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ORIGINAL ARTICLE

Antiproliferative and Antiangiogenic Effects of Zerumbone from *Zingiber zerumbet* L. Smith in Sprague Dawley Rat Model of Hepatocellular Carcinoma

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ABSTRACT

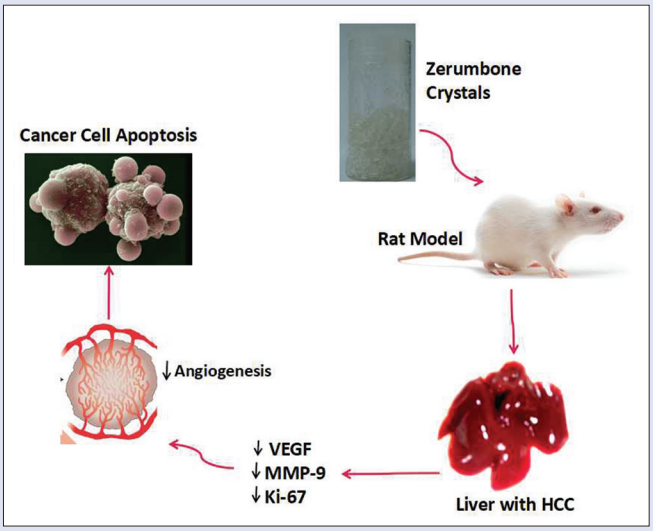
Context: Zerumbone (ZER) is known to exhibit anticancer properties on various cancer cells both *in vitro* and *in vivo*. However, the anti-angiogenesis effect of ZER on liver cancers *in vivo* is not yet addressed clearly. **Aims:** This study aimed to investigate the *in vivo* antiproliferative and antiangiogenesis effects of ZER using rats with diethylnitrosamine-induced hepatocellular carcinoma (HCC). **Materials and Methods:** The antiproliferative and anti-angiogenesis effects of ZER were determined using the hepatosomatic index, vascular endothelial growth factor (VEGF) immunoassay, terminal deoxynucleotidyl transferase dUTP nick end labeling assay, histopathology, and immunohistochemistry analysis. **Results:** Nontreated rats with HCC had higher median liver weight than those treated with ZER or suramin. The expression of VEGF, matrix metalloprotease, and Ki-67 that were high in nontreated HCC rats was down-regulated with ZER or suramin treatments. **Statistical Analysis Used:** Statistical analyses were performed using the Statistical Package for Social Science version 21.0 (SPSS Inc, IBM, Maryland, USA). The data were expressed as the mean ± standard deviation and analyzed using a one-way analysis of variance. $P < 0.05$ was considered statistically significant. **Conclusion:** ZER has the potential to be developed as the pro-apoptotic and antiangiogenic agent in the treatment of HCC.

Key words: Active compound, angiogenic biomarkers, antiproliferative, hepatocellular carcinoma, *in vivo* study, natural product

SUMMARY

- Cancer has the ability to spread to adjacent or distant organs through the formation of a new blood, and lymphatic vessels in a process called angiogenesis and lymphangiogenesis, respectively. Thus, studying the compounds that derived from natural products with antiangiogenic abilities are of great importance to reduce the chances of life-threatening diseases.

Abbreviations used: ZER: Zerumbone; HCC: Hepatocellular carcinoma; MMP-9: Matrix metalloproteinase-9; VEGF: Vascular endothelial growth factor; DEN: Diethylnitrosamine; PBS: Phosphate-buffered saline; ELISA: Enzyme-Linked Immunosorbent Assay; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; FFPE: Formalin-fixed and paraffin-embedded; H and E: Hematoxylin and Eosin; DAB: Dako Envision®+Dual Link System; HRP: Horseradish peroxidase; H_2O_2: Hydrogen peroxide; PBST: PBS in Tween 20; SPSS: Statistical Package for Social Science; RUGS: Research University Grant Scheme.



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INTRODUCTION

Zerumbone (ZER), a monocyclic sesquiterpene with chemical formula $C_{12}H_2O$ and molecular weight of 218.340 daltons, is most abundantly found in *Zingiber zerumbet* (L.) Smith. ZER was shown to have antiproliferative properties on cancer cells by inducing apoptosis and G2/M cell cycle arrest.[1]

Angiogenesis and neovascularization are tightly controlled by the intricate balance between pro- and anti-angiogenic factors.[2,3] In the

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development and growth of tumors, during angiogenesis, new blood vessels invade the surrounding tissues. In tumor angiogenesis, there is a shift in the equilibrium between angiogenic and anti-angiogenic factor, alteration in endothelial cell morphology, secretion of proteolytic enzymes, migration of endothelial cells, generation of new endothelial cells, and formation of new blood vessels. The proteolytic enzymes cause degradation of the vascular wall and extracellular matrix to facilitate the proliferation of new blood vessels. In this process, the endothelial cells migration resulting in sprouting and elongation of new blood vessels.[4]

Primary liver cancers, such as hepatoma and hepatocellular carcinoma (HCC) are among the common malignancies in humans. HCC is the most deadly form of cancers accounting for up to 90% of all primary liver cancers.[5] The cancer is heterogeneous, and the etiologies include hepatitis and hepatitis C virus infections, alcohol abuse, aflatoxin B1, and other hepatotoxic substances.[6] HCC is a hypervascular tumor and is highly dependent on angiogenesis to grow, spread, and metastasis.[7,8] Cancer can be treated with liver transplantation or surgical resection if diagnosed at the early stage. However, since most patients are presented with the advanced stage of the disease, only approximately 15% of patients can be cured by interventions.[5] Even after surgical resection, the recurrence rate of the tumor in these patients may still be as high as 50% after 2 years and 76% after 10 years.[9]

Consequently, active compounds derived from plant parts pose a candidate for anti-angiogenic agents due to their potent activity in causing the death of cancerous cells. Alternatively, extensive studies have been conducted to assess the role of pure compounds in the prevention of cancer growth and development through their antiangiogenic activities. Considering these perspectives, there have been extensive studies on natural product compounds and extracts that showed potent anti-angiogenic activity to overcome the adverse effect of synthetic agents on human health,[10] such as resveratrol from grape,[11] curcumin from turmeric,[12] Honokiol from magnolia,[13] silymarin from milk thistle,[14] and rhein from *Rhizoma rhei*.[15]

Regarding the molecular mechanism in initiating and developing the angiogenic switch, tumors can produce vascular endothelial growth factor (VEGF) and matrix metalloprotease-9 (MMP-9) to aid angiogenesis.[16] VEGF is mainly implicated in early dissemination of malignant cells,[17] whereas MMP-9 plays a vital role in the digestion of extracellular matrix and basement membrane during angiogenesis in which it degrades Type IV collagen, a major constituent of the basement membrane,[18] to facilitate angiogenesis.

HCC highly expresses MMP-9, a characteristic associated with the tendency for relapse and metastasis.[19] For that reason, the expression of MMP-9 in HCC is of prognostic significance.[20] The activation of MMP-9 is linked to up-regulation of other angiogenic stimulators including VEGF.[21] On the other hand, Ki-67, a nuclear protein that presents only in proliferating cells,[22] and is widely used as a marker for tumor cell proliferation. Thus, in HCC, the determination of expression of this antigen would be a good indicator of prognosis and overall survival of cancer patients.[23]

Very recently, we have confirmed that ZER suppresses angiogenesis in HepG2 liver cancer cells through inhibition of MMP-9, VEGF, and VEGFR expressions.[21] Thus, the present study investigated the anti-angiogenic effect of ZER in diethylnitrosamine (DEN)-induced rat hepatocarcinogenesis model through the determination of angiogenic factors and histopathological changes.

MATERIALS AND METHODS

Materials

Zerumbone

Pure ZER crystals were extracted from the essential oil of *Z. zerumbet* (L.) Smith rhizome according to the method described previously.[1] Briefly, fresh rhizomes were sliced, and placed in a steam distillation containing tap water and heated. Then, the distillate containing volatile oil was collected in Dienstag glassware that was connected to the outlet of the device. The volatile oil was crystallized using absolute n-hexane (Sigma-Aldrich), and the solution was evaporated in a fume hood (Novaire, Newton, MA, USA). Recrystallization was performed 3 times using absolute n-hexane to obtain pure colorless ZER crystals that were collected in clean glass vials and kept at 4°C until further analysis.

Animals

Thirty adult male Sprague-Dawley rats weighing 150 ± 5.0 g and aging 7–9 weeks were used in this experiment. The rats were maintained in a humidified room under 12 h light/12 h dark cycle for 1 week before experimentation. All rats were given normal rodent diet and water *ad libitum*.

Ethical approval

All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Faculty of Medicine and Health Sciences (UPM/FPSK/PADS/BR-UUH/00467), Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Preparation of solutions and cancer induction

DEN (Sigma-Aldrich) solution was prepared by dissolving 1.0 mL DEN in 2.33 mL corn oil. ZER solution was prepared by dissolving 100 mg of ZER crystals in 10 mL of corn oil. All rats except those in control negative group were treated with the single intraperitoneal injection of DEN in corn oil at 200 mg/kg body weight to induce hepatocarcinoma.[24]

Experimental design

The rats were in five groups of six animals each; Group A was untreated and served as the negative control. Group B was not treated and served as the positive cancer control. On day 12 post-DEN injection, Groups C and D were treated intraperitoneally with 30 and 60 mg/kg body weight ZER in corn oil respectively, 4 times a week for 3 weeks. Group E was treated intraperitoneally with 5 mg/kg body weight suramin in water twice a week for 3 weeks. The body weights were recorded, and blood collected through cardiac puncture to obtain serum for biochemical analyses. The animals were humanely sacrificed, and the liver removed. Each liver was weighed and the hepatosomatic index (liver weight/body weight ×100) for each rat calculated. Tissue samples were washed in ice-cold phosphate-buffered saline (PBS) and dried with tissue paper. Liver tissues for histological examination were preserved in 10% buffered formalin.[25]

Serum vascular endothelial growth factor

The serum VEGF concentration was determined in triplicates using the Enzyme-Linked Immunosorbent Assay Quantikine Rat VEGF Immunoassay kit (R and D Systems, Minneapolis, MN, USA) according to the instructions of the manufacturing company and standard VEGF curve was generated.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Dead EndTM fluorometric TUNEL system, Promega, Fitchburg, MI, USA) was used to determine apoptosis in

the liver tissues. The formalin-fixed and paraffin-embedded (FFPE) liver tissue sections were first deparaffinized, rehydrated, fixed in formaldehyde, and equilibrated. rTdT incubation buffer was then added and the sections covered with glass slips and incubated in the dark at 37°C for 60 min in a humidified chamber. After terminating reactions by immersing in 2 × SSC, the slides were stained with freshly prepared PI in PBS solution. The slides were washed with PBS between each step. Samples mounted on glass coverslips were microscopically viewed for green fluorescence at 520 ± 20 nm and red fluorescence at >620 nm.

Histopathology

FFPE liver tissues were stained with hematoxylin and eosin according to the standard method.[26] The tissues were examined to determine cell size, nuclear chromatin content, and presence of cytoplasmic granulation intracytoplasmic and violaceous material.

Immunohistochemistry of liver tissue

This assay was performed with the Dako Envision®+Dual Link System-HRP (DAB+) kit (Dako K4965, USA) with slight modification. The FFPE liver tissues on glass slides were heated in an oven at 60°C for 1 h before deparaffinization and rehydration by immersion twice in xylene followed in sequence with 100, 90, 70, and 50% (v/v) ethanol for 3 min each, at room temperature. The slides were rinsed at trice under slow running tap water. The sections were immersed in boiling sodium citrate buffer (pH 6.0) for 5 min, to expose the antigenic sites, and rinsed with distilled water, washed twice with PBS in 0.1% Tween-20 (PBST) for 3 min each before incubation with 3% (v/v) hydrogen peroxide (H_2O_2) for 10 min at room temperature to quench endogenous peroxide. The sections were then incubated overnight at 4°C with primary antibodies either to VEGF (Santa Cruz Biotechnology, Santa Cruz, Palo Alto, CA, USA; 1:250 dilution), MMP-9 (Santa Cruz Biotechnology, Santa Cruz, Palo Alto, CA, USA; 1:200 dilution) or Ki-67 (Abcam, UK; 1:100 dilution). After rinsing twice with PBST and distilled water for 3 min each time, 500 µL secondary antibodies were added and the section incubated for 1 h at room temperature. The sections were again rinsed twice with PBST and distilled water for 3 min each time. Peroxidase activity shown by the presence of a brown precipitate was determined by incubating sections with 0.6% H_2O_2 and 300 µL of 3, 3'Diaminobenzidine (DAB) chromogen substrate for 10 min at room temperature. The tissue sections were counterstained with 500 µL hematoxylin for 3 min and dehydrated xylene followed in order by 50, 70, 90, and 100 ethanol for 3 min each before mounting. Dried slides were examined under light microscopy at ×40 magnification.[27]

RESULTS AND DISCUSSIONS

Body and liver weight

The body and liver weights of treated rats are shown in Table 1. There was no difference in mean body weight gain among groups of rats. The average liver weight was significantly ($P < 0.05$) higher in the DEN-treated than negative control, ZER-treated or suramin-treated rats. There was no significant ($P > 0.05$) difference in either body or liver weights between ZER-treated and suramin-treated rats. This result indicates that the administration of ZER as an angiogenesis inhibitor does not have any effect on either body weight or liver weight which is in agreement with the results of other researchers that done on tumor-bearing male Japanese White rabbits.[28]

Table 1: Body and liver weights of zerumbone-treated diethylnitrosamine-induced hepatocellular carcinoma in rats

Group	Final body weight (g)	Liver weight (g)
Normal control	255^{a}±2.90	7.5^{a}±2.8
DEN control	238^{a}±1.19	11.5^{b}±0.5
ZER (30 mg/kg body weight)	241^{a}±2.18	$8.2^{a,b}$±0.21
ZER (60 mg/kg body weight)	250^{a}±2.09	$8.4^{a,b}$±0.35
Suramin (5 mg/kg body weight)	254^{a}±1.52	$8.0^{a,b}$±1.7

Values are mean±SD. ^{a,b}Means within columns with different superscripts are significantly different at P=0.05. DEN: Diethylnitrosamine; ZER: Zerumbone; Normal control: Rats without HCC; DEN control: Untreated rats with HCC. Rats with HCC were treated with ZER and suramin; HCC: Hepatocellular carcinoma; SD: Standard deviation

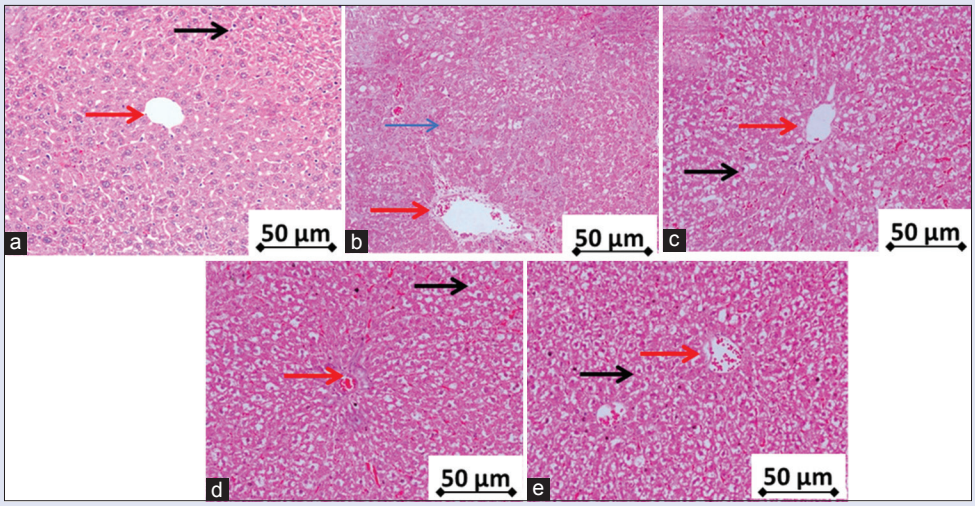

Figure 1: Liver tissues of rats with hepatocellular carcinoma and treated with zerumbone and suramin. (a) Normal untreated rat liver showing centrally located central vein (red arrow) with normally arranged hepatocytes (black arrow), (b) Diethyl nitrosamine control showing pleomorphic cancerous cells (blue arrow) with that are abnormally arranged giving the section anaplastic appearance (black arrow) and the vein is not centrally located (red arrow), (c) Zerumbone (30 mg/kg body weight)-treated liver section showing slightly normally oriented hepatocytes (black arrow) around the central vein (red arrow) with reduced number of cancerous cells, (d) Zerumbone (60 mg/kg body weight)-treated liver section showing significant reduction of cancer cells with regularly arranged hepatocytes (black arrow) around the central vein (red arrow) and (e) Suramin (5 mg/kg body weight)-treated liver showing profound reduction in cancerous cells with normal oriented hepatocytes (black arrow) around the central vein (red arrow) (×400)

Histopathology

Histopathological analysis showed DEN had induced HCC in the rats [Figure 1] with the appearance of anaplastic tissue with pleomorphic cells. ZER treatment had reduced the level of the cancerous rat liver tissues to approach normal with normally oriented hepatocytes around the central veins, and like suramin treatment also reduced the number of cancer cells. This result is in agreement with the outcomes of Taha *et al*. 2010.[24] Furthermore, a previous study revealed that ZER treatment alone did not significantly affect the liver histological changes, suggesting this compound is a safe therapeutic compound.[29]

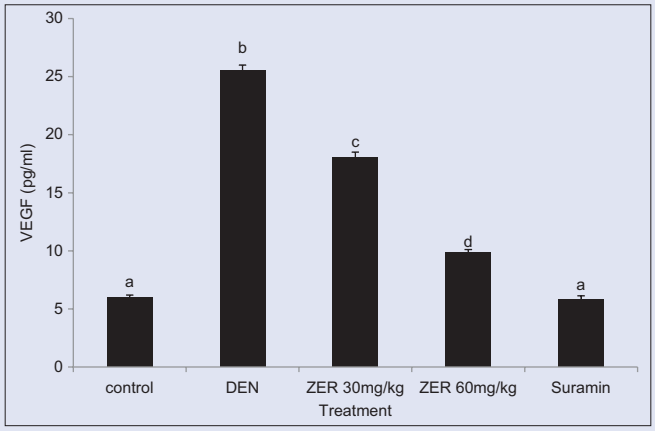

Figure 2: Serum vascular endothelial growth factor concentration of rats with hepatocellular carcinoma and treated with zerumbone and suramin. Values are mean ± standard deviation (error bar). DEN = diethyl nitrosamine rats. Means with different alphabets are significantly different at $P < 0.05$. The serum vascular endothelial growth factor of untreated rats was significantly higher than the control, zerumbone and suramin treated rats with hepatocellular carcinoma. Zerumbone treatment significantly increased serum vascular endothelial growth factor above that of control and suramin treated rats with hepatocellular carcinoma

Serum vascular endothelial growth factor

Many endogenous proangiogenic factors are expressed in HCC and these factors are suggested to play a role in HCC pathogenesis. For instance, the serum VEGF increases with advancing HCC stages, being the highest in patients with metastatic cancers.[30,31] Serum VEGF is closely associated with VEGF expression in livers with HCC. In fact, elevated serum VEGF levels are also associated with poor prognosis and response to therapy.[32] In our study, the serum VEGF concentrations increased in untreated rats with HCC. On treatment with ZER, the serum VEGF in these rats decreased significantly [Figure 2]. It is presumed that the decrease in serum VEGF in the treated rats is due to decrease in angiogenic activity in the tumor tissues. Since the expression of VEGF in HCC tissue is highly linked with growth, malignancy, and metastasis of the tumor, this biomarker can be also used to determine prognosis in HCC.[33,34] Thus, with the downregulation of VEGF expression in ZER-treated rat HCC suggests that this compound is effective in inhibiting the growth of liver cancers. This outcome is confirming the *in vitro* VEGF expression in HepG2 cells that recently done by our group.[21]

Apoptosis

The liver tissue sections of rats with HCC treated with ZER and suramin showed the increased number of apoptotic cells [Figure 3] while nontreated rat livers with HCC did not show apoptosis. ZER is known to cause apoptosis of various tumor cells to include human mammary adenocarcinoma,[35] human lymphoblastic leukemia,[1] and human colorectal adenocarcinoma.[36] These studies show that ZER does not only just prevent growth and spread of tumors but also induces death of cancer cells.

Liver vascular endothelial growth factor, matrix metalloprotease-9, and Ki-67 expressions

VEGF, MMP-9, and Ki-67 have suggested being useful biomarkers for HCC. MMP-9 over-expression seems to promote liver tumor development,[37] and is associated with malignancy, easy recurrence, and

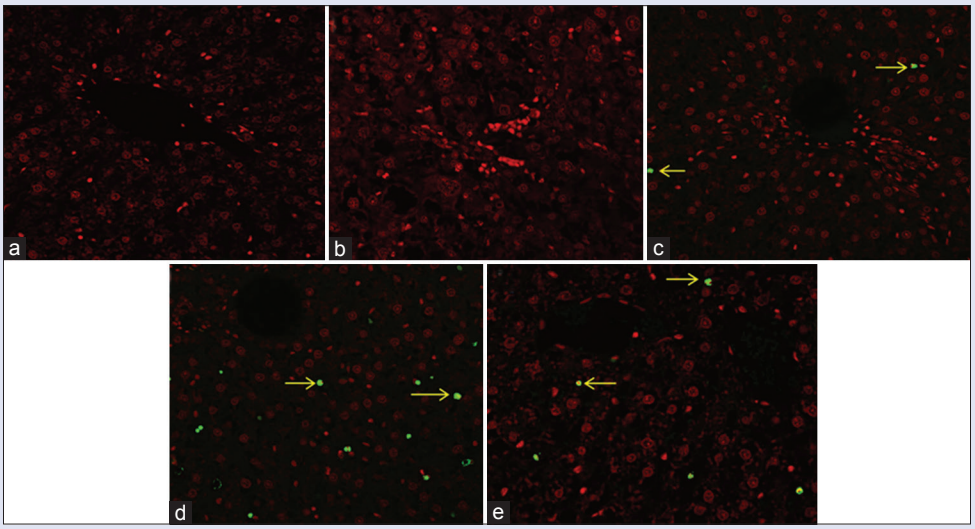

Figure 3: Terminal deoxynucleotidyl transferase dUTP nick end labeling assay of zerumbone -treated hepatocellular carcinoma rats. Liver tissues of rats with hepatocellular carcinoma treated with zerumbone and suramin. (a) Normal (control), (b) Untreated, (c) Treated with 30 mg/kg body weight zerumbone, (d) Treated with 60 mg/kg body weight zerumbone and (e) Treated with suramin (5 mg/kg body weight). Zerumbone caused the increase in apoptotic cells (yellow arrow) in a dose-dependent manner. Effect of zerumbone in inducing apoptosis of hepatocellular carcinoma is greater than suramin. Cells with green stained nuclei are apoptotic cells (×400 magnification)

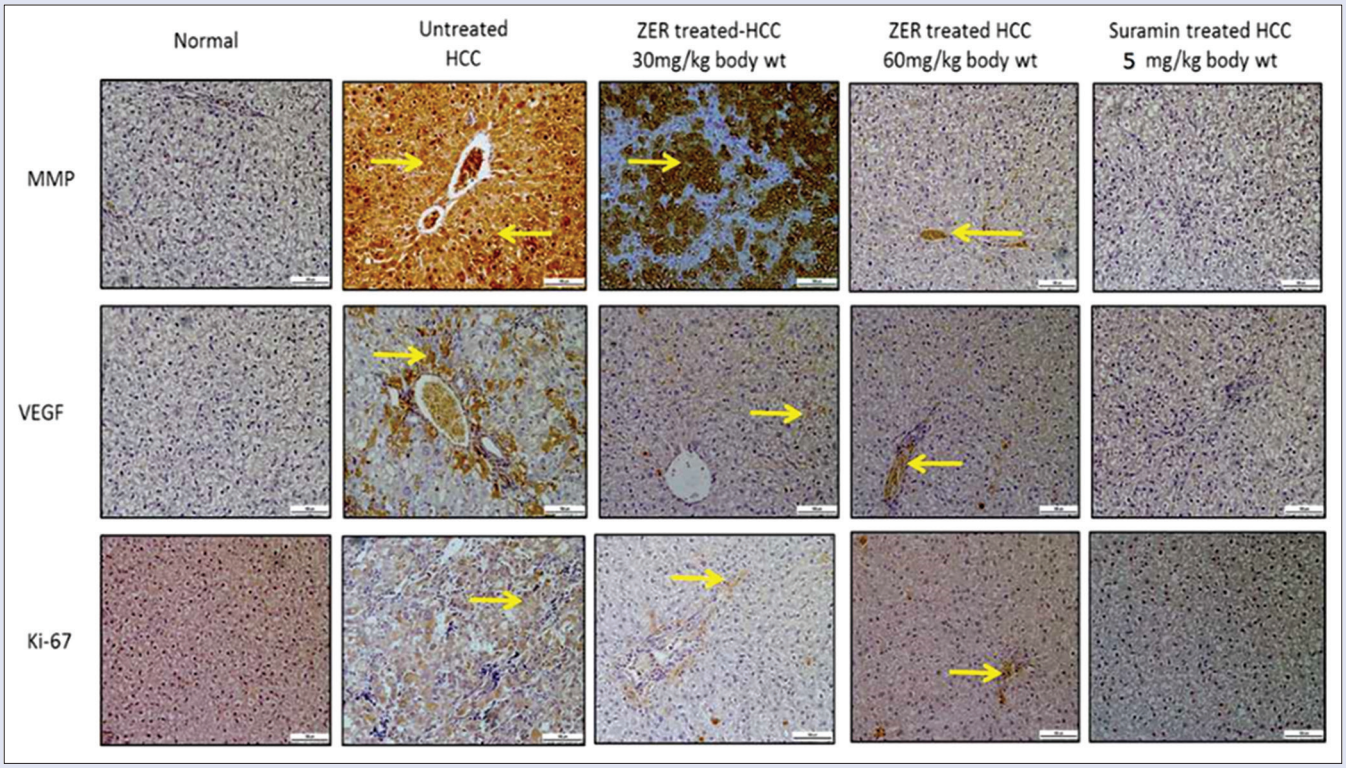

Figure 4: Immunohistochemical staining of rat liver tissues stained for vascular endothelial growth factor, matrix metalloprotease-9, and Ki-67. Normal group (untreated control group without vascular endothelial growth factor, matrix metalloprotease-9 and Ki-67 expression), untreated hepatocellular carcinoma (positive cancer control) showing strong vascular endothelial growth factor, matrix metalloprotease-9 and Ki-67 expression, 30 mg/kg body weight zerumbone showing reduced vascular endothelial growth factor, matrix metalloprotease-9 and Ki-67 expression compared to positive control and 60 mg/kg body weight zerumbone showing greater reduction of vascular endothelial growth factor, matrix metalloprotease-9 and Ki-67 expression than that produced by 30 mg/kg body weight zerumbone. Positive vascular endothelial growth factor, matrix metalloprotease-9 and Ki-67 expression are shown by the brownish staining of cells (Yellow arrows) (×400)

cancer metastasis.[38] High serum VEGF, presumably from high tissue expression,[39] and high Ki-67 expression in the HCC tissues indicates the poor prognosis.[40,41] High serum and tissue VEGF is associated with growth and metastasis of the tumor.[42] In our study, immunochemistry analysis showed that VEGF, MMP-9, and Ki-67 were expressed in the liver of rats with HCC but not in normal rats. Rats with HCC and treated with ZER showed ZER decreased the expression of VEGF, MMP-9, and Ki-67 in the liver tissues. The effect was particularly striking in rats treated with high ZER dose and with suramin [Figure 4].

Tumor cells mostly consist of proliferating cells and some temporarily nonproliferating cells at the G0 stage of the cell cycle.[43] Ki-67 is a nuclear antigen that is highly expressed in proliferating cells but not those in G0 stage.[44] However, Ki-67 is rapidly degraded, or its antigenic determinant disappears after mitosis making it a good indicator of cell proliferation, particularly in cancers. Thus, in HCC, high cellular Ki-67 labeling index suggest low cellular differentiation and poor prognosis.[45] In rats with HCC treated with high dose ZER, unlike in nontreated, the liver tissues no longer showed expression of Ki-67 antigen.

Regarding the biomarker expression by the effect of ZER in tumor angiogenesis, other researchers indicated that ZER inhibits tumor angiogenesis by blocking NF-κB in gastric and pancreatic cancers respectively.[46,47]

CONCLUSION

ZER was shown *in vivo* to have anti-HCC properties. VEGF, MMP-9, and Ki-67 are mediators of angiogenesis. These mediators were downregulated in the HCC liver tissues after treatment with ZER. The anticancer properties of ZER on HCC is both by inhibition of angiogenesis and stimulating cancer cell apoptosis suggesting it has great potential to be developed into an anti-liver cancer chemotherapeutic.

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Conflicts of interest

There are no conflicts of interest.

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