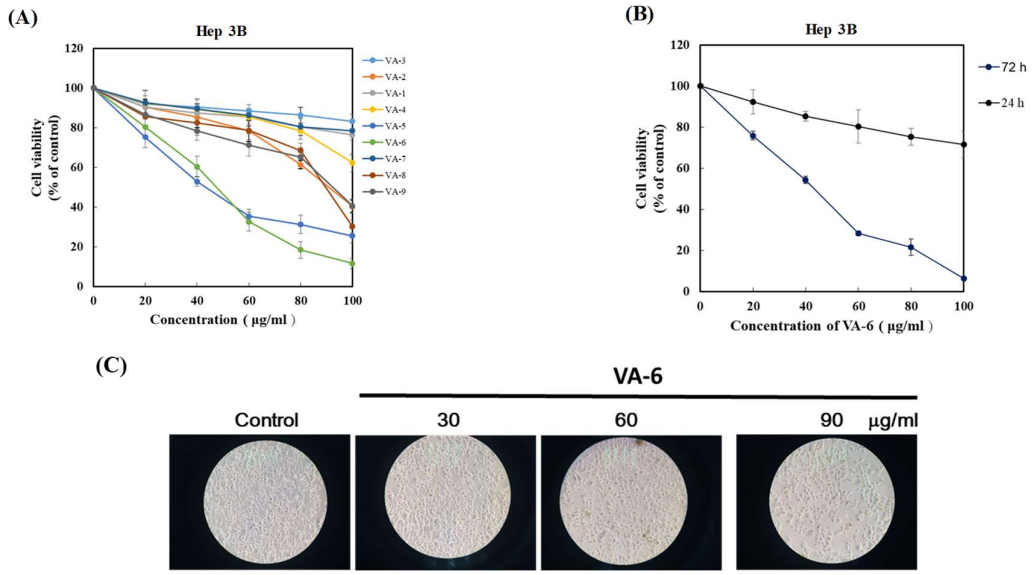


Fig. 1. The extraction process of *Vernonia amygdalina* (VA). VA stem water extract (VA-1), VA stem chloroform extract (VA-2), VA stem *n*-butanol extract (VA-3), VA stem hexane extract (VA-4), VA stem ethanol extract (VA-5), VA leave chloroform extract (VA-6), VA leave *n*-butanol extract (VA-7), VA leave hexane extract (VA-8), and VA leave ethanol extract (VA-9).



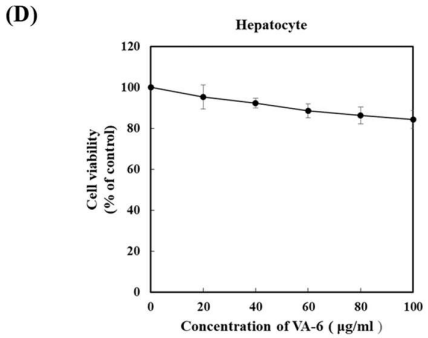


Fig. 2. VA-6 inhibited Hep3B cell proliferation and has no cytotoxicity to hepatocytes. (A) Hep3B cells were treated with various concentrations of VA extracts for 48 h. (B) Hep3B cells were treated with various concentrations of VA-6 for different times. (C) Phase contrast photomicrographs of HepG2 cells after a 48 h treatment with various concentrations of VA-6. (D) Hepatocyte cells were treated with various concentrations of VA-6 for 48 h. Cell viability was measured using an MTT assay. Each experiment was performed in triplicate, and the error bars represent the standard deviation

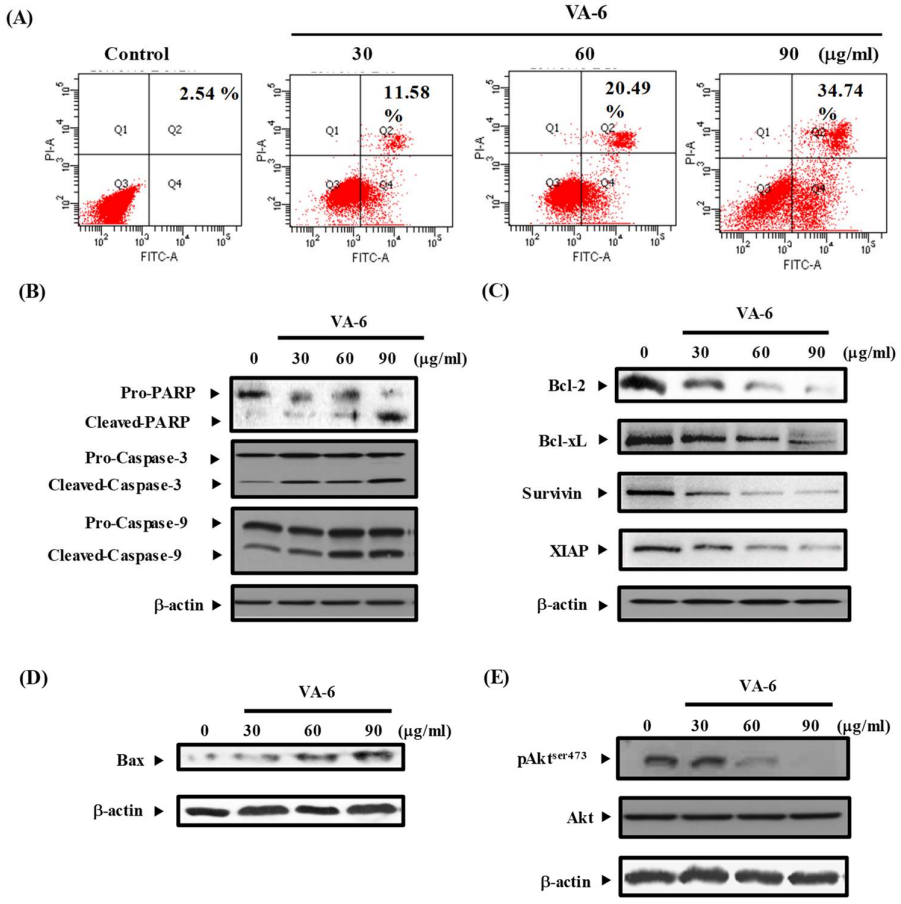


Fig. 3. VA-6 induces apoptosis by inhibiting the PI3k / Akt signaling pathway. (A) Hep3B cells treated with various concentrations of VA-6 for 48 h were subjected to flow cytometry. Cells were stained with Annexin V-FITC/PI for 10 min. Hep3B cells were treated with various concentrations of VA-6 for 48 h. (B) The protein levels of PARP, Caspase-3, Caspase-9, and β-actin were assayed by Western blot. (C) Protein levels of Bcl-2, Bcl-xL, Survivin, XIAP, and β-actin were assayed by Western blot. (D) The protein levels of Bax, and β-actin were assayed by Western blot. (E) Protein levels of $pAkt^{ser473}$, Akt, and β-actin were assayed by Western blot. Each experiment was performed in triplica

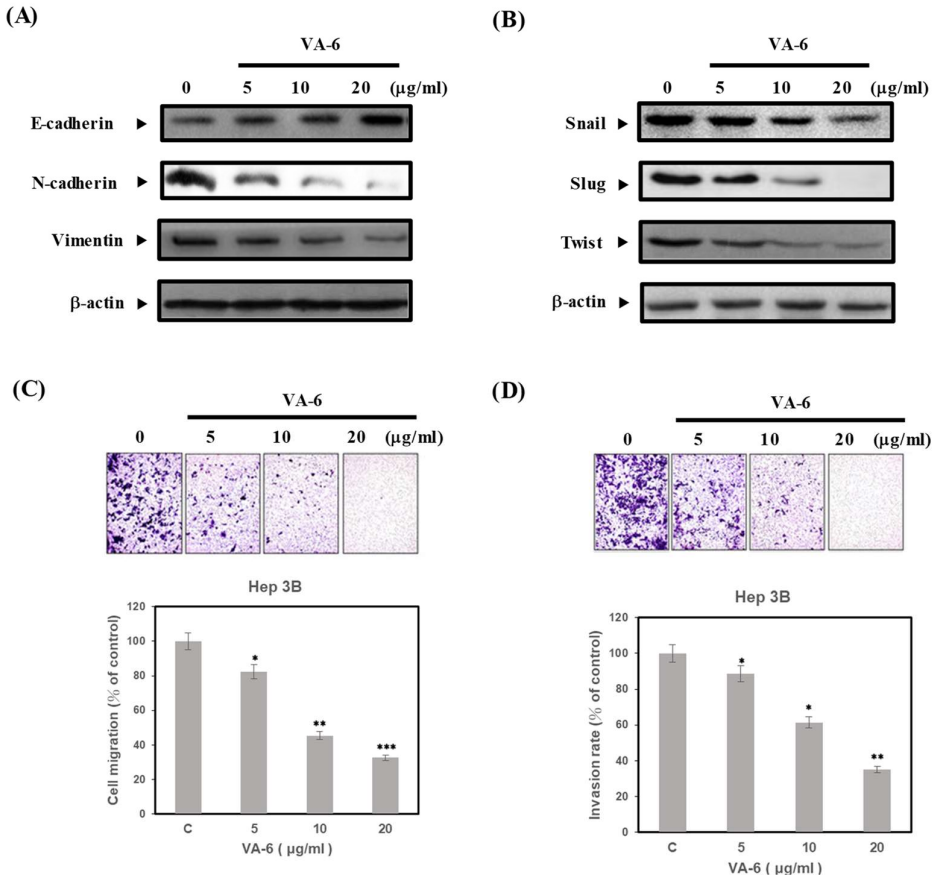


Fig. 4. VA-6 inhibits the migration and invasion capacities of Hep 3B cells by inhibiting EMT. (A) Hep3B cells were treated with various concentrations of VA-6 for 48 h. Protein levels of E-cadherin, N-cadherin, vimentin, and β-actin were assayed by Western blot. (B) Hep3B cells were treated with various concentrations of VA-6 for 48 h. Protein levels of snail, slug, Twist, and β-actin were assayed by Western blot. Hep3B cells were treated with various concentrations of VA-6 for 24 h. The migration ability of Hep3B cells with or without VA-6 stimulation was detected by the Transwell migration (C) and invasion (D) assay. Each experiment was performed in triplicate, and the error bars represent the standard deviation. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

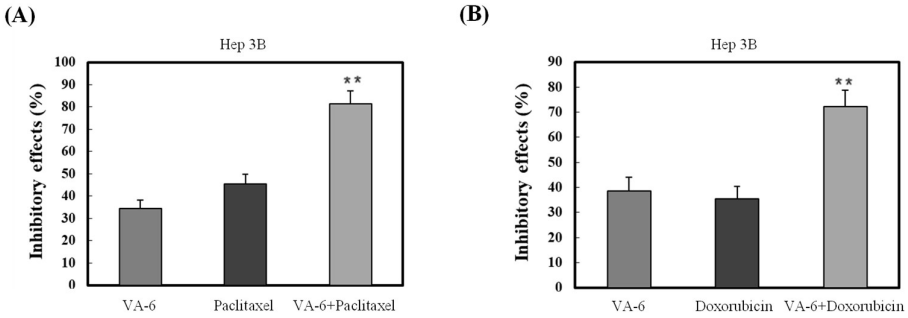


Fig. 5. Effect of Paclitaxel, and Doxorubicin alone or in combination with VA-6 on the proliferation of Hep 3B cells. Hep 3B cells were treated with 30 μg/mL VA-6 alone or in combination with (A) 400 nM Paclitaxel, (B) 2.5 μM Doxorubicin at 37°C for 48 h. Cell viability was measured using an MTT assay. Each experiment was performed in triplicate, and the error bars represent the standard deviation. $p < 0.01$ (**). The inhibitory effect was calculated as 100% minus the percentage of cell proliferation.

3.3 VA-6 Induces Apoptosis Through Inhibition of The Akt Survival Signaling Pathway

Given the important role of the phosphatidylinositol 3'-kinase/protein kinase B (PI3K/Akt) pathway, a classical oncogenic signaling pathway, in controlling cell survival/death of cancer cells. We determined whether Akt activity is associated with the apoptotic effects of VA-6. As can be seen from the data, the VA-6 suppressed the phosphorylated levels of Akt in Hep3B cells without significantly altering the levels of total Akt was observed (Fig. 3E). This indicated that VA-6 cytotoxicity may be related to inhibition of the PI3K / Akt pathway.

3.4 VA-6 Inhibited EMT

Recent studies have shown that EMT is an important process in tumor invasion and metastasis (Giannelli et al., 2016; Mikesh et al., 2010). To determine whether VA-6 inhibited EMT and prevented the development of metastasis in Hep 3B cells, Western blotting was performed to analyze the effects of VA-6 on proteins associated with EMT. The non-toxic doses of VA-6 were then used in the following experiments. The experimental results demonstrated that increasing VA-6 concentrations significantly increased the expression of the epithelial marker E-cadherin but decreased the expression of the mesenchymal cell marker Vimentin and N-cadherin (Fig. 4 A). Interestingly, treatment of Hep 3B cells with VA-6 significantly reduced the expression of EMT-promoting transcription factors such as Snail, Slug and Twist in a dose-dependent manner (Fig. 4B). To test whether VA-6 could inhibit the migration and invasion of HCC cells, we conducted a transwell migration and invasion assay. The results showed that treatment of Hep 3B cells with increasing concentrations of VA-6 significantly inhibited cancer cell migration (Fig. 4C) and invasion (Fig. 4D). Thus, these results confirmed that VA-6 could inhibit HCC migration and invasion by suppressing EMT in HCC cells.

3.5 VA-6 Enhances the Sensitivity of Paclitaxel and Doxorubicin to the Growth of Hep 3B Cells

The search for a new combination of drugs for HCC is important to improve the therapeutic efficacy. To sensitize Hep 3B cells to this candidate chemotherapeutic drug, the MTT assay was performed to determine the synergistic effect of VA-6 combined with paclitaxel or doxorubicin. The combination of VA-6 and paclitaxel (Fig. 5A), or doxorubicin (Fig. 5B) resulted in more significant tumor inhibition was observed, compared to the individual sample. The study may provide a new combination of VA-6 and paclitaxel or doxorubicin for the treatment of HCC.

4. DISCUSSION

To evaluate the effectiveness potential of VA extracts on the proliferation of HCC cells and to determine its anticancer activity potential, the MTT assay and cell morphology analysis were performed. The data generated from these series of experiments suggested that VA extracts inhibit Hep 3B cell growth significantly in a dose-dependent manner. Among these, VA-6 exhibited the most potent ability. Tumor cells spread and proliferated in the control group, as seen from cell morphology observation. Likewise, the results of Western blotting demonstrated that VA-6 could induce apoptosis through inhibition of the PI3k / Akt signaling pathway. However, the experimental data also revealed that VA-6 inhibited cancer migration and invasion through the inhibition of EMT in HCC. VA-6, indeed, has the synergistic effect of being combined with paclitaxel, or doxorubicin. Therefore, it suggests that VA-6 could also be applied in conjunction with chemotherapeutic drugs for the treatment of HCC.

To the best of our knowledge, no study was available on the potential for apoptosis of VA extracts in HCC. In this study, we found that the cytotoxic efficacy of VA extracts in Hep 3B cells may be associated with apoptosis. The findings of this study are consistent with previous studies that found that VA extract treatment induces apoptosis in human breast cancer MCF-7 cells (Fachrunisa et al., 2019; Yedjou et al., 2008). Yedjou et al. (2018) suggested that induction of DNA damage and apoptosis are involved in the therapeutic efficacy of VA in acute promyelocytic leukemia. Johnson et al. (2017) found that VA caused growth-inhibitory effects in PC-3 cells by inducing cell growth arrest, DNA damage, and apoptosis. Nowadays, cancer cells have evolved defense mechanisms in order to dodge from the apoptosis-inducing signaling pathway for survive. Therefore, targeting apoptosis in tumor cells may be an effective treatment approach for all types of cancer therapy.

In this study, we found that VA-6 inhibits Hep3B cell migration *in vitro* by transwell migration and invasion assays. Accumulating evidence has shown that EMT has been identified as crucial drivers of tumor progression and is widely and intensely studied in the fields of HCC invasion and metastasis. Therefore, we speculate that VA-6 inhibits Hep3B cell migration and metastasis and may also be related to inhibition of EMT. This is indeed supported by our hypothesis from the results of the Western blot experiments. Our study found that VA-6 could up-regulate epithelial markers, E-cadherin, and down-regulate mesenchymal markers, Vimentin (a cytoskeletal protein) as well as N-cadherin (a cell surface protein). To our knowledge, this is the first study to reveal that VA might inhibit tumor migration and metastasis by impeding the EMT progression of HCC cells.

Theoretically, clinical surgery or chemotherapy is the preferred therapeutic strategy for advanced HCC. However, patients with advanced HCC are not suitable for surgery due

to the metastasis and drug resistant nature of terminal HCC. Actually, there have been few effective chemotherapeutic agents available for HCC treatment. Among them, doxorubicin and paclitaxel are commonly used chemotherapeutic drugs for the treatment of HCC (Jin et al., 2010). Our study demonstrated that VA-6 can synergize with doxorubicin and paclitaxel, together with their chemopreventive actions. The data presented provide hope for better cancer treatment, the absence of severe side effects, and improved survival rates for HCC patients. This supports the potential of VA extract as a powerful treatment for HCC, a health problem that disproportionately affects humans and reinforces the idea that VA extracts deserve more attention for further development as a phytoceutical and anticancer drug entity. Consistent with our findings, VA exhibits synergism when combined with doxorubicin, suggesting that it can complement current chemotherapy (Wong et al., 2013). Further *in vivo* studies may provide a new combination of cytotoxic drugs for HCC chemotherapy.

ACKNOWLEDGEMENTS

Experiments and data analysis were performed in part through the use of the Medical Research Core Facilities Center, Office of Research & Development at China medical University, Taichung, Taiwan, R.O.C.

CONFLICTS OF INTEREST

The Authors declare no conflict of interest in regard to this study.

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