Candidone inhibits migration and invasion, and induces apoptosis in HepG2 cells

Chantana Boonyarat, a Kanlaya Sangchavee, b, Kusawadee Plekratoke a, Chavi Yenjai, c Prasert Reubroycharoen, d Rawiwun Kaewamatawong b and Pornthip Waiwut, b*

aFaculty of Pharmaceutical Sciences, Khon Kean University, Khon Kean 40002, Thailand,
bFaculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani 34190, Thailand,
cNatural Products Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002 Thailand,
dDepartment of Chemical Technology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

*Corresponding author

Pornthip Waiwut, PhD
Faculty of Pharmaceutical Sciences, Ubon Ratchathani University,
Ubon Ratchathani 34190, Thailand
E-mail: porntip.w@ubu.ac.th
Summary

The aim of the study was to investigate the inhibitory activity of candidone, the active constituent of *Derris (D.) indica*, on the proliferation, migration, and invasiveness of human hepatoblastoma (HepG2) cells. Cancer cell death was assessed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, and apoptosis-associated morphological changes were observed by phase contrast microscopy. Additionally, western blotting was used to study protein expression following treatment with candidone, and transwell migration and invasion assays were used for observing cancer cell migration and invasiveness, respectively. The results suggest that candidone possesses potent inhibitory activity against HepG2 cells (concentration, 100 µM; 24 h treatment). Cancer cells treated with candidone exhibited apoptosis-associated changes, including detachment, cell shrinkage and death. Furthermore, candidone was shown to promote cell death by activating caspase-3 and -9, and decreasing the expression of antiapoptotic proteins, including p65, induced myeloid leukemia cell differentiation protein Mcl-1, B-cell lymphoma 2 (Bcl2), Bcl2-associated agonist of cell death and survivin. Moreover, candidone inhibited the migration and invasion abilities of HepG2 cells and decreased the levels of proteins associated with these processes, including phospho-p38 and active matrix metallopeptidase 9. Collectively, the results of the present study indicate that candidone is able to inhibit the proliferation, migration and invasive potential of HepG2 cells.

**Keywords:** Cancer; *Derris indica*; candidone; apoptosis; migration; invasion.
1. Introduction

Cancers of the liver predominantly occur in patients with chronic liver disease.\textsuperscript{1)} Chronic viral hepatitis infections such as hepatitis B and C, and exposure to the toxic metabolites of alcohol and/or aflatoxin are increasingly recognized as risk factors for liver cancer progression.\textsuperscript{2)} The prevalence of liver cancer is on the increase in Thailand, and is most evident in men.\textsuperscript{1)} Generally speaking, patients with liver cancer have a poor prognosis due to examination, and hence detection, most often occurring at a late disease stage. The therapeutic benefits and long-term survival rates of patients are limited due to the negative side effects of chemotherapy, which include fatigue, diarrhea, pain, nausea and vomiting, hair loss and cancer cell resistance to treatment.\textsuperscript{3)} To alleviate these side effects, alternative traditional plant medicines are currently being used in combination with pharmaceutical therapies.

\textit{Derris (D.) indica} is a plant that belongs to the Leguminosae family.\textsuperscript{4)} The tree is widely planted throughout Southeast Asia and is distributed across the southern part of Thailand.\textsuperscript{5)} Several parts of this plant have been used as traditional medicines for the treatment of cancer, ulcers, rheumatic arthritis, wounds and diabetes.\textsuperscript{6)} It has also been reported that various parts of \textit{D. indica} contain biologically active substances, such as flavonoids rotenoids and chalcones.\textsuperscript{7)} Additionally, the seeds consist of 27-39 \% oil which is used for the treatment of inflammatory and infectious diseases, as well as for the tanning of leather.\textsuperscript{8)} The oil itself consists of unsaturated and saturated fatty acids including oleic, stearic and palmitic acids.\textsuperscript{9)} A previous study revealed that \textit{D. indica} possessed cytotoxicity against the human HepG2 cancer cell line and the cholangiocarcinoma M156 cell line.\textsuperscript{7)} Furthermore, \textit{D. indica} has been shown to reduce the proliferative capacity of cancer cells by activating cleaved caspase 3, which is increased in the apoptotic cell by both extrinsic (death receptor)
and intrinsic (mitochondrial) pathways. A decrease in the B-cell lymphoma 2 (Bcl2) anti-apoptotic protein was also demonstrated.

The aim of the present study was to investigate the effects of candidone (Fig. 1), a flavanone isolated from *D. indica*, on the proliferation, migration, and invasion abilities of HepG2 cells. The mechanisms underlying the anti-tumor properties of candidone were also investigated using western blot analysis.

2. Materials and methods

Candidone was extracted from the seed of *D. indica* which was collected from Krabi Province, Thailand. The plant was identified by Pranom Chantaranothai, Faculty of Science, Khon Kaen University. A botanically identified voucher specimen (KKU0042012) was deposited at the herbarium of the Department of Chemistry, Faculty of Science, Khon Kaen University, Thailand. The isolation, structural elucidation and percentage yield were reported in a previous study.10)

2.1. Cell culture.

The HepG2 cell line was purchased from the American Type Culture Collection (ATCC) and authenticated using short tandem repeat analysis (also conducted by the ATCC). The cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10 % fetal bovine serum and 100 IU/ml penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA) and incubated in incubator at 37˚C (5% CO₂).

2.2. Cell cytotoxicity assay.

The cytotoxicity of candidone towards HepG2 cells was determined using an MTT assay.10) The cells were initially added to 96-well plates at a concentration of $1 \times 10^5$ cells/well, and incubated at 37˚C for 48 h. The cells were then incubated with various concentrations of candidone (1, 10 and 100 µM) for 24 h, using DMSO (dimethyl sulfoxide) as the negative control and 10 µg/ml doxorubicin as the positive control. The culture medium
was subsequently removed and 100 µl DMEM containing 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich; Merck KGaA] solution was added. The plates were incubated at 37°C for 2 h, after which the medium was discarded. The formazan product was determined by adding 100 µl DMSO to each well. Absorbance was measured at 600 nm using a microplate reader; the percentage of viable cells was then calculated using the following formula: % cell survival = [absorbance of treated cells/absorbance of control (untreated) cells] x 100.

2.3. Phase contrast microscopy.

HepG2 cells were cultured in a 96-well plate at a concentration of 1x10^5 cells/well and incubated at 37°C for 48 h. The cells were then incubated with 1, 10 and 100 µM candidone for a further 24 h. Following treatment, morphological changes were observed under a 20x phase contrast microscope.

2.4. Preparation of cell extracts.

In order to observe the mechanism of the candidone-induced apoptotic pathway, HepG2 cells were treated with different concentrations of candidone for 4 h. Preparation of whole cell lysates was accomplished by adding lysis buffer (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM dithiothreitol. Following centrifugation at 12,000 xg at 4 °C for 10 min, the supernatant fraction was collected, and the protein concentration was determined using a Bradford assay.

2.5. Western blot analysis.

Cell lysate samples 15 µl were loaded into each lane of a 10 or 12% gel and separated using SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membranes and blocked with a 5% (w/v) BSA blocking solution. The membranes were incubated with primary antibodies including anti-phospho p65 (3033), Bcl2 (3498), survivin (2808), Mcl-1 (94296), Bcl-xl (2764), caspase-9 (9502), caspase-3 (9662), cleaved caspase-3 (9664),
PARP-1 (9532), phosphor JNK (9255), phosphor p38 (4511), and actin (3700) (1:1,000; Cell Signaling Technology, Inc.) at 25 °C for 2 h with continuous shaking. The membranes were washed three times with washing buffer (0.01% Tween-20 in PBS), after which a horseradish peroxidase-conjugated secondary antibody (1:2,000) was added 25 °C for 45 min. After being washed another three times, the membranes were treated with ECL substrates (Life Science Technology) for 1 min and then visualized by X-ray film exposure.

2.6. Migration and invasion assays.

The cancer cell migration and invasion capacities were determined using a transwell assay. A total of 1x10^6 cells/well in serum-free DMEM were added into the top chambers of a 24-well insert (pore size, 8-mm; Corning Life Sciences) and incubated for 4 h to allow the cells to attach. The cells were treated with candidone and incubated for 24 h at 37°C. For the invasion assays, the membranes were pre-coated with collagen for 4 h at 37°C. The lower chambers were filled with DMEM supplemented with 10% fetal bovine serum as a chemoattractant. After 4 h, the migratory/invasive cells were fixed with a 10% formalin solution for 30 min and then stained with 0.05% crystal violet solution for 30 min, after which the stained cells were counted under a 20x inverted microscope.

2.7. Statistical analysis.

The data was analyzed using IBM SPSS statistics Version 24. The statistical techniques used for the analysis is one-way analysis of variance (ANOVA). Analysis was performed in triplicate and the values were expressed as mean ± SD. The P-values less than 0.05 were considered statistically significant.

3. Results

Candidone induces cell death in HepG2 cells. The effects of candidone on HepG2 cancer cell viability were assessed using an MTT assay. The results demonstrate that candidone induced cell death in a concentration-dependent manner (Fig. 2A). To investigate changes in cellular...
morphology, the cells were incubated for 24 h various concentration of candidone; 10 µM doxorubicin was used as a positive control. The results of phase contrast microscopy revealed that candidone and doxorubicin induced morphological changes in HepG2 cancer cells, including cell rounding and shrinkage (Fig. 2B). This result indicates that candidone markedly induces cell death in HepG2 cells.

_Candidone induces HepG2 cell apoptosis by inhibiting the p65 pathway._ To determine the molecular mechanism of candidone-induced apoptosis, HepG2 cancer cells were incubated with various concentrations of candidone for 4 h. The apoptotic effect was determined by observing caspase-9 and -3 cleavage. Candidone induced the cleavage of caspase-9 and 3 (Fig. 3A), which was associated with decreased expression of phopho-p65 (comparing with PD98059 inhibitor treatment), Bcl2, survivin, Bcl2-associated agonist of cell death (Bcl-xl) and induced myeloid leukemia cell differentiation protein Mcl-1, compared with doxorubicin positive control (Fig 3B). These results suggest that candidone induces apoptosis by inhibiting p65 phosphorylation.

*Effects of candidone on the migration and invasion abilities of HepG2 cells.* HepG2 cells were treated with various concentrations of candidone for 24 h, and the results revealed that candidone significantly decreased cellular migration at concentrations of 1 and 10µM (Fig. 4A and B). The inhibitory effect of candidone on HepG2 cancer cell invasion was assessed by cell penetration through the collagen-coated polycarbonate filter of a Boyden chamber. The results revealed that candidone decreased the invasiveness of HepG2 cells at concentrations of 1 and 10 µM, compared with the control (Fig. 5A and B). These findings indicate that candidone is able to suppress both the migration and invasion capacities of HepG2 cancer cells.

_Candidone suppresses MMP-9 expression._ Matrix metalloproteinases are a major class of enzymes that can cleave extracellular matrix (ECM) components, and thus play important
roles in the migration and invasion of cancer cells. Therefore, the inhibitory effects of candidone on MMP-9 protein expression were demonstrated using western blot analysis. The results illustrate that active MMP-9 expression was significantly decreased in HepG2 cells treated with candidone, at concentrations 1, 10 and 100 µM, was associated with decreased phospho-p38 (comparing with PD98059 inhibitor treatment) and increased levels of JNK phosphorylation (Fig. 6A and B). This result illustrates that candidone inhibits the migration and invasion abilities of HepG2 cells by decreasing the expression of MMP-9.

4. Discussion

Candidone is a flavanone that was isolated and identified from the fruit of *D. indica*. Candidone has been shown to exert significant cytotoxic activity against human cancer cells, and to possess antimicrobial potential for the treatment of multiple drug resistant bacterial infections. However, candidone showed cytotoxic activity in normal cells (Vero cells). Further studies may be required to determine the effects of candidone on other different kinds of human normal cells.11-13)

Cancer cells are transformed via a combination of genetic alterations, which ultimately result in a malignant phenotype. The evasion of apoptosis is one of the most important characteristics of cancer cells.14) Furthermore, cancer cell migration and invasion are the primary factors of metastasis, which is considered to be the most significant cause of cancer-associated death. Hence, understanding the regulatory mechanisms of the migration, invasion and apoptosis of cancer cells is key to the development of effective treatments.

The current study investigated the effects of candidone on apoptosis, and the migration and invasion abilities of HepG2 cancer cells. The intrinsic pathways of apoptosis are regulated by NF-κB (p50/p65), a nuclear transcription factor that regulates the expression of numerous apoptosis-associated genes. NF-κB activation was reported to induce the expression of the anti-apoptotic proteins Bcl-xL, Bcl2, Mcl-1, survivin and X-linked IAP,
which suppress the activation of caspase-9 and -3, resulting in apoptosis evasion.\textsuperscript{15-18} It has been reported that candidone showed antitumor effect on human cholangiocarcinoma (CCA) cells and sensitized MDR cells to daunorubicin and mitoxantrone.\textsuperscript{19-20} In the current study, the candidone-activated cleavage of caspase-9, caspase-3 and PARP-1 was associated with a decrease in the expression of antiapoptotic proteins, including Bel2, survivin, Bcl-xl, Mcl-1, as well as the inhibition of p65 phosphorylation. The apoptosis process was controlled by many apoptotic proteins. At 10 µM, candidone suppressed the expression of phospho-p65, Bcl-2, and survivin. Apart from phospho-p65, Bcl-2, and survivin, other anti-apoptotic proteins may control survival process and the concentration of candidone at 10 µM is not sufficient to inhibit all of anti-apoptotic proteins. However, the confirmation of apoptosis should be made by other methods including Annexin V and PI staining.

Candidone was also found to inhibit both the migration and invasion abilities of HepG2 cells. Figure 2A shows that candidone at concentration 100 µM induced cell death more than 50%. Therefore, the migration and invasion assay are required to be used at the concentration that is lower than 100 µM, so that the cancer cell will not die before migrating and invasion. MMP-9 degrades various ECM proteins to regulate tissue remodeling, and has been extensively investigated in association with the migration, invasion, metastasis and angiogenesis of a variety of cancer types.\textsuperscript{21} The upregulation of MMP-9 is accompanied by activation of the p38 mitogen-activated protein kinase and ERK pathways,\textsuperscript{22} though JNK suppresses MMP-9 expression.\textsuperscript{23} Candidone has been tested cytotoxicity on Vero normal cells (\textit{Cercopithecus aethiops} kidney normal cells).\textsuperscript{24} PD98059 is a selective inhibitor of MAP kinase kinase (MEK). It has been reported that PD98059 inhibited phosphorylation of p65 and p38.\textsuperscript{25-26} This study found that the suppression of p65 phosphorylation resulted in activation of cleaved of caspase-3 (Fig 3B) and decreased MMP-9 level that was correlated with phosphorylation of p38 inhibition (Fig 6A). The results of the present study revealed...
that candidone inhibits both the phosphorylation of p38 and the expression of active MMP-9, but activates the phosphorylation of JNK. Candidone was also indicated to inhibit the migration and invasion, and induce the apoptosis of HepG2 hepatoblastoma cells in a way that may be developed as a potential anticancer agent.

5. Conclusion

The current study indicated that candidone induced apoptosis in HepG2 cancer cell through inhibiting the expression of p65 phosphorylation, Bcl2, survivin, Bcl-xl, and Mcl-1 which resulting in activation of caspase-9, 3. Candidone was also found to inhibit the migration and invasion of HepG2 cell by decreased the levels of proteins associated with these processes, including phospho-p38 and active matrix metallopeptidase 9. The results of the present study indicate that candidone is able to inhibit the proliferation, migration and invasive potential of HepG2 cells. Overall, the efficacy of candidone support it as a promising agent for the prevention and treatment of cancers.

Acknowledgements

This work was supported by the National Research Council of Thailand and Thailand Research Fund (grant no. DBG6080006), Thailand.

Authors’ contributions

Conceptualization, P.W., and C.B.; methodology, P.W. and C.B; formal analysis, C.B. and P.W.; investigation, K.S., P.W. and C.B; resources, P.W., P.R. and C.Y.; writing—original draft preparation, C.B., K.S. and P.W.; writing—review and editing, P.W. and C.B.; project administration, P.W. All authors have read and agreed to the published version of the manuscript.
Conflict of interest

The authors declare no conflict of interest.
References

1. Somboon K, Siramolpiwat S, Vilaichone RK. Epidemiology and survival of hepatocellular carcinoma in the central region of Thailand. *Asian Pac J Cancer Prev.*, 15: 3567-3570 (2014).

2. Mittal S, El-Serag HB, Epidemiology of HCC: Consider the population. *J Clin Gastroenterol.*, 47, S2-S6 (2013).

3. Safarzadeh E, Shotorbani SS, Baradaran B. Herbal medicine as inducers of apoptosis in cancer treatment. *Adv Pharm Bull.*, 4 (Suppl 1), S421-S427 (2014).

4. Sagwan S, Rao DV, Sharma RA. Biochemical estimation of primary metabolites from *Pongamia pinnata* (L.): an important biodiesel plant. *Inter J Pharm Sci Rev Res.*, 5, 146–149 (2010).

5. Koysomboon S, Altena I, Kato S, Chantrapromma K. Antimycobacterial flavonoids from *Derris indica*. *Phytochemistry.*, 67, 1034–1040 (2006).

6. Yadav PP, Ahmad G, Maurya R. Furanoflavonoids from *Pongamia pinnata* fruits. *Phytochemistry.*, 65, 439–443 (2004).

7. Li L, Li X, Shi C, Deng Z, Fu H, Proksch P, Lin W. Pongamone A–E, five flavonoids from the stems of a mangrove plant, *Pongamia pinnata*. *Phytochemistry.*, 67, 1347–1352 (2006).

8. Rahman MS, Islam MB, Rouf MA, Jalil MA, Haque MZ. Extraction of alkaloids and oil from Karanja (*Pongamia pinnata*) seed. *J Sci Res.*, 3, 669–675 (2011).

9. Khatri P, Patel R. A phytochemical overview of various parts of *Pongamia pinnata* (Karanj). *World J Pharm Res.*, 2,146–165 (2013).

10. Sribuhom T, Saraphon C, Decharchoochart P, Boonyarat C, Yenjai C. Acetylcholinesterase inhibition and cytotoxicity of flavonoids and chalcones from *Derris indica*. *ScienceAsia.*, 42, 247–251 (2016).
11. Blatt CT, Chávez D, Chai H, Graham JG, Cabieses F, Farnsworth NR, Cordell GA, Pezzuto JM, Kinghorn AD. Cytotoxic flavonoids from the stem bark of *Lonchocarpus aff. fluvialis*. *Phytother Res.*, **6**(4),320-325 (2002).

12. Mbaveng AT, Sandjo LP, Tankeo SB, Ndifor AR, Pantaleon A, Nagdjui BT, Kuete V. Antibacterial activity of nineteen selected natural products against multi-drug resistant Gram-negative phenotypes. *Springerplus.*, **4**, 823 (2015).

13. Decharchoochart P, Suthiwong J, Samatiwat P, Kukongviriyapan V, Yenjai C. Cytotoxicity of compounds from the fruits of *Derris indica* against cholangiocarcinoma and HepG2 cell lines. *J Nat Med.*, **68**(4), 730-736 (2014).

14. Wong RS. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res.*, **30**(1), 87 (2011).

15. Shukla S, MacLennan GT, Fu P. Nuclear factorkappaB/p65 (Rel A) is constitutively activated in human prostate adenocarcinoma and correlates with disease progression. *Neoplasia.*, **6**, 390-400 (2004).

16. Dai Y, Rahmani M, Dent P. Kinase 1 Activation Downregulation, and c-Jun N-terminal mediated by oxidative damage, XIAP leukemia cells through a process NF-kB activation potentiates apoptosis in inhibitor-induced RelA/p65 acetylation and blockade. *Mol Cell Biol.*, **25**, 5429 (2005).

17. Mendoza FJ, Espino PS, Cann KL. Anti-tumor chemotherapy utilizing peptide-based approaches- apoptotic pathways, kinases, and proteasome as targets. *Arch Immunol Ther Exp.*, **53**, 47-60 (2005).

18. Namba H, Saenko V, Yamashita S. Nuclear factor-κB in thyroid carcinogenesis and progression: a novel therapeutic target for advanced thyroid cancer. *Arq Bras Endocrinol Metab.*, **51**, 843-51 (2007).
19. Kurasug B, Kukongviriyapan V, Prawan A, Yenjai C, Kongpetch S: Antitumor effects of candidone extracted from *Derris indica* (Lamk) Bennet in cholangiocarcinoma cells. *Trop J Pharm Res.*, **17**(7), 1337-1343 (2018).

20. Darzi S, Mirzaei SA, Elahian F, Shirian S, Peymani A, Rahmani B, Dibazar SP, Aali E. Enhancing the therapeutic efficacy of daunorubicin and mitoxantrone with bavachinin, candidone, and tephrosin. *Evid Based Complement Alternat Med.*, **7**, 3291737 (2019).

21. Huang H. Matrix metalloproteinase-9 (MMP-9) as a cancer biomarker and MMP-9 biosensors: recent advances. *Sensors (Basel)*., **18**(10), 3249 (2018).

22. Cho A, Graves J, Reidy MA. Mitogen-activated protein kinases mediate matrix metalloproteinase-9 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.*, **20**(12), 2527-2532 (2000).

23. Lee YS, Tran HTL, Quang VT. Regulation of expression of matrix metalloproteinase-9 by JNK in Raw 264.7 cells: presence of inhibitory factor(s) suppressing MMP-9 induction in serum and conditioned media. *Exp Mol Med.*, **41**(4), 259–268 (2009).

24. Sribuhom T, Saraphona C, Decharchoocharta P, Boonyarat C, Yenjai C. Acetylcholinesterase inhibition and cytotoxicity of flavonoids and chalcones from *Derris indica*. *ScienceAsia*, **42**(4), 247-251 (2016).

25. Yeh PY, Yeh KH, Chuang SE, Song YC, Cheng AL. Suppression of MEK/ERK signaling pathway enhances cisplatin-induced NF-kappaB activation by protein phosphatase 4-mediated NF-kappaB p65 Thr dephosphorylation. *J Biol Chem.*, **279**(25):26143-26148 (2004).

26. Huynh H, Nguyen TT, Chow KH, Tan PH, Soo KC, Tran E. Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular
carcinoma: its role in tumor progression and apoptosis. BMC Gastroenterol., 19(3), 1-24 (2003).
Figure 1. Structure of candidone.
Figure 2. Effects of candidone on the viability of HepG2 cancer cells. (A) HepG2 cells were treated with various concentrations of candidone and 10 μM doxorubicin (reference compound) for 24 h. Cell viability was determined using an MTT assay. (B) Cells were treated with 100 μM candidone and 10 μM doxorubicin for 24 h. Cell morphology was investigated by 20x phase contrast microscopy. *P<0.05 and **P<0.01.
Figure 3. Effects of candidone on apoptotic signaling pathways. Cells were treated with various concentrations of candidone for 4 h comparing with PD98059 inhibitor. Cell lysates were separated by SDS-PAGE and detected by western blotting using (A) anti-caspase-9, PARP-1, cleaved caspase-3 and actin antibodies and (B) anti-pp65, Bcl-2, survivin, Bcl-xl, Mcl-1, β-actin. Arrows indicate the cleavage of caspase-9 and -3. pp65, phospho p65; Bcl2, B-cell lymphoma 2; Bcl-xl, Bcl2-associated agonist of cell death; Mcl-1, induced myeloid leukemia cell differentiation protein Mcl-1. PARP-1, Poly [ADP-ribose] polymerase 1.
Figure 4. Candidone inhibits the migration of HepG2 cancer cells. (A) HepG2 cells were treated with 1 and 10 µM candidone, as well as 10 µM doxorubicin. Cell migration was observed using a Transwell migration assay. Arrows indicate the migration of HepG2 cancer cells. (B) Histogram illustrating HepG2 cell migration, determined by cell counting. *P<0.05 and **P<0.01.
Figure 5. Candidone inhibits the invasiveness of HepG2 cancer cells. (A) HepG2 cells were treated with 1 and 10 µM candidone, as well as 10 µM doxorubicin. Cellular invasiveness was investigated using a Transwell invasion assay. Arrows indicate the invasive capacity of HepG2 cells. (B) Histogram illustrating HepG2 cell invasiveness, determined by cell counting. *P<0.05 and **P<0.01.
A.

Candidone (µM)

|   | 0 | DMSO | 10 | 100 | Dox10 | Dox20 |
|---|---|------|----|-----|-------|-------|
| pJNK1/2 (54 kDa) |   |      |    |     |       |       |
| JNK1/2 (54 kDa)  |   |      |    |     |       |       |
| Pp-38 (43kDa)    |   |      |    |     |       |       |
| p-38 (43kDa)     |   |      |    |     |       |       |
| MMP-9 (92kDa)    |   |      |    |     |       |       |
| Actin (35kDa)    |   |      |    |     |       |       |

B.

Relative protein levels (pJNK1/2)

|   | 0 | DMSO | 10 | 100 | Dox10 | Dox20 |
|---|---|------|----|-----|-------|-------|

Relative protein levels (Pp-38:p-38)

|   | 0 | DMSO | 10 | 100 | Dox10 | Dox20 |
|---|---|------|----|-----|-------|-------|

Relative protein levels (MMP-Actin)

|   | 0 | DMSO | 10 | 100 | Dox10 | Dox20 |
|---|---|------|----|-----|-------|-------|
**Figure 6.** Effects of candidone on cell migration and invasion signalling proteins. (A) HepG2 cells were treated with various concentrations of candidone and doxorubicin for 4 h comparing with PD98059 inhibitor. Cell lysates were separated by SDS-PAGE and detected by western blotting using anti-pJNK, pP38, MMP-9 and β-actin antibodies. (B) Relative phospho JNK, p38 and active MMP-9 expression were normalized to that of JNK, p38 and β-actin respectively. pJNK, phospho JNK; Pp38, phospho-p38; MMP-9, matrix metallopeptidase 9. *P<0.05 and **P<0.01.