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ORIGINAL ARTICLE

Zerumbone Suppresses Angiogenesis in HepG2 Cells through Inhibition of Matrix Metalloproteinase-9, Vascular Endothelial Growth Factor, and Vascular Endothelial Growth Factor Receptor Expressions

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ABSTRACT

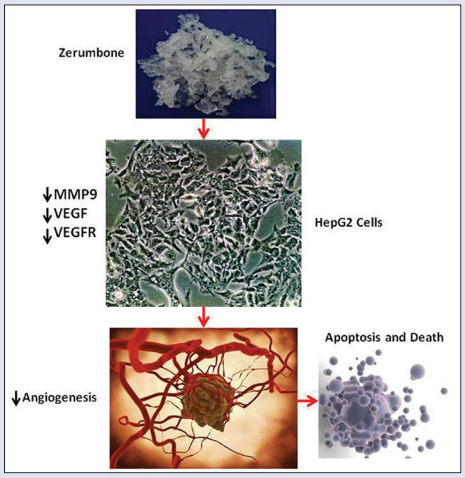
Context: Due to increase in the number of patients with impaired immunity, the incidence of liver cancer has increased considerably. **Aims:** The aim of this study is the investigation the *in vitro* anticancer effect of zerumbone (ZER) on hepatocellular carcinoma (HCC). **Materials and Methods:** The anticancer mechanism of ZER was determined by the rat aortic ring, human umbilical vein endothelial cells (HUVECs) proliferation, chorioallantoic membrane, cell migration, and proliferation inhibition assays. **Results:** Our results showed that ZER reduced tube formation by HUVECs effectively inhibits new blood vessel and tissue matrix formation. Western blot analysis revealed that ZER significantly ($P < 0.05$) decreased expression of molecular effectors of angiogenesis, the matrix metalloproteinase-9, vascular endothelial growth factor (VEGF), and VEGF receptor proteins. We found that ZER inhibited the proliferation and suppressed migration of HepG2 cell in dose-dependent manner. **Statistical Analysis Used:** Statistical analyses were performed according to the Statistical Package for Social Science (SPSS) version 17.0. The data were expressed as the mean ± standard deviation and analyzed using a one-way analysis of variance. A $P < 0.05$ was considered statistically significant. **Conclusion:** The study for the first time showed that ZER is an inhibitor angiogenesis, tumor growth, and spread, which is suggested to be the mechanisms for its anti-HCC effect.

Key words: Angiogenesis, matrix metalloproteinase-9, vascular endothelial growth factor, vascular endothelial growth factor receptor, zerumbone

SUMMARY

- Tumor angiogenesis has currently become an important research area for the control of cancer growth and metastasis. The current study determined the effect of zerumbone on factors associated with angiogenesis that occurs in tumor formation.

Abbreviations used: ZER: Zerumbone, MMP-9: Matrix metalloproteinase-9, VEGF: Vascular endothelial growth factor, VEGFR: Vascular endothelial growth factor receptor, HUVECs: Human umbilical vein endothelial cells, HCC: Hepatocellular carcinoma, HIFCS: Heat inactivated fetal calf serum, DMSO: Dimethyl sulfoxide, EDTA: Ethyldiaminetetraacetic acid, Ig: Immunoglobulin, CAM: Chorioallantoic membrane, HRP: Horseradish peroxidase, NIH: National Institutes of Health, MTT: Microtetrazolium, SPSS: Statistical Package for Social Science.



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INTRODUCTION

Cancer is a complex disease that develops as a result of genetic errors in cells.[1] These genetic errors may be caused by chemicals, viruses, or physical assaults to the DNA.[2] Among the consequences, these errors are alterations in the cellular signaling pathways that lead to cancer formation. Since there are numerous signaling pathways involved in cancer formation, the biological and chemical profiles of this

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disease can differ from one cancer to another.[3] Cancer growth and spread are dependent and facilitated by angiogenesis in the tumorous tissues. Angiogenesis is controlled through the balance between pro- and anti-angiogenesis factors, which are vital in the triggering the angiogenesis switch.[4] Thus, among approaches used to curb and control tumor growth is through the inhibiting angiogenesis. In fact, several cytotoxic chemotherapeutic drugs currently in use, including those in HCC chemotherapies, exhibit antitumor activities through antiangiogenesis.[5,6] There are structural variations in angiogenesis receptors in organs and tissues and microenvironments at the tumor sites that present great challenges and limitations to the use of specific receptor-targeted compounds, such as monoclonal antibody-based compounds, as therapeutic agents. Consequently, an antitumor agent may be effective in one organ but not in another.[7]

Hepatocellular carcinoma (HCC) is a typical hypervascular tumor and among the most common liver malignancies. The occurrence of the disease is often plagued with metastasis, even after resection. Treatment of this type of carcinoma is difficult because most patients are diagnosed at the advanced stage,[4,8] and the disease becomes nonamenable toward curative therapy.[9] Several natural compounds are being used for the treatment of HCC, include resveratrol, silibinin, and tanshinone IIA.[10-12] Resveratrol is a polyphenol found in grape skin, peanuts, berries, and red wine. This compound has growth inhibitory effects toward various human cancer cells including HCC, through cell proliferation inhibition and induction of apoptosis. It was postulated that the effect of resveratrol is mediated through the downregulation of Bcl-2 and upregulation of Bax expression.[13] Silibinin (INN), or silybin, is the major active constituent of silymarin (milk thistle). The silymarin extract contains a mixture of flavonolignans to include silicristin, silidianin, isosilibinin, and silibinin.[14] These compounds are known for its hepatoprotective properties.

There are numerous other natural compounds with potential anti-HCC effect. Among them is zerumbone (ZER), a phytochemical isolated from *Zingiber zerumbet* Smith. Conventionally, ZER is used to treat many illnesses, most probably because of its antioxidant and anti-inflammatory activities.[13,15] Currently, this compound has been shown to have anticervical,[16] anti-colon, anti-lung, and anti-leukemia properties.[17] In this study, we investigated the anti-HCC activities of ZER. The choice of ZER as an anti-HCC primarily centers on its reported antiangiogenic and anti-inflammatory properties.[14] In this study, the mechanism of anticancer properties of ZER inhibition was determined on HepG2 cells.

MATERIALS AND METHODS

Zerumbone preparation

Pure colorless ZER crystals were prepared in our laboratory from fresh *Zingiber zerumbet* rhizomes extracted by steam distillation according to a method described earlier.[18]

Animal preparation

All procedures were carried out in accordance with the guidelines of Universiti Putra Malaysia Institutional Animal Care and Use Committee (No: UPM/FPSK/PADS/BR-UUH/00467). A 10- to 12-week-old male Sprague Dawley rats were used in this study. The rats were housed under normal room temperature with free access to food and water.

Ex vivo rat aortic ring assay

The angiogenesis assay was conducted according to the method described by Brown *et al.* with slight modifications.[19] Briefly, freshly excised thoracic tissues harvested from 10- to 12-week-old rats were rinsed and flushed with Hanks balanced salt solution containing 2.5 μg/mL amphotericin B. After careful removal of the fibro-adipose tissue, each aorta was cut under a dissecting microscope into 1 mm long cross-sections and placed in a 48-well tissue culture plate (Coster Corning, USA) containing 500 μL of 3 mg/mL fibrinogen in serum-free M199 growth medium with 5 mg/mL aprotinin. The tissue sections were placed in the center of the wells, and 15 μL of thrombin (0.15M in NaCl) added. The aortic rings were then fed with 0.5 mL medium M199 supplemented with 20% HIFCS, 0.1% ε-aminocaproic acid, 1% L-glutamine, 1% amphotericin, and 0.6% gentamycin and cultured in a humidified incubator at 37°C under 5% CO_2. On day 5, sprouting blood vessels from aortic explants was measured according to the technique described by Nicosia *et al.*[20] Suramin, a well-known antiangiogenesis agent, purchased from Sigma-Aldrich, Germany, was used as positive control and DMSO as a negative control (NC). The blood vessel growth was quantified and examined under an inverted microscope (Olympus, Japan) (×4 magnification), fitted with a camera (Lieca CCD, Japan). The data are represented as mean ± standard deviation. The experiment was repeated three times using six replicates per sample.

HepG2, human umbilical vein endothelial cell and WRL68 proliferation inhibition

3-4-5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell proliferation using the method described earlier.[21] The MTT solution was prepared by adding 5 mg/mL MTT in PBS (phosphate buffered saline). HepG2 (passage 4–7), human umbilical vein endothelial cell (HUVEC), and WRL68 cells (1×10^4) purchased from ATCC (Rockville, MD, USA) were treated with the HepG2 cell IC50 concentration (6.2 μg/mL) of ZER for 72 h. Approximately 20 μL MTT solution was placed in each well and the plates incubated at 37°C under 5% CO_2 for 5 h. DMSO (1%) was used as NC. The absorbance at 570 nm and the reference at 630 nm were determined using a microplate reader (BIORAD, USA). The viability of treated cells represented was determined as a percentage of untreated control, which are cells cultured in control media. Each concentration was tested in triplicate, and the experiment repeated trice.

Endothelial cell tube formation assay

HUVECs (ATCC, Rockville, MD, USA) were maintained in ECM-2 media (Science cell. the USA). The cells were washed, trypsinized (Trypsin-EDTA, Gibco, Life technology, UK), centrifuged at 1000 rpm (Hettich Zentrifugen, Germany) for 5 min, and resuspended in basal media containing 0.1% bovine serum albumin. The cell (5×10^4) in 100 μL media was then seeded into BD BioCoat™ Angiogenesis System Endothelial Cell Tube Formation plates (BD Biosciences and incubated for 4–6 h at 37°C, under 5% CO_2 to promote endothelial cell tube formation. Suramin (Sigma, Aldrich, Germany) (1.0 mg/mL), dissolved in DMSO to a concentration of 10 μg/mL, was used as positive control.

After 4–6 h, the media was discarded, the cells washed twice with Hank's Balanced Salt Solution and viewed under an inverted light microscope (Olympus, Japan) at × 4 magnification. The number of tube junctions formed were determined[22] and expressed as percentage inhibition.

Chorioallantoic membrane assay

The anti-neovascularization effect of ZER was investigated by the *in vivo* chorioallantoic membrane (CAM) assay, using chicken embryos.[23] A 5-day-old fertilized eggs, on initiation of embryogenesis, were incubated under constant humidity at 37°C. Albumin (5 mL) was aspirated and the eggs incubated horizontally to allow for detachment of CAM from the shell. The ZER samples at 25 and 50 μM in 1.2% agarose were

incorporated into the disks. The disk with 1.2% agarose only was the NC. A square window was made on the eggshell, and the disks directly applied onto the CAM. The window was then covered with sterilized surgical tape and the embryos incubated for 24 h. The CAMs were photographed under a dissecting microscope and blood vessels in each CAM photograph quantitated. The results are presented as a mean blood vessel count per CAM.

Vascular endothelial growth factor-induced tyrosine phosphorylation of vascular endothelial growth factor receptor-2 (human umbilical vein endothelial cells)

Serum-starved HUVECs were treated with 12.5, 6.25, 3.125, and 1.56 μg/mL of ZER for 30 min, followed by 50 ng/mL VEGF for 5 min. The phosphorylation state of VEGR receptor-2 (VEGFR-2) was assessed by Western blot using anti-phosphoVEGFR2 antibody.

Cell migration assay

The assay determined the effect of ZER on HepG2 cell migration. The assay was performed according to a previously described method with minor modifications.[24] Briefly, confluent monolayers of HepG2 cells were plated in a 6-well plate. The cells were treated with 6.25, 12.5, and 25 μg/mL ZER for 0, 12, 24, 30, 48, 54, 72, 78, and 96 h. The image was photographed and distances between scratches measured under inverted microscopy (×20 magnification). Ten fields for each treatment were captured, and a minimum of 10 distance readings for each field were obtained. The area of cell-free zone was determined using the Leica QWin software and results presented as percentage wound closure.

Human cytokine array

The expression of angiogenesis-related proteins of ZER-treated HepG2 cells was evaluated by a semiquantitative technique (Proteome Profiler™, Human Angiogenesis Array Kit, RandD Systems, USA) according to the manufacturer's instructions. Duplicate spots on the nitrocellulose membranes were excised and the samples diluted and mixed with a cocktail of biotinylated detection antibodies. The protein components of the mixture was determined by Human Angiogenesis Array kit (Proteome Profiler™, Human Angiogenesis Array Kit, RandD Systems, USA). The protein-antibody complexes present were bound to the membrane cognate-immobilized capture antibody. After washing to remove unbound materials, streptavidin-HRP and chemiluminescent detection reagents were sequentially added. The light intensity of the spots is proportional to the amount of bound analyte. The image of the spots was captured on X-ray films and analyzed using Image J software (version 1.46d; US National Institutes of Health [NIH]). The results are expressed as fold changes above or below the unexposed cell cultures. Only proteins of interest were quantified and reported.

Western blot analysis

The protein contents of cell cultures were determined by Immunblot Kit Goat Anti-Mouse IgG (H + L)-AP Assay Kit (Bio-Rad, USA), according to the manufacturer's protocol The cells were first lysed, and protein concentrations of the lysate determined using the Bradford protein assay. The lysates were also subjected to Western blot assay, and the protein bands were visualized using the Gel Doc XR system (Bio-Rad, USA).

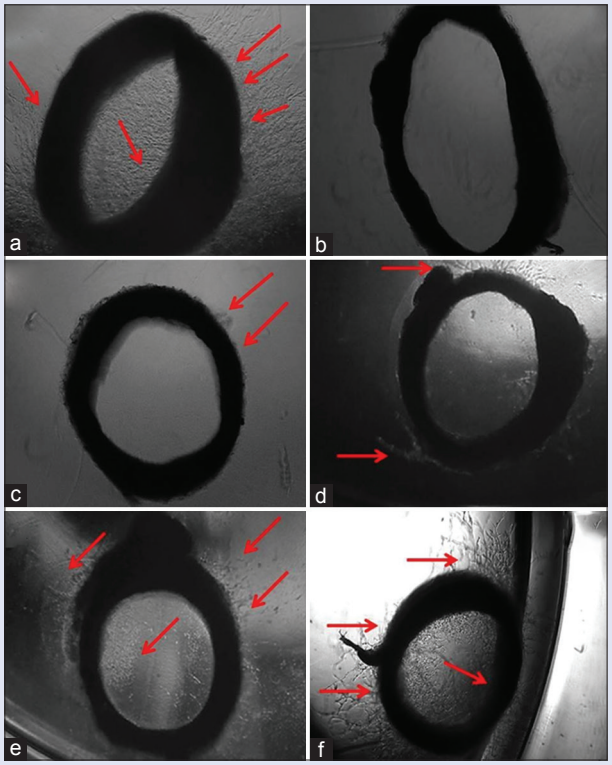

Figure 1: Rat aorta angiogenesis assay. (a) negative control (1% dimethyl sulfoxide), suramin (b) 12.5 μg/mL zerumbone (c), 6.25 μg/mL zerumbone (d), 3.125 μg/mL zerumbone (e), and (f) 1.56 μg/mL zerumbone. The antiangiogenesis activity was quantified by measuring the cell sprouting area around the aorta. Arrows indicate the sprouting of microvessel from the rat aorta, magnification (×4)

RESULTS

Ex vivo rat aortic ring assay

As shown in Figure 1, the microvessel outgrowth from the untreated aortic rings was inhibited by ZER treatment. Suramin as a positive control showed almost 100% inhibition of microvessels outgrowth at 100 μg/mL. At 12.5 μg/mL the anti-neovascularization of ZER was not significant ($P > 0.05$) different from that of suramin. At 6.25 μg/mL, ZER inhibited neovascularization by 70% and doubling the dose to 12.5 μg/mL led to almost complete inhibition.

Cell proliferation inhibition

The MTT assay showed that the IC50 of ZER on HepG2 cells was 6.20 ± 0.7 μg/mL [Table 1]. ZER was noncytotoxic toward HUVECs with IC50 of >50 μg/mL.

Endothelial cell tube formation assay

In the matrigel matrix under a normal microenvironment, endothelial cells will form a network of tube-like structures within 6 h. Treatment of HUVECs with ZER inhibited the formation of endothelial cell tube formation in a dose-dependent manner [Figure 2]. At low concentrations, ZER (3.125 μg/mL) reduced the intensity of network structures and caused the formation of incomplete and broken tubules. At 12.5 μg/mL, ZER completely abrogated endothelial tube formation by 89.00% ± 0.72%. Suramin (100 μg/mL) inhibited endothelial tube formation by 96% ± 0.56%.

Chorioallantoic membrane Assay

The CAM assay provides a rapid method for investigating the release of ZER in a biological system and penetration of ZER through the cell membrane and comparative assessment of the anti-angiogenic activity of the formulation. The assay showed that control CAM exhibited normal capillary network structure [Figure 3]. Chorioallantoic membrane treated with ZER showed vascular regression, which was devoid of capillary network, clearly indicative of the angiostatic activity of ZER.

vascular endothelial growth factor-induced tyrosine phosphorylation of vascular endothelial growth factor receptor-2

ZER was shown to downregulate phosphorylation of VEGFR-2 in HUVECs in a dose-dependent manner [Figure 4]. The greatest downregulation of phopho-VEGFR-2 occurred at 12.5 μg/mL ZER.

Table 1: IC_{50} of zerumbone on human cell lines

Cell lines	IC_{50}±SD (μg/mL)
HepG2	6.20±0.7
WRL68	150±0.4
HUVEC	62.0±1.3

SD: Standard deviation; HUVEC: Human umbilical vein endothelial cell

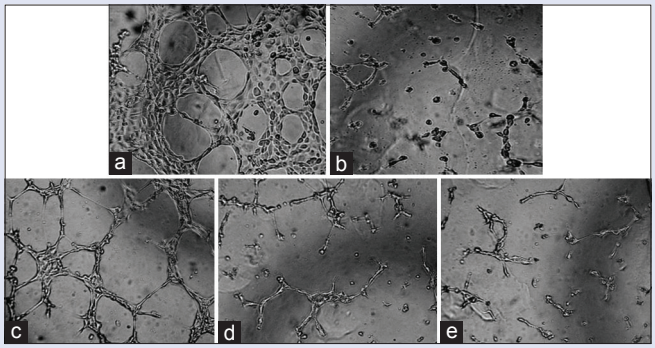

Figure 2: Inhibition of human umbilical vein endothelial cell tube formation by zerumbone. (a) Negative control (b) positive control (c) 3.125 μg/mL zerumbone, (d) 6.25 μg/mL zerumbone and (e) 12.5 μg/mL zerumbone. The ability of cells to form a matrix scaffold of the endothelial tube structure was noticeably inhibited by 6 h of exposure, magnification (×4)

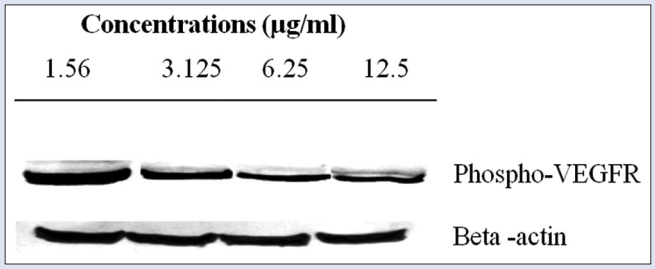

Figure 4: Phosphorylation of vascular endothelial growth factor receptor-2 was assessed by Western blot with antiphospho-vascular endothelial growth factor receptor-2 antibody. Zerumbone inhibited phosphorylation of Vascular endothelial growth factor receptor-2 in human umbilical vein endothelial cells in a dose-dependent manner

Cell migration assay

The effect of ZER on HepG2 cell lines cell migration was also examined. After creation a scratch wound on the membrane monolayer, cells on the edge of the newly created gap moved toward the opening to close the "scratch" until new cell–cell contacts were again established. The wound

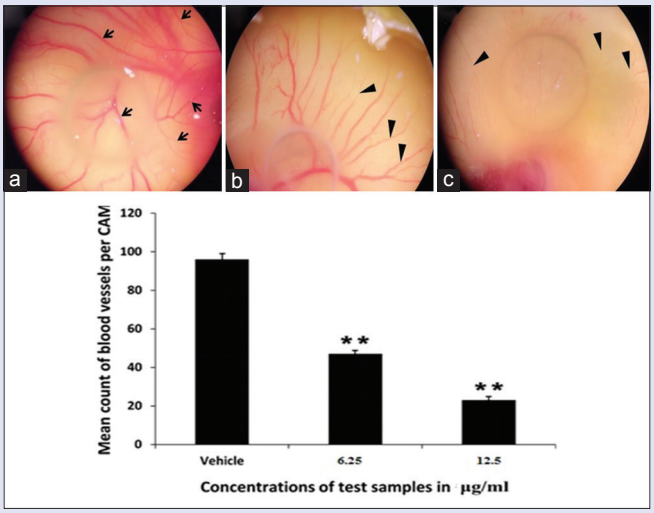

Figure 3: Neovascularisation in chick chorioallantoic membrane after treatment with zerumbone. (a) Vehicle (1% agarose) (b) 6.25 μg/mL zerumbone and (c) 12.5 μg/mL zerumbone. The number of blood vessels are expressed as count per chorioallantoic membrane. **indicates significant difference with vehicle at $P < 0.05$

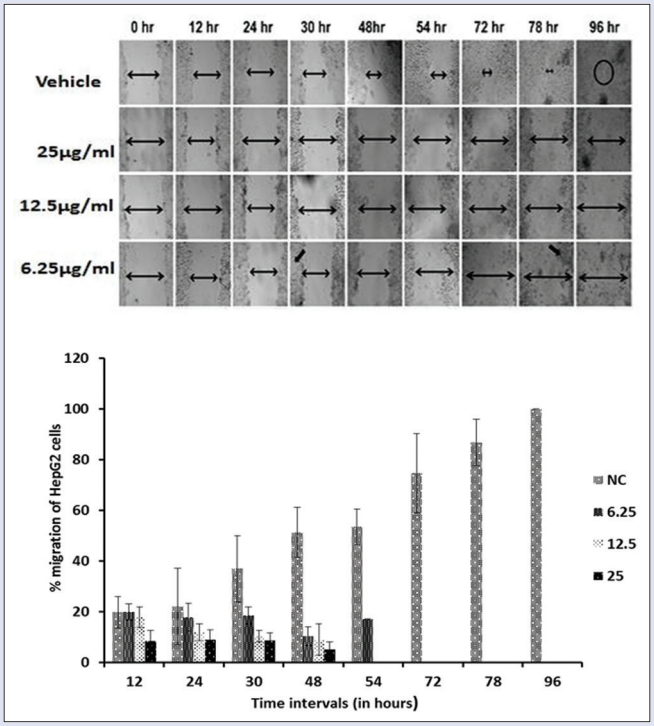

Figure 5: HepG2 cells migration after treatment with 6.25 μg/mL zerumbone for 12, 24, 30, 48, 54, 72, 78 and 96 h. Cells treated with 1% dimethyl sulfoxide served as negative control. The effect of zerumbone was time- and dose-dependent

closure was the result of cell migration [Figure 5]. For untreated cells the scratch closed after 96 h, while for ZER-treated cells, there was not scratch closure. Reduction in HepG2 cell movement to close the scratch was achieved with all concentrations of ZER in time-dependent manner.

Human cytokine array

ZER treatment inhibited expression of VEGF, MMP, angiogenin, and angiopoietin while increasing expression of endostatin and thrombospondin [Figure 6]. The most marked effect of ZER seems to be on the downregulation of MMP and upregulation of thrombospondin.

Western blot

As shown in Figure 7, there is a significant ($P < 0.05$) downregulation in VEGF, Matrix metalloproteinase-9 (MMP-9), and NF-κB expressions. The result suggests that ZER down-regulates these proteins in a dose-dependent manner, with greater effect at higher doses.

DISCUSSIONS

Tumor angiogenesis has currently becoming an important research area for the control of cancer growth and metastasis.[7] The current study determined the effect of ZER on factors associated with angiogenesis that occurs in tumor formation. The rat aortic ring assay is widely used to determine the angiogenesis activity of potential therapeutic compounds. This assay is convenient because angiogenesis can be induced within a short period. This assay is quantitative and the microvessel outgrowths from the aorta, on treatment with chemicals and compounds, can conveniently be quantitated.[20] ZER caused inhibition of microvessel outgrowth from the rat aorta, an effect similar to that produced by the antiangiogenic drug, suramin.[25] Fortunately, ZER although has antiproliferative effect on the HepG2 cells were relatively harmless to the normal HUVECs and human WRL68 cell lines. New blood vessel formation requires a conducive microenvironment that includes presence of tissue extracellular matrix for new vessels to anchor. ZER impeded matrix formation for the endothelial cell growth, suggesting that ZER is antiangiogenic. The antiangiogenic effect was confirmed by the CAM assay that showed that ZER exerted strong an inhibitory effect on neovascularization.[19,20] ZER had dose-dependently caused severe disruption in the vasculature pattern affecting primary vessels and subsequent reduction in number of new secondary and tertiary vessels. The anti-neovascularization effect of ZER is particular evident at the higher concentrations.

VEGF is a signalling protein regulating angiogenesis and vasculogenesis. The VEGF binds to and activates the receptor tyrosine kinase, VEGFR through transphosphorylation and the formation of VEGF-VGFR complex induces angiogenesis. Antiangiogenic therapies based on inhibition of VEGF-VEGFR signalling were reported to be effective therapeutic strategies in oncology and ophthalmology.[26] Our study showed that ZER suppresses phosphorylation of VEGFR-2 in HUVECs. Evidently, ZER had effectively inhibited the *in vitro* release of key angiogenic factor, VEGF and the VEGF-VEGFR binding through phosphorylation.

On the HepG2 cells, ZER demonstrated both dose- and time-dependent inhibition of cell migration. The HepG2 cells on treatment with ZER at its IC50 concentration were very slow to migrate, while the cells not treated completed migration by 96 h. In fact, at concentrations higher than its IC_{50}, ZER caused dislodgement of HepG2 cells, almost completely inhibiting cell migration that is essential for cancer to grow and spread.

The VEGF, angiogenin, MMP, angiopoietin, endostatin, and thrombospondin are among positive effectors of angiogenesis. In this study, ZER was shown to downregulated VEGF, angiogenin, MMP, and angiopoietin while upregulating endostatin and thrombospondin expressions in HepG2 cells. Western blot analysis showed that ZER suppressed VEGF, MMP-9 and NF-κB expression in the HepG2 cells in time-dependent manner, with the effect getting stronger with time of exposure. This phenomenon may be due to the fact that tumors are not all angiogenic from the beginning. It is only after a certain mass of tumor has established and will the tumor begin inducing angiogenesis to facilitate growth and spread. Tumors also require capillary networks to grow and for the supply of oxygen and metabolites.[6,21,23] Initiation of angiogenesis requires an increase in expression of angiogenic cytokine, such as VEGF.[21,22] The VEGF is one of the most potent and specific angiogenic factors in tumour-induced angiogenesis,[20,22,24] stimulating endothelial cell permeability, proliferation and angiogenesis.[27] In addition, VEGF also induces expression of MMP-9,[28] which are enzymes implicated in the degradation basement membrane and extracellular matrix. These effects allow for new blood vessels to sprout from existing ones.[29,30]

The metastatic potential of tumor cells is also directly correlated to the level of VEGF expression, and tumor growth and invasion can be suppressed through inhibition of VEGF expression.[31,32] While VEGF

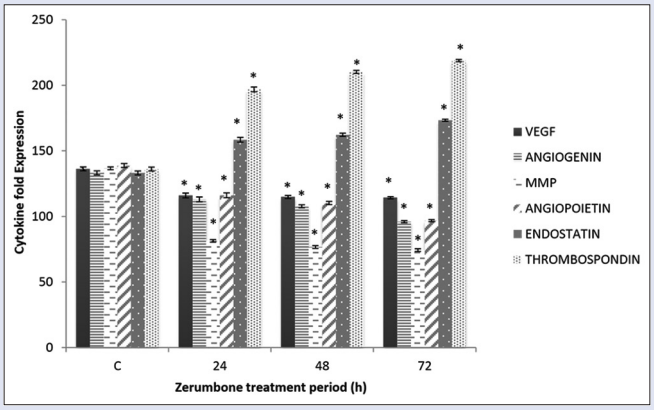

Figure 6: Differentially expressed cytokines. Differentially expressed cytokines in HepG2 cells treated with 6.25 µg/mL zerumbone. The results are expressed as cytokine fold changes above or below the control (untreated cell cultures). * indicates significant differences at $P < 0.05$

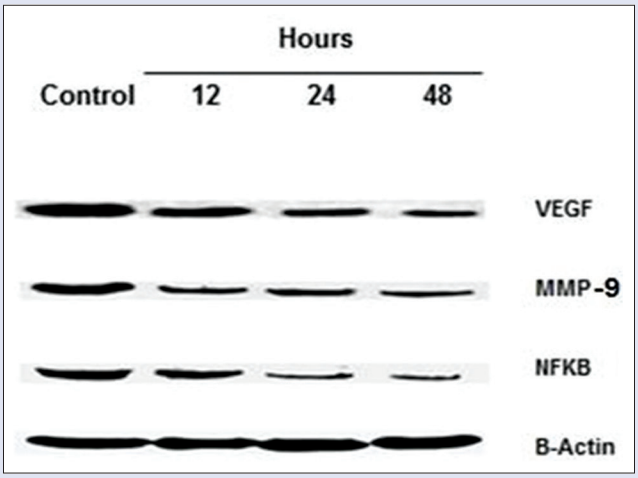

Figure 7: Expression Western blot analysis study of zerumbone 6.25 µg/mL on vascular endothelial growth factor, matrix metalloproteinase-9 and NF-κB proteins shown by Western blot analysis. zerumbone downregulates vascular endothelial growth factor, matrix metalloproteinase-9 and NF-κB in dose-dependent manner

and MMP are angiogenic, the NF-κB plays a key role in cellular response toward chemical and organic stimuli. The NF-κB is responsible for cytokine production and cell survival; its downregulation would impede cell survival. The expressions of VEGF and MMP proteins are positively correlated with NF-κB gene expression.[33] Thus, the downregulations of VEGF, MMP, and NF-κB proteins in the ZER-treated HepG2 cells are among the mechanisms responsible for growth prevention, migration, and metastasis of hepatocellular carcinoma.

CONCLUSION

The study showed that ZER induces antiangiogenesis in HCC through the downregulation of positive mediators of angiogenesis, especially MMP, VEGF, and VEGFR proteins. Thus, ZER is an effective anti-liver cancer compound.

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Conflicts of interest

There are no conflicts of interest.

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