RESEARCH ARTICLE

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Clausenidin Induces Caspase 8-Dependent Apoptosis and Suppresses Production of VEGF in Liver Cancer Cells

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Abstract

Clausena excavata Burm f. is used by traditional healers to treat cancer patients in South East Asia. The use of the plant and its compounds is based on Asian folklore with little or no scientific evidence supporting the therapeutic efficacy The current study aimed to determine the effect of pure clausenidin isolated from *C. excavata* on caspase-8-induced cell death as well as angiogenesis in the HepG2 hepatocellular carcinoma cell line. Caspase-8 and extrinsic death receptor protein expression was determined using spectrophotometry and protein profile arrays, respectively. Ultrastructural analysis of clausenidin-treated cells was conducted using transmission electron microscopy. In addition, anti-angiogenic effects of clausenidin were investigated by Western blot analysis. Clausenidin significantly ($p<0.05$) increased the activity of caspase-8 and expression of protein components of the death inducing signaling complex (DISC) in HepG2 cells. Ultrastructural analysis of the clausenidin-treated HepG2 cells revealed morphological abnormalities typical of apoptosis. Furthermore, clausenidin significantly ($p<0.05$) decreased the expression of vascular endothelial growth factor (VEGF). Therefore, clausenidin is a potential anti-angiogenic agent which may induce apoptosis of hepatocellular carcinoma cells.

Keywords: Clausenidin- caspase 8- death receptor- apoptosis- angiogenesis

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Introduction

Clausena excavata Burm. f is a medicinal plant used for the treatment of cancer in Asia (Huang et al., 1997; Manosroi et al., 2004). There is no obvious evidence that *C. excavata* is commonly used in diets, but it is well-known as a tree with numerous medicinal properties that includes anti-cold, anti-snake venom, anti-fungal, anti-malarial and anti-HIV (Wu et al., 1994; Kongkathip et al., 2005). In Thailand for instance, the *C. excavat*a extract is administered to treat cancer patients (Manosroi et al., 2004; Arbab et al., 2013). The use of *C. excavata* is based on hearsay and folklore with little or no scientific evidence supporting its therapeutic use. However, the compounds in *C. excavata* have been found to be cytotoxic and lethal to cancer cell lines (Sharif et al., 2011). Clausenidin, dentatin and nordentatin are among compounds in *C. excavata* that has been shown to be toxic to cancer cell lines (Su et al., 2009; Sharif et al., 2011; Arbab et al., 2013). Previously, we found that clausenidin isolated from *C. excavata* induced apoptosis in hepatocellular carcinoma (HepG2) cells via mitochondrial signaling (Waziri et al., 2016), but we did not know if clausenidin has any effect on caspase-8 activity or angiogenesis, hence this study was conducted.

In spite of the advances in the conventional treatment of cancers, liver cancer remains a leading cause of death in developing countries and its treatment is largely ineffective (Bakiri and Wagner, 2013; Yin et al., 2013). Therefore, the current treatment options for liver cancer is aimed at improving the survivability and quality of life of patients. In cancer patients, chemotherapy involves the use of compounds that can induce apoptosis of tumor cells (Fesik, 2005). Apoptosis can occur via the intrinsic or extrinsic (death receptor) pathways. An impairment of the extrinsic pathway involving caspase-8 signaling contributes to carcinogenesis and evasion of apoptosis (Fulda, 2010; Stupack, 2013). Currently, natural products are more becoming the choice method for the treatment of cancers because of relative lack of side-effects.

In the current study, we examined the effect of

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clausenidin (Figure 1), a pyranocoumarin, isolated from the root extract of *C. excavata*, on apoptosis and vascular endothelial growth factor (VEGF) production by the HepG2 cells. This work is a first description of the potential anti-angiogenic effect of clausenidin in hepatocellular carcinomas.

Materials and Methods

Plant material and isolation of clausenidin

The fresh roots of Clausena excavata were collected from University Putra Malaysia and assigned a voucher specimen number of 2991/16. Clausenidin isolated as pure crystals from the chloroform extract of the roots of *C. excavata* was characterized in our earlier report (Waziri et al., 2016).

Cell line and cell culture

Hepatocellular carcinoma (HepG2) cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium (Gibco, Life Technologies Corporation) supplemented with 10% FBS. The cells were grown in an incubator at 37°C and 5% CO_2.

Caspase 8 assay

The Caspase-8 assay was performed using the caspase-8 IETD-R110 fluorimetric and colorimetric assay kit (Biotium Inc., USA). The cells were seeded in a 6-well plate at a density of 1 x 10^6 cells/well and treated with either 5, 15, 30, 40, or 50 µg/mL clausenidin for 24 h while vehicle control cells were treated with 0.1% (v/v) DMSO. After treatment, cells were harvested and suspended in 50 µL of chilled lysis buffer for 10 min. The cell lysate was centrifuged at 14000 × g for 5 min at 4°C and the supernatants transferred to fresh tubes. Approximately 50 µL of assay buffer and 5 µL of caspase-8 substrate was added to each sample. The samples were mixed and incubated in a water bath at 37°C for 2 h before transferring to a 96-well plate. The absorbance of samples was read at 450 nm in an ELISA plate reader (BioTek, USA).

Scanning electron microscopy

Cells were seeded overnight in T-25 mL flask at a density of 1 × 10^6 cells/flask and treated with the IC50 of clausenidin (7.7 µg/mL) for 24, 48, or 72 h. Vehicle control cells were treated with 0.1% (v/v) DMSO. At the end of the treatment, the cells were washed twice with PBS and centrifuged at 1000 × g for 10 min at 4°C and the supernatant discarded after each wash. The cells were fixed with 4% glutaraldehyde and 1% osmium tetraoxide for 6 and 2 h respectively at 4°C, washed three times with 0.1M sodium cacodylate buffer (10 minutes each) after each fixing by centrifuging at 3000 × g for 5 minutes to obtain cell pellet. After discarding the supernatant, the cells were dehydrated with 35, 50, 75, and 95% acetone, in succession, for 10 min each. Further dehydration was done trice with 100% acetone for 15 min each and the cells dried on a critical dryer for approximately 30 min. The cell pellets were mounted on stubs, coated with gold particles before viewing under the JSM 6400 scanning electron microscope (Jeol, USA).

Transmission electron microscopy (TEM)

Cells were seeded overnight in T-25mL flask at a density of 1 × 10^6 cells/flask and treated with 7.7 µg/mL of clausenidin for 24, 48 or 72 hours while vehicle control cells were treated with 0.1% (v/v) DMSO. After the treatment period, the cells were washed twice with PBS and centrifuged at 1000 × g for 10 minutes at 4°C and the supernatant discarded after each wash. The cells were fixed with 4% glutaraldehyde and 1% osmium tetraoxide and then dehydrated with increasing concentrations of acetone as described in the scanning electron microscopy analysis. Then, the cells were then infiltrated with acetone:resin mixture at 1:1 for 60 min and then at 1:3 for 120 min before infiltration again with 100% resin, overnight. The cells were then embedded by inserting into a resin-filled beam capsule and polymerized for 2 h in an oven at 60°C, before cutting with an ultramicrotome into 1 µm thick sections. The sections were stained with toluidine blue and further cut into thinner sections of 60 to 90 nm. The thin sections were stained with uranyl acetate and lead for 15 and 10 min, respectively before viewing under H-7100 TEM (Hitachi, Japan) transmission electron microscope.

Reactive oxygen species assay

The intracellular production of ROS was determined by the Total ROS assay kit 520nm (Ebioscience Inc., Affymetrix) according to manufacturer's protocol. The HepG2 cells were seeded overnight in a 6-well plate at a density of 2 × 10^5 cells/well. The cells were then treated with either 5, 15, 30 or 40 µg/mL of clausenidin for 24 h before harvesting for the assay. The harvested cells were washed with PBS and centrifuged at 1000 × g for 5 minutes at 4°C and the supernatant discarded. Staining of cells was done with 100 µL ROS assay stain solution (Ebioscience, Affymetrix) and incubated at 37°C under 5% CO_2 for 60 min. Determination of ROS-producing cells was by flow cytometry (BD FACS, USA).

Protein expression studies

Protein profile assay

Protein profile array was done to determine expression of some apoptotic proteins of the extrinsic pathway in clausenidin-treated HepG2 cells using the proteome profiler antibody array kit (R and D systems, USA). Briefly, cells were seeded overnight at a density of 1 × 10^6 cells/well and treated with 15 µg/mL clausenidin while vehicle control cells treated with 0.1% (v/v) DMSO, both for 24 h. The protein concentrations were determined by Bradford's assay. Primary antibody-coated membranes (R and D systems, USA) were incubated with protein samples from HepG2 cells at a concentration range of 200 to 400 µg/mL overnight at 4°C and washed with wash buffer before incubating with secondary antibody conjugated with streptavidin-bound horseradish peroxidase (R and D systems, USA) for 30 min on a rocking platform. The membranes were again washed and chemireagent mix was slowly pipetted onto the membrane to ensure that the membrane surface is fully covered. The images were then

captured on a ChemiDocTM Imaging system (Biorad, USA) and the data obtained was analyzed using the Image Lab software 5.

Protein assay

The cells were seeded in a 6-well plate overnight at a density of 5 × 10^5 cells/well before treating with either 5, 15, 30 or 40 µg/mL of clausenidin and vehicle control cells were treated with 0.1% (v/v) DMSO, both for 24 h. After the treatment period, cells were harvested using a cell scraper and then washed with PBS by centrifugation at 5000 × g for 5 mins at 4°C. The cell pellets were suspended in 200 µL RIPA buffer (Thermo Fisher scientific, USA) for 30 min, on ice with vortexing for 20 secs at 10 min intervals. The cell suspension was then centrifuged at 14000 × g for 25 mins at 4°C and the supernatant collected and transferred to fresh tubes. To quantify protein, 250 µL Bradford reagent was added to 5 µL sample in a 24-well plate and the plate incubated for 5 min at room temperature in the dark. A protein standard curve was prepared with bovine serum albumin in PBS. The absorbance was determined at 570 nm in an ELISA plate reader (BioTek, USA) and protein concentration of samples obtained from the standard curve.

SDS PAGE

Protein samples were separated by electrophoresis for 90 min at 120 V on a 10% sodium doedecyl sulphate-polyacrylamide gel. The samples were run using Tris running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3) medium.

Western Blot

The resolved proteins from SDS-PAGE gel were transferred to the polyvinylidene difluoride (PVDF) membrane (Biorad, USA) using the wet transfer method (Gels, 2001). The transfer of proteins was done with the use of transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100 V for 90 min. The membrane was then blocked for 2 h with 5% skim milk in an orbital shaker (Heidolph, Germany) before washing trice with PBS-Tween 20 on an orbital shaker (Heidolph, Germany) for 10 min each time. Primary and secondary antibodies (Santa Cruz, USA) were prepared in 5% skim milk at a concentration of 1:2000 and 1:3000, respectively. The membrane was incubated with the primary antibodies at 4°C for 24 h followed by secondary antibody at 25°C for 1 h. After incubation with either primary or secondary antibodies, the membrane was washed trice for 10 min each time with PBS-Tween 20. The membranes were developed using the chemiluminescent substrate (Thermo Fisher Scientific, USA) according to manufacturer's protocol. Solutions A and B were mixed in equal proportions and 1 mL of the mixture was added to the membrane while ensuring the surface is completely covered with the substrate. The membranes were incubated for 5 min and then viewed on a ChemiDocTM imaging system (Biorad, USA) and the protein expressions determined.

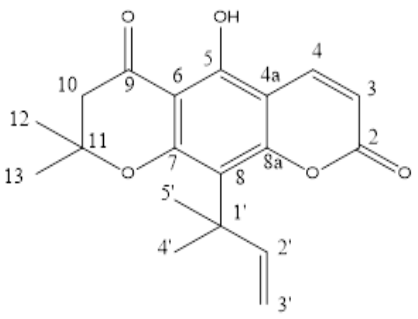

Figure 1. Clausenidin

Statistical Analysis

The data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) on SPSS 22 software (SPSS Inc, Chicago IL, USA) was used to determine significance between means at 95% confidence interval.

Results

Caspase 8 activity

Clausenidin treatment increased the activity of caspase 8 in HepG2 cells in a dose dependent manner (Figure 2). The activity of caspase 8 is significant ($p<0.05$) after treatment with 30, 40 and 50 µg/mL of clausenidin.

Scanning electron microscopy

Clausenidin caused the apoptosis of HepG2 cells that increased with time. Membrane blebbing and cytosolic shredding are the main morphological damage observed in the clausenidin-treated cells (Figure 3).

Transmission electron microscopy

The ultrastructural examination using TEM showed morphological abnormalities in the HepG2 cells consistent with apoptosis. The morphological abnormalities include nuclear shrinkage, chromatin condensation and margination (Figure 4). Lipid droplets became more evident after 48 and 72 h of clausenidin treatment (Figures 4C-D). Convolution of nuclear outline was observed at all treatment points and this is the change that precedes nuclear fragmentation. Clausenidin also caused development of vacuoles in the treated HepG2 cells.

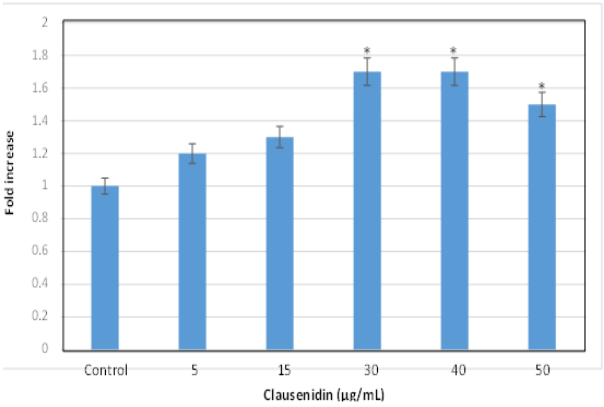

Figure 2. Caspase 8 Activity in Clausenidin- Treated HepG2 Cells at 24 Hours. *Means significantly ($p<0.05$) different from control.

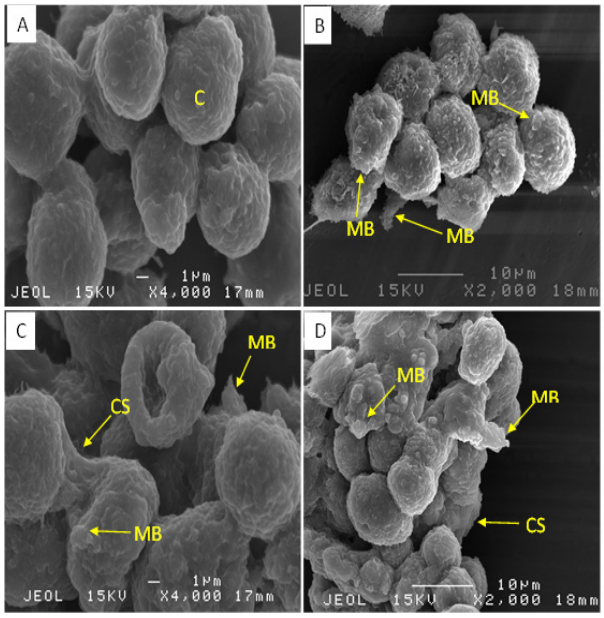

Figure 3. SEM Micrograph of Clausenidin-Treated HepG2 Cells at (B) 24, (C) 48 and (D) 72 hours respectively. (A) is the control; C, Intact cell; MB, membrane blebbing ; CS, cytosolic shredding. (Magnification × 5000).

Reactive oxygen species

Clausenidin treatment caused a significant ($p<0.05$) increase in the number of active ROS-producing HepG2 cells and the effect was concentration-dependent (Figure 5). However, the number of ROS-producing cells decreased after treatment with 40 µg/mL clausenidin. This phenomenon is attributed to the death of most active ROS-producing cells at this point of treatment. The number of non-active ROS-producing cells decreased significantly ($p<0.05$) after treatment with 15, 30 and 40 µg/mL of clausenidin.

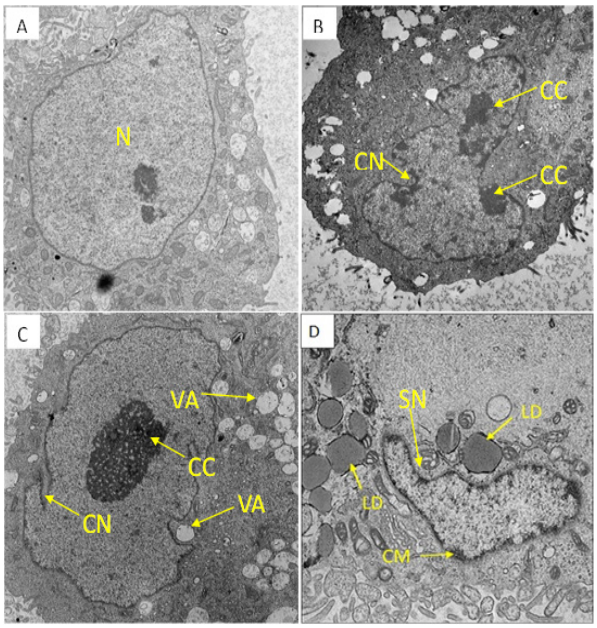

Figure 4. Ultrastructural Micrograph of Clausenidin-Treated HepG2 Cells at (B) 24, (C) 48 and (D) 72 hours respectively. (A) is the control; N, Intact nucleus; CC, chromatin condensation; CM, chromatin margination; CN, nuclear convolution; LD, lipid droplet; SN, shrinkage of nuclei. (Magnification × 5000).

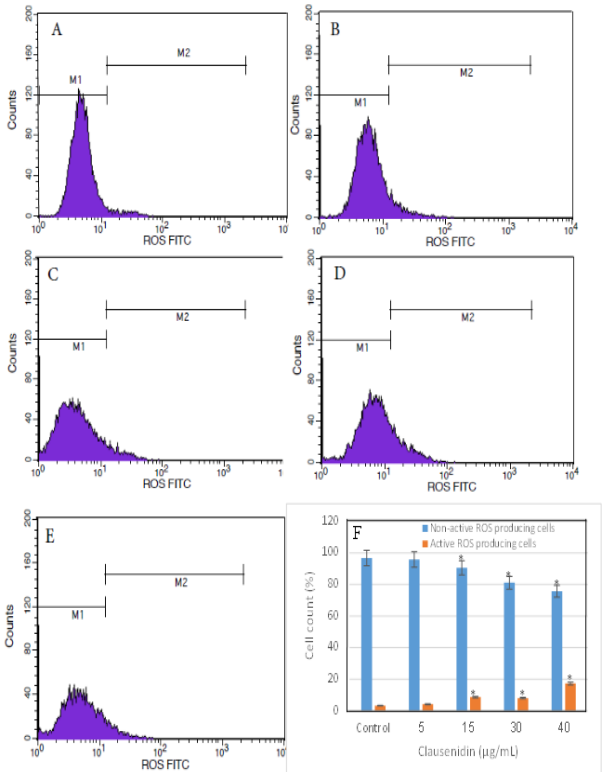

Figure 5. Reactive Oxygen Species-Producing HepG2 Cells after Treatment with Clausenidin at (B) 5, (C) 15, (D) 30 and (E) 40 µg/mL. (A) is the control. (F) Distribution of active and non-active ROS producing cells. Error bars represent standard deviation. *Means significantly ($p<0.05$) different from control.

Protein expression studies

Clausenidin caused significant ($p<0.05$) increases in expression of TNFR1, Fadd, TRAIL R1 and TRAIL

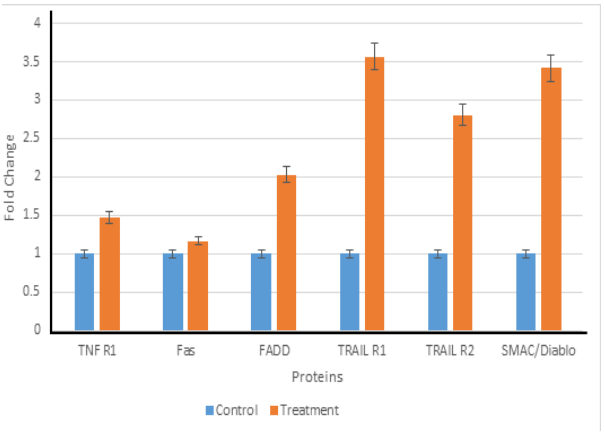

Figure 6. Expression of Death Receptor Pathway Proteins in Clausenidin-Treated HepG2 Cells. TNF R1 – Tumor necrosis factor Receptor 1, Fas – Fas Receptor, FADD – Fas associated death domain, TRAIL R1 – Tumor necrosis factor related apoptosis inducing ligand Receptor 1, TRAIL R2 – Tumor necrosis factor related apoptosis inducing ligand Receptor 2, SMAC/Diablo – Second mitochondria-derived activator of caspase/ Direct inhibitor of apoptosis binding protein with low pI. Error bars represent standard deviation. * Means significantly ($p<0.05$) different from control.

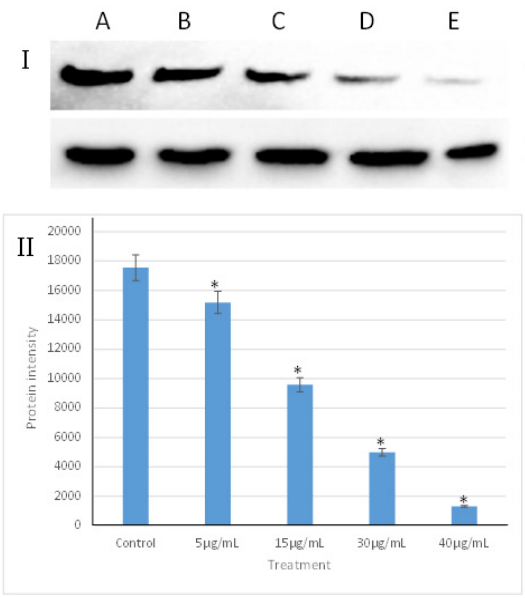

Figure 7. VEGF Protein Expression in HepG2 Cells Treated with Clausenidin at (B) 5, (C) 15, (D) 30 and (E) 40 µg/mL. (A) is untreated control. (I) Western blot; (II) Relative protein expression. β-actin protein was used for normalization. *Means significantly ($p<0.05$) different from control.

R2, which are the pro-apoptotic proteins of the extrinsic pathway (Figure 6). Also, the expression of SMAC/Diablo increased significantly ($p<0.05$) following treatment of HepG2 cells with clausenidin. Conversely, the expression of vascular endothelial growth factor (VEGF) decreased significantly ($p<0.05$) in the clausenidin-treated HepG2 cells (Figures 7I and II).

Discussion

Clausenidin treatment of HepG2 cells increased caspase-8 activity and expression of death receptor proteins and suppressed VEGF production. The decreased expression of the death receptors is known to be associated with increased survival of tumor cells (Stupack, 2013). Induction of apoptosis is the preferred mode of cell death in cancer treatments, because impairment of apoptosis causes tumor cells to become immortal (Kamesaki, 1998; Fesik, 2005; Stupack, 2013). Apoptosis may occur either through the intrinsic or extrinsic pathways. The extrinsic pathway of apoptosis is activated when death receptors such as TNFR1 and Fas bind to their receptors to form death-inducing signaling complex (DISC) (O'Brien and Kirby, 2008). In the HepG2 cells after treatment with clausenidin, the protein profile showed increased expression of TNFR1, Fas, and the Fas-associated death domain (FADD). This led to the formation of DISC and subsequent activation of caspase-8. Caspase-8 is an upstream caspase of the extrinsic pathway that activates the executioner caspase-3 (Karp, 2008). The Caspase 8 is also known to activate the pro-apoptotic protein, bid that plays a major role in mitochondrial pathway of apoptosis (Li et al., 2002). In our earlier report (Waziri et al., 2016), which is similar to the current study, it was observed that significant upregulations of caspase 3 and bid in HepG2 cells are triggered by clausenidin-induced activation of caspase 8. We also observed that clausenidin caused significant increased expressions of TRAIL R1 and R2, which are known to activate the caspase pathways.

The ultrastructural analysis of clausenidin-treated HepG2 cells showed morphological abnormalities that represent apoptosis. These abnormalities include membrane damage, chromatin condensation, chromatin margination, and nuclear shrinkage; features that are hallmarks of apoptosis (Kroemer, 2005; Mohan et al., 2010). We also observed cellular vacuolation that indicates that clausenidin may also induce non-apoptotic death of the HepG2 cells. Apoptosis is characterized by the extrusion of phosphatidyl serine component from the inner to the outer membrane of cells and exposing this component to the exterior of the cell (Hengertner, 2001). This effect of clausenidin was evident from the TEM study, where droplets of phospholipids were present in the clausenidin-treated cell samples.

Clausenidin induced production of ROS by HepG2 cells that exceeded the capacity of the anti-oxidant defense system. Increased ROS production is one of the mechanism that causes mitochondrial membrane damage of tumor cells (Avendaño and Menendez, 2015). The increased production of ROS has also been associated with caspase activation (Simon et al., 2000). In our study, clausenidin treatment did in fact activated caspase-3 and -8 in the HepG2 cells purportedly through increased production of ROS.

Development of new blood vessels or angiogenesis is essential for tumors to grow and spread. Tumor cells produces vascular endothelial growth factor (VEGF) to enhance angiogenesis. Angiogenesis is also facilitated by secretion of matrix degrading proteinases (Zhong et al., 2006) and the VEGF induces sprouting and branching of blood vessels. Angiogenesis is used by tumors to invade and spread in the tissues (Zhong et al., 2006). Through angiogenesis tumors can metastasize to other organs. Thus, several drugs were developed including Semaxinib and vitalanib that target VEGF receptors for their anti-tumor effect (Mendel et al., 2000; Hecht et al., 2005). As a potential anticancer agent, clausenidin on the HepG2 cells, also exerts its effect through inhibition of the production VEGF. It was shown in the in vitro study that the reduction of VEGF expression in the HepG2 induced by clausenidin occurred in a dose-dependent manner. Although it is yet to be shown in vivo, it is postulated that among the anti-tumor effects of clausenidin is through its anti-angiogenic effect.

In conclusion, the increased activity of caspase-8 induced by clausenidin suggests that it has pro-apoptotic effect on cancer cells. This is reflected by the ultrastructural analysis of the clausenidin-treated HepG2 cells that showed morphological evidences of apoptosis. Furthermore, clausenidin was found to suppress VEGF expression of the HepG2 cells that suggests that it can potentially inhibit tumors. The findings from this study clearly demonstrates the potential of clausenidin as a potent anticancer agent particularly in the treatment of liver cancers.

Abbreviations

DMSO – Dimethyl sulfoxide, RPMI – Rosewell

Park Memorial Institute, HepG2 – liver hepatocellular carcinoma, DNA - Deoxyribonucleic acid, ROS – reactive oxygen species, VEGF – vascular endothelial growth factor, DISC – death inducing signaling complex, TEM – Transmission electron microscopy.

Conflict of interest

The authors declare that there are no conflict(s) of interest pertaining this study.

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