Anti-tumor potential of ethanol extract of Curcuma phaeocaulis Valeton against breast cancer cells

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A R T I C L E   I N F O

Keywords:
Curcuma phaeocaulis Valeton
MCF-7 cell
Proliferation
Apoptosis

A B S T R A C T

Curcuma phaeocaulis Valeton is a commonly prescribed Chinese medical herb for tumor therapy. In this study, an extract of Curcuma phaeocaulis Valeton referred as Cpv was prepared and its anti-tumor effect was evaluated with MCF-7 and MDA-MB-231 cells. Curcuma phaeocaulis Valeton power was extracted with ethanol and the main components of the extract (Cpv) were analyzed with HPLC. The effect of Cpv on MCF-7 cells proliferation, intracellular reactive oxygen species (ROS) formation, mitochondrial membrane potential (ΔΨm), apoptosis, apoptotic related proteins, MDA-MB-231 cell migration, and integrins expression were determined. Furthermore, the effect of Cpv on some key signal transduction molecules was also investigated. Furanodienone, germacrone and furanodiene were identified as the main components of Cpv. Cpv treatment significantly inhibited cell proliferation, induced LDH release, induced intracellular ROS formation, and decreased ΔΨm in a dose-dependent manner in MCF-7 cells.

Introduction

According to the Chinese pharmacopoeia, three species of Rhizoma Curcumae (Curcuma phaeocaulis Valeton, Curcuma kwangsiensis S.G. Lee et C.F. Liang, and Curcuma Wenyujin Y.H. Chen et C. Ling) are officially approved as Chinese medicines. In clinical practice, Rhizoma Curcumae is commonly prescribed for cardiovascular and tumor therapy alone or in combination with other herbs. Recent studies discovered that the main bioactive constituents of Rhizoma Curcumae are its essential oils, which possess anti-inflammatory (Makabe et al. 2006), anti-tumor (Wang et al. 2009), and neuroprotective properties (Dohare et al. 2008). Actually, the Rhizoma Curcumae essential oil has already been approved by State Food and Drug Administration (SFDA) of China as a therapeutic drug for multiple disorders. At present, many volatile oil components from Rhizoma Curcumae, such as germacrone, β-elemene, curcumol, curdione, neoecdione, etc., have been isolated and identified (Dang et al. 2010). However, pharmacological evaluation of these pure compounds revealed that only a few of them such as β-elemene showed relative higher biological activities. Some studies also suggested that there is synergistic effect in Chinese medicine formulas and herbs (Wang et al. 2008). In view of the complicated components in Chinese medical herbs, it seems reasonable that drug–drug interaction is inevitable. Therefore, some researchers preferred to study Chinese medical herb extracts. Using MDA-MB-231 breast cancer line cells, our previous study demonstrated that a Curcuma Wenyujin Y.H. Chen et C. Ling extract showed synergistic anti-tumor activity with Rhizoma Corydalis by inhibiting cell proliferation and reducing the cell invasion (Gao et al. 2009). In present study, we hypothesized that other species of Rhizoma Curcumae might also show anti-tumor activities. To test the hypothesis, an ethanol extract of Curcuma phaeocaulis Valeton referred as Cpv was prepared. The chemical components and the in vitro anti-tumor effect of Cpv were determined. Furthermore, the underlying mechanisms were explored as well.
Materials and methods

Reagents

Curcuma phaeocaulis Valeton crude was purchased from Sichuan province (China) and identified by Prof. Zhongzhen Zhao (School of Chinese Medicine, Hong Kong Baptist University). Furanodienone, germacrone, and furanodienone were kindly provided by Prof. Shaoping Li (University of Macau). The RPMI-1640 culture medium was obtained from Gibco. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin (PS), 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA), and 0.2% (w/v) trypsin/1 mM EDTA were purchased from Invitrogen. Propidium iodide (PI), 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33342, 5′,6′-tetrachloro-1′,3′,5′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1), phenylmethanesulfon fluoride (PMSF), protease inhibitor cocktail were purchased from Molecular Probes. RIPA lysis buffer was obtained from Santa Cruz. A cytotoxicity detection kit (lactate dehydrogenase, LDH) was purchased from Roche Diagnostics. Tert-butyl-hydroperoxide (TB-H2O2) was obtained from Sigma. Primary antibodies against Bcl-2, Bcl-xL, Bax, Bad, Bik, Bim, Caspase-3, 7, PARP, cleaved PARP, integrin α5, β1, β3, NF-κB, IKKα, IKKβ, p38MAPK, p42/44MAPK, JNK1, p-p42/44MAPK, β-actin, and secondary antibodies were obtained from cell signaling.

Cpv preparation

The dry Curcuma phaeocaulis Valeton was cut into small pieces, ground into a fine powder, and extracted with 95% alcohol 1:8 (v/v) for three times, each 2 h. After the alcohol had been retrieved, the extract was freeze-dried, producing a powdery and dissolved in DMSO to make a stock solution (100 mg/ml). The stock solution was filtered with 0.22 μm membrane.

HPLC assay

The HPLC assay for Cpv was performed with Agilent Series 1100 as our previous report (Yang et al. 2006). Cpv and standard substances (furanodienone, germacrone, and furanodienone) were dissolved in acetone. An ODS column (250 mm × 4.6 mm I.D., 5 μm) was used. Solvents that constituted the mobile phase were A (water) and B (acetone). The mobile phase was A (water) and B (acetone). The elution conditions applied were: 0–15 min, linear gradient 30–47% B; 15–30 min, isocratic 47% B; 30–40 min, linear gradient 47–60% B; 40–50 min, linear gradient 60–90% B; 50–60 min, linear gradient 90–100% B. The flow-rate was 1.0 ml/min and the temperature was controlled at 25 °C. The absorbance was monitored with a DAD detector at 256 nm.

Cell culture

Human breast cancer cell lines, MCF-7 and MDA-MB-231 were obtained from ATCC. Cells were cultured in medium containing RPMI-1640, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 10% (v/v) heat-inactivated FBS at 37 °C under 5% CO₂.

MTT assay and LDH assay

Exponentially growing MCF-7 (1.5 × 10⁴) cells in 100 μl medium were seeded in 96-well plates. When cells reach approximately 80% confluency, the medium was replaced with a serum-free medium, and the cells were incubated for another 24 h and then treated with different concentrations (10, 25, 50, 75, and 100 μg/ml) of Cpv. The cell viability was determined after 24 h of incubation by MTT assay. Cells were treated with 50 μg/ml of Cpv for 4, 12 and 24 h, the cell viability was determined. After treated with 25, 50, and 75 μg/ml Cpv for 24 h, the LDH release from the cells was evaluated with the commercial kit according to the manufacturers’ protocol. TB-H₂O₂ (50 μM) was used as the positive control in LDH assay.

CM-H₂DCF-DA assay

Intracellular ROS formation was determined with CM-H₂DCF-DA as previously described (Hamel et al. 2008) with minor revision. MCF-7 cells were pretreated with CM-H₂DCF-DA (2 μM) for 15 min followed by co-incubation with different concentrations (10, 25, 50, and 75 μg/ml) of Cpv for another 30 min and washed with ice-cold PBS for three times. The fluorescence was observed with a fluorescent microscopy. TB-H₂O₂ (50 μM) was used as the positive control. Images were captured with an electronic camera.

JC-1 assay

The mitochondrial membrane potential (Δψm) was monitored by JC-1 staining (Bedner et al. 1999) with minor modification. MCF-7 cells were seeded in 96-well plates as described above and treated with different concentrations of Cpv (10, 25, 50, and 75 μg/ml) for 2 h. The culture medium was removed and incubated with 100 μl PBS (containing 2 μg/ml JC-1) at 37 °C for 15 min. The JC-1 fluorescence was observed with a fluorescence microscope. TB-H₂O₂ (50 μM) was used as the positive control. Images were captured with an electronic camera.

Hoechst 33342/PI double staining

The Hoechst 33342/PI double staining was performed as previously reported (Kelly et al. 2003) with minor revision. In brief, after 24 h treatment of Cpv (25, 50, and 75 μg/ml), the culture medium was gently removed and the MCF-7 cells were stained with Hoechst 33342 (final concentration 1 μg/ml) and PI (final concentration 1.5 μg/ml) for 20 min. The fluorescence was also observed with a fluorescence microscope. Images were captured with an electronic camera.

Transwell cell migration assay

Cell migration assays were performed through an 8 μm pore size polycarbonate membrane. Briefly, cells (5 × 10⁴/200 μl) suspended in serum-free RPMI-1640 was added to the upper wells and induced to migrate for 12 h with 1% FBS, migrated cells were stained, fixed, and quantified as described in our previous study (Gao et al. 2008).

Western blotting assay

To determine the effect of Cpv on Bcl-2 family proteins, caspas, and PARP, the MCF-7 cells were treat with Cpv (25, 50, and 75 μg/ml) for 24 h. To test the effect of Cpv on intergrins expression, MDA-MB-231 cells were treat with Cpv (5, 10, and 20 μg/ml) for 24 h. For studying the effect of Cpv on JNK, MAPK and NF-κB pathways, the MCF-7 cells were treated with Cpv (25, 50, and 75 μg/ml) for 2 h.

After cells were treated with Cpv, the total proteins were extracted with RIPA lysis buffer containing 1% PMSF and 1% protease inhibitor cocktail, and their concentrations were determined with a BCA protein assay kit. Equivalent amounts of proteins from each group were separated by SDS-PAGE, followed by transferring onto a PVDF membrane. After blocking for 1 h in 5% non-fat dried milk, the membrane was incubated with a specific primary
antibody (1:1000), followed by incubation with the corresponding second antibody (1:5000). Specific protein bands were visualized using an ECL advanced Western blotting detection kit. Densitometric measurement of band intensity was performed with the Quantity One Software.

Data analysis

All data were presented as mean ± SD. The significance of intergroup differences was evaluated by one-way analyses of variance (one-way-ANOVA) using the SPSS 11.5 software. Statistical differences were considered significant at P<0.05.

Results

The chemical content of Cpv

Under our experimental conditions, the HPLC chromatogram for Cpv was shown as Fig. 1A. Three peaks were identified as furanodienone, germacrone and furanodiene. At 100 μg/ml of Cpv, the calculated concentrations for furanodienone, germacrone, and furanodiene are 50 μM, 12.5 μM, and 12.5 μM, respectively.

Cpv inhibited MCF-7 cells proliferation

The cytotoxicity of Cpv was firstly assessed by MTT assay. Compared with the control group, 10 and 25 μg/ml Cpv treatments showed no obvious effect on MCF-7 cell proliferation (Fig. 1B). While 50, 75 and 100 μg/ml Cpv could significantly inhibit cell proliferation in a dose-dependent manner after 24 h (Fig. 1B). Furthermore, the inhibitory effect was also observed to occur in a time-dependent manner at 50 μg/ml Cpv (Fig. 1C).

Cpv increased LDH release from MCF-7 cells

After treatment with Cpv for 24 h, the LDH release from MCF-7 cells were significantly increased as determined by the absorbance (Fig. 1D). A dose-dependent manner was observed. Compared with the control group, a more than 50% increase of absorbance at 75 μg/ml Cpv was observed. TB-H2O2 (50 μM) showed more potency than Cpv and dramatically increased the amount of LDH released.

Cpv increased ROS formation and decreased ΔΨm in MCF-7 cells

As shown in Fig. 2A, sporadic green fluorescence was observed in control group suggesting the low production of ROS in intact cells. TB-H2O2 (50 μM) treatment induced bright green fluorescence showing the large amount of ROS formation (Fig. 2B). 10 μg/ml Cpv showed no effect on ROS formation (Fig. 2C) while 25, 50, and 75 μg/ml Cpv incubation induced enhanced ROS formation (Fig. 2D–F). JC-1 staining showed similar results (Fig. 2G–L). In control group, there were intensive red fluorescence and little green fluorescence (Fig. 2G) suggesting the high ΔΨm. While after TB-H2O2 (50 μM) treatment, the green fluorescence was greatly increased suggesting the significant decreased of ΔΨm (Fig. 2H). 10 μg/ml of Cpv showed no obvious effect on ΔΨm (Fig. 2I). The green fluorescence was increased in a dose-dependent manner in 25, 50, and 75 μg/ml Cpv treated cells (Fig. 2J–L). Furthermore, the JC-1 fluorescence ratio (red/green) was dramatically decreased after Cpv treatment (Fig. 2J–L).

Cpv increased DNA fragmentation in MCF-7 cells

The Hoechst 33342/PI double staining showed that no DNA fragmentations were observed in control group and 25 μg/ml
Cpv treated group. No PI positive cell was observed as well (Fig. 3A and B). While in 50 and 75 μg/ml of Cpv treated groups, both the DNA fragmentations and PI positive cells were recorded (Fig. 3C and D).

Cpv showed no effect on MDA-MB-231 cell migration

In the transwell migration model, both the Cpv treated and untreated group showed significant cell migration (Fig. 3E–H). Sta-
Fig. 4. Effect of Cpv on protein expressions. MCF-7 cells were treated with Cpv (25, 50, and 75 μg/ml) for 24 h, apoptotic related protein expression were determined (A). MDA-MB-231 cells were treated with Cpv (5, 10, and 20 μg/ml) for 24 h, integrins expressions were determined (B). MCF-7 cells were treated with Cpv (25, 50, and 75 μg/ml) for 2 h, JNK1, MAPK, NF-κB, etc. expression were determined (C).

statistical analysis revealed that compared with the control group, treatment MDA-MB-231 cells with different concentrations of Cpv (5, 10, and 20 μg/ml) showed no effect on breast cancer cell migration (Fig. 3).

Cpv regulated apoptotic related proteins expression

To explore the potential mechanisms of Cpv, the expression of several proteins related to apoptosis were determined in MCF-7 cells. Compared with the untreated cells, 50 μg/ml Cpv significantly increased protein expression of Bax, Bik, cleaved PARP, PARP, caspase-3 and 7 and decreased Bcl-2, Bim, Bad without altering Bcl-xL. While 75 μg/ml Cpv incubation significant increased protein expression of Bax, cleaved PARP, PARP, and caspase-3 and decreased Bcl-2, Bcl-xL, Bim, Bad without affecting Bik, caspase-7 (Fig. 4A).

Cpv regulated expression of integrins in MDA-MB-231 cell

Compared with the control group, Cpv (5, 10, and 20 μg/ml) treatment showed no obvious effect on both integrin α5 and β1 protein expressions but significantly decreased integrin β5 expression (Fig. 4B).

Cpv activated multiple signaling pathways

Cpv treatment (25, 50, and 75 μg/ml) for 2 h significantly increased protein expression of JNK1, p-p42/44MAPK, IKKα, IKKB, and decreased p42/44MAPK expression without affecting p38MAPK expression (Fig. 4C).

Discussion

As a common prescribed herb, Curcuma phaeocaulis Valeton has been used in China from ancient times. However, its pharmacological effect has not been systematically evaluated. In the present study, we prepared an ethanol extract termed as Cpv. HPLC analysis revealed that the main chemical components are furanodienone, germacrone and furanodienone. Previous studies reported that furanodienone induced G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma cells (Xiao et al. 2007), HL60 leukemia cells (Mao et al. 2008) and inhibited Hela, HL-60, PC3, SGC-7901 and HT-1080 cells proliferation (Sun et al. 2009). Our studies also revealed that germacrone showed anti-tumor activity in MDA-MB-231 cells (Zhong et al. 2011). Therefore, germacrone and furanodienone might partly contribute to the anti-tumor activity of Cpv as described in this study.

Both the MTT and LDH assays confirmed that Cpv significantly suppressed MCF-7 cells proliferation in a dose- and time-dependent manner. In MTT assay, 25 μg/ml Cpv showed no obvious effect on MCF-7 proliferation, while in LDH assay, 25 μg/ml Cpv significantly induced LDH release, suggesting that 25 μg/ml Cpv is toxic. The cell viability is only about 30% of the control after 100 μg/ml Cpv treatment for 24 h. Therefore, 25, 50, and 75 μg/ml Cpv were chosen as the experimental concentrations. ROS is proposed as "redox messengers" in intracellular signaling at physiological levels while uncontrolled ROS production might involved in apoptosis (Circu and Aw, 2010). Furthermore, activation of MAPK, NF-κB, JNK by ROS was also documented (Han et al. 2009; Pan et al. 2009; Pantano et al. 2006). Our results found that Cpv induced both quick and robust ROS formation and p-p42/44MAPK, NF-κB, JNK activation suggesting that ROS might mediate Cpv induced p-p42/44MAPK, NF-κB, JNK activation in MCF-7 cells. It is well known that p42/44MAPK signaling is related to oncogenesis while the p38MAPK pathway contributes to tumor suppression (Pan et al. 2009). NF-κB inhibition might be an important new approach for the treatment of certain malignancies (Orlowski and Baldwin, 2002). However, in present study we discovered that Cpv showed no effect on p38MAPK but induced p42/44MAPK phosphorylation and NF-κB activation. These results suggested that the
pharmacological effect of Cpv is multiple, probably results from its complex composition. The reduction or loss of $\Delta V_m$ is an early indicator of apoptosis and a key indicator of cellular viability. Our results revealed that Cpv treatment significantly induced MCF-7 $\Delta V_m$ loss suggesting that Cpv might induce apoptosis in these cells, which was further confirmed by Hoechst 33342 staining and western blotting assay. Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into fragments. After Cpv treatment for 24 h, DNA fragmentation and condensation events were clearly observed in Hoechst 33342 stained cells suggesting the presence of apoptosis.

To explore the potential mechanisms of Cpv induced apoptosis, apoptosis-related protein expression was examined. The key biochemical event involved in the apoptotic process was the up-regulation of pro-apoptotic proteins and/or the down-regulation of anti-apoptotic protein molecules. Among these proteins, the Bcl-2 family of proteins, including Bcl-2, Bcl-xL, Bim, and Bax proteins, serve as critical regulators of the mitochondrial pathway involved in apoptosis, acting to either inhibit or promote cell death. The Bcl-2 and Bcl-xL proteins have been identified as anti-apoptotic proteins, which bind to the outer membrane of the mitochondria and prevent the release of cytochrome c. Bax and Bim are thought to be pro-apoptotic effector proteins and are responsible for permeabilizing the membrane due to damaging cellular stress. While Bad and Bik can bind and regulate the anti-apoptotic Bcl-2 proteins to promote apoptosis (Youle and Strasser, 2008). Our results showed that Cpv exert potent effects on Bcl-2 family proteins expressions. It decreased anti-apoptotic protein Bcl-2, Bcl-xL expression and increased pro-apoptotic protein Bax expression. In the same time, it also disturbs Bad and Bik expressions. The regulation of Bcl-2 family proteins involved in Cpv induced MCF-7 cell apoptosis.

Caspase-3 is a critical executioner of apoptosis and is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which is an important protein that appears to be involved in DNA repair, maintenance of chromosomal stability, and programmed cell death. PARP induction may be an early signal of apoptosis in human tumor (Bursztajn et al. 2000). Caspase-7, another member from caspase family, is also a critical executioner of apoptosis (Kuribayashi et al. 2006). The activation of caspase-3, 7, and the induction of PARP cleavage suggest that Cpv induced MCF-7 cell apoptosis was caspase-dependent.

To evaluate the effect of Cpv on metastasis, cell migration was performed in a transwell migration model with MDA-MB-231 cells. Results showed that Cpv treatment could not inhibit cell migration but significantly decreased integrin $\beta 5$ expression. The integrin family of cell adhesion receptors regulates a diverse array of cellular functions crucial to the initiation, progression and metastasis of solid tumors (Desgrosellier and Cheresh, 2010). Thus, the role of Cpv in metastasis need further study to elucidate.

In summary, our results showed that Cpv containing furanodiene, germacrone, and furanodiene significantly inhibits MCF-7 cell proliferation possibly through the induction of apoptosis mediated by inducing ROS formation, decreasing $\Delta V_m$, regulating Bcl-2 family proteins expression, and activating caspases. Furthermore, Cpv treatment also activated several signaling transduction pathways.

Acknowledgments

This study was supported by the Macao Science and Technology Development Fund (029/2007/A2) and Research Fund of the University of Macau (SRG013-ICMS511-CPX; UL016/09-Y3/CM/WYTO1/ICMS). The authors would like to thank Prof. Shaoping Li (University of Macau) for providing furanodiene, germacrone, and furanodieneone and Prof. Zhongzhen Zhao for identifying the herb.

References