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Malaysian *Tualang* Honey Suppresses the Angiogenic Events in Endothelial Cells Induced by Vascular Endothelial Growth Factor

(Madu Tualang Malaysia Menindas Keadaan Angiogenik dalam Sel Endotelium Teraruh oleh Faktor Pertumbuhan Endotelium Vaskular)

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ABSTRACT

Anti-VEGF therapy has been used as the anti-angiogenic agent in cancer treatment. However, the treatment often couples with severe complications. Complementary natural products that offer similar anti-angiogenic potency with less side effects are sought as alternatives. Study showed that Malaysian *Tualang* Honey (MTH) can inhibit inflammation-induced vascular hyperpermeability *in vitro* and *in vivo*, but the effect on the angiogenesis process remains unclear. Thus, this study aims to determine the anti-angiogenic effects of MTH. Effects of MTH ranging from 0.3% to 0.9% on VEGF-induced human umbilical vein endothelial cells (HUVEC) angiogenesis was determined by proliferation, migration and tube-formation assays. Matrix metalloproteinase-2 (MMP-2) secretion from HUVEC and VEGF production from MCF7 cancer cells in response to MTH were also quantified by using ELISA kits. Suramin, an angio-suppressive agent was used as positive control. MTH significantly suppressed HUVEC proliferation (from 155% proliferation rate to 54%); migration (~ 50% inhibition rate) and tube-formation (69% reduction) induced by VEGF. These findings were explained by the significant reduction ($p < 0.05$) of VEGF-induced MMP-2 secretion in HUVEC with 39% suppression exhibited by 0.9% of MTH. MTH also significantly ($p < 0.05$) reduced VEGF secretion (19% reduction compared to control) in MCF7 breast cancer cells. Our findings suggest that the anti-angiogenic effects of MTH mainly targets endothelial cell through inhibition cell proliferation, migration and tube formation capacity, via suppression of MMP-2 secretion by the endothelial cells. However, MTH has less prominent effect on suppressing VEGF secretion by the MCF7 cancer cells.

Keywords: Angiogenesis; human umbilical veins endothelial cells; matrix metalloproteinases-2; migration; proliferation; tube formation

ABSTRAK

Terapi anti-VEGF telah digunakan sebagai agen anti-angiogenik dalam rawatan kanser. Walau bagaimanapun, rawatan ini menyebabkan komplikasi yang teruk. Produk pelengkap semula jadi yang memberikan potensi anti-angiogenik serupa dengan kurang kesan sampingan dicari sebagai alternatif. Kajian menunjukkan bahawa Madu Tualang Malaysia (MTH) boleh menghalang kebolehtelapan vaskular yang disebabkan oleh keradangan secara *in vitro* dan *in vivo*, tetapi kesan ke atas proses angiogenesis masih kurang jelas. Oleh itu, kajian ini bertujuan untuk menentukan kesan anti-angiogenik MTH. Kesan MTH antara 0.3% hingga 0.9% pada angiogenesis sel endotelium vena umbilikus manusia (HUVEC) yang disebabkan oleh VEGF telah ditentukan melalui ujian percambahan, penghijrahan dan pembentukan tiub. Rembesan matriks netaloproteinase-2 (MMP-2) daripada pengeluaran HUVEC dan VEGF daripada sel kanser MCF7 sebagai

tindak balas kepada MTH juga dihitung dengan menggunakan kit ELISA. Suramin, agen penekan angio digunakan sebagai kawalan positif. MTH telah menyekat percambahan HUVEC teraruh VEGF dengan ketara (daripada 155% kadar percambahan kepada 54%); penghijrahan (~ 50% kadar perencatan) dan pembentukan tiub (69% pengurangan). Penemuan ini dijelaskan oleh pengurangan ketara ($p < 0.05$) rembesan MMP-2 yang disebabkan oleh VEGF dalam HUVEC dengan 39% penindasan ditunjukkan oleh 0.9% daripada MTH. MTH juga secara signifikan ($p < 0.05$) mengurangkan rembesan VEGF (pengurangan 19% berbanding kawalan) dalam sel kanser payudara MCF7. Penemuan ini mencadangkan bahawa kesan anti-angiogenik MTH mensasarkan sel endotelium secara utama dengan percambahan sel perencatan, penghijrahan dan kapasiti pembentukan tiub, melalui penindasan rembesan MMP-2 oleh sel endotelium. Walau bagaimanapun, MTH mempunyai kesan yang kurang menonjol dalam menyekat rembesan VEGF oleh sel-sel kanser MCF7.

Kata kunci: Angiogenesis; matriks metaloproteinase-2; pembentukan tiub; penghijrahan; percambahan; sel endotelium urat umbilikus manusia

Introduction

Cancer remains the major cause of mortality worldwide. According to the Global Cancer Observatory database by World Health Organization (Globocan 2018), cancers related to breast, colorectum, lung, nasopharynx and liver cancers are the 5 most common types of cancer in Malaysia in 2018 regardless of gender. About 43,837 new cases was reported and total number of deaths reported was 26,395 in the same year. About 90% of the death was due to cancer metastasis, a phenomenon which is characterized by spreading of malignant cells from the primary site to surrounding tissue or distant organ (Guan 2015). It is known that the growth of vascular networks, or also known as angiogenesis, is key to tumour growth and its transition to malignancy, as the process enables more efficient oxygen supply and nutrient delivery through rapid branching of new blood vessels from pre-existing vasculature to enrich and support the tumour microenvironment (Teleanu et al. 2020).

Several endogenous angiogenic factors have been identified which are involved in new blood vessels formation, namely the vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), epidermal growth factor, and basic fibroblast growth factor (bFGF) (Rajabi & Mousa 2017). Under normal physiological conditions, angiogenesis is regulated by a balance between activators and inhibitors. However, the disruption of the physiological balance between the angiogenic activators and inhibitors occurs in tumour cells promote angiogenesis and ultimately causes neoplasm (Rajabi & Mousa 2017). The VEGF family and its receptors (VEGFR) are the potent angiogenic factors and have been the attractive target to achieve anti-angiogenic effects through its inhibition. Hence, molecular therapeutic targeting VEGF or VEGFR has emerged as an important strategy in combating cancers (Casey et al. 2015).

Numbers of anti-angiogenic drugs, particularly the anti-VEGF agents have been approved by the U.S. Food and Drug Administration (FDA) to treat cancer, some of which have entered the clinical armamentarium against cancer. However, anti-angiogenic therapy has been associated with unexpected toxicities and could potentially be life-threatening. One of the most severe and difficult-to-manage side effects is bleeding (Elice & Rodeghiero 2012). For instance, treatment with Bevacizumb, a humanized monoclonal antibody works by interfering with the process of angiogenesis targeting VEGF, has been linked to several bleeding complications, including epistaxis, hemoptysis and brain haemorrhage (Elice & Rodeghiero 2012). The underlying cause of bleeding induced by the therapy is still unclear, but it was hypothesized that inhibition of VEGF may decrease the renewal capacity of damaged endothelial cells (Elice & Rodeghiero 2012). In addition, anti-VEGF agents could also cause hypertension, proteinuria, gastrointestinal perforation, impaired wound healing and arterial and venous thromboembolism (Keefe et al. 2011). Therefore, complementary natural products with anti-angiogenic properties with lesser side-effects may serve a better alternative regimen to the conventional anti-angiogenic therapy.

Malaysian *Tualang* Honey (MTH), a natural product collected from the honeycombs produced and built on the *Tualang* tree (*Koompassia excelsa*, from the family of Fabaceae) by *Apis dorsata* (giant rock bees) (Devasvaran & Yong 2016). MTH consume by Malaysian for the health benefits, and allergy to MTH is uncommon and rare, although it is possible to develop an allergy to either the pollen or the bee protein in the honey especially among the children. Previous study has shown that MTH exhibited anti-cancer properties on

oral squamous cell carcinoma, osteosarcoma cell lines (Ghashm et al. 2010), leukemia cell lines (Nik Man et al. 2015) and breast cancer *in vitro* and *in vivo* (Ahmed & Othman 2017; Fauzi et al. 2011; Kadir et al. 2013; Yaacob et al. 2013). MTH also exhibited inhibitory effect on oxidative stress-induced vascular hyperpermeability *in vitro* (Devasvaran et al 2019). As de-stabilizating blood vessels to increase vascular permeability is a pivotal step in the initiation of VEGF-mediated angiogenesis (Melincovici et al. 2018), hence, this experiment aimed to examine if MTH-mediated inhibitory effect could also affect VEGF-angiogenesis.

Materials and Methods

CHEMICALS AND REAGENTS

Human umbilical vein endothelial cells (HUVEC), EndoGRO culture media, EndoGRO-LS complete culture media kit, human VEGF165, Millicell cell culture inserts (pore size 8.0 µm) and dimetyhl sulfoxide (DMSO) were purchased from Merck Milipore Malaysia. Human breast cancer cell line MCF7 and its media, Eagle's Minimum Essential Medium (EMEM were purchased from American Type Culture Collection (ATCC). Human VEGF Quantikine ELISA kit (Cat# DVE00) and Total MMP-2 Quantikine ELISA kit (Cat# MMP200) were purchased from R&D Systems, Inc.

PREPARATION OF MTH

The MTH used in this study was from the Federal Agriculture Marketing Authorities of Malaysia (FAMA) as stated in the previous study (Devasvaran et al. 2019). MTH solutions were prepared freshly before any experiment by diluting it to 10% (v/v) with culture media as a stock and sterilized by using a syringe filter (0.22 µm). MTH was then diluted further from the stock to the desired concentrations for the cell culture.

CELL CULTURE

The EndoGRO™ human umbilical vein endothelial cells (HUVEC) were cultured in T-25 cell culture flasks with EndoGRO complete culture media which consists of a basal medium and its supplement kit. MCF7 cells were maintained in EMEM supplemented with 0.01 mg/mL human recombinant insulin and fetal bovine serum (10%). All the cells were incubated at 37 °C in an incubator with 95% humidified air and 5% CO_2. HUVEC between passage MTH three to six were used in the experiments to maintain its originality.

CELL PROLIFERATION ASSAY

Cell proliferation assay was performed as described previously (Ng et al. 2018). Briefly, HUVEC were seeded at 1×10^4 cells/well in a 96-well plate. Cells were treated with different concentrations of MTH ranging from 0.3 - 0.9% for 72 h of incubation and the plates without treatment were incubated for 24, 48, and 72 h in separate well plates to determine the growth curve. The concentration chosen was based on the previous study (Devasvaran et al. 2019) where it documented that MTH was not cytotoxic to HUVEC at concentrations below 1%. After indicated times, 10 µL of MTT (5 mg/mL stock concentration) was added into each well and incubated for another 4 h. Next, the MTT solution was removed and the Formazan product was dissolved in 100 µL of DMSO. The absorbance was measured using a microplate reader (Tecan M200 Infinite) at a wavelength of 570 nm. Experiment was performed in triplicates and three independent tests.

CELL MIGRATION ASSAY

Effect of MTH on spreading and migration capabilities of HUVEC were assessed using a scratch wound assay which measured the expansion of a cell population on surfaces. The assay was carried out according to Ng et al. (2018). Briefly, the HUVEC was grown until confluence on 6-well plates. Then, a linear scratch gap was generated in the monolayer with a sterile 100 µL pipette tip. All detached/dead cells/cell debris was removed by washing with culture media. Cells were then co-treated with $VEGF_{165}$ and MTH. The cell migration was observed by using Olympus inverted microscope (Olympus CKX31) at 40× magnification and quantified at 0 h (baseline) and 12 h or as soon as the gap in VEGF-treated groups was completely covered by cell by using the formula: (initial wound distance minus - wound distance)/2.

TUBE FORMATION ASSAY

Briefly, growth factor-reduced Matrigel™ was pipetted into pre-chilled 96-well plates and polymerized at 37 °C for 30 min. Approximately 1.5×10^4 cells per well of HUVEC were seeded onto the plate that coated with Matrigel previously together with MTH. The tube formation ability of HUVEC was measured after 3 h and Suramin was used as an inhibitor (Ng et al. 2018). The tube length was using an Image J with integrated angiogenesis analyzer plugin (Gilles Carpentier, Faculté des Sciences et. Technolo-gie, Université Paris Est, Creteil Val de Marne, France) (Arnaoutova & Kleinman 2010).

MATRIX METALLOPROTEINASE-2 (MMP-2) ELISA ASSAY

Briefly, HUVEC were seeded onto 96-well plates overnight and the media was replaced with media containing MTH. Then, the supernatant was obtained after 72 h of incubation with VEGF. MMP-2 was quantified using the commercially available Total MMP-2 Quantikine ELISA kit based on the manufacturer's recommendations.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) ELISA ASSAY

Effect of MTH on suppression of VEGF production from MCF-7 cancer cell lines was determined by using Human VEGF Quantikine ELISA kits and the procedure was according to the manufacturer. Briefly, MCF-7 cells were seeded onto 96-well plates at a cell density of 1×10^4 cells per well overnight. The media was replaced with media containing desired concentration of MTH for another 72 h. Production of VEGF in MCF-7 cells were measured by reading at 450 nm and corrected to 570 nm by using a microplate reader.

STATISTICAL ANALYSIS

All the experiments were repeated three times with triplicate and the data were expressed as the mean ± SEM. Statistical analysis was performed using IBM SPSS 22.0. One-way analysis of variance (ANOVA) was performed followed by Tukey's test as the *post-hoc* test. *p* value less than 0.05 was considered statistically significant.

RESULTS

MTH SUPPRESS HUVEC PROLIFERATION, MIGRATION AND TUBE FORMATION

HUVEC was treated with MTH at 0.3%, 0.6%, and 0.9% in the presence of VEGF for 72 h. VEGF significantly increased HUVEC growth by 55% as compared to untreated control ($p<0.001$) (Figure 1(A)). MTH successfully decreased VEGF-induced HUVEC proliferation from 155.13% to 104.85% ($p<0.001$) and 54.30% ($p<0.001$), at the concentrations of 0.6% and 0.9%, respectively. Similarly, HUVEC migration was significantly reduced after treatment with 0.6% and 0.9% MTH ($p<0.001$) (Figure 1(B)). HUVEC tube formation was also significantly ($p<0.01$) suppressed in the presence of MTH and the suppression potency was comparable to reference drug, Suramin, at 0.6% and 0.9% (Figure 1(C)).

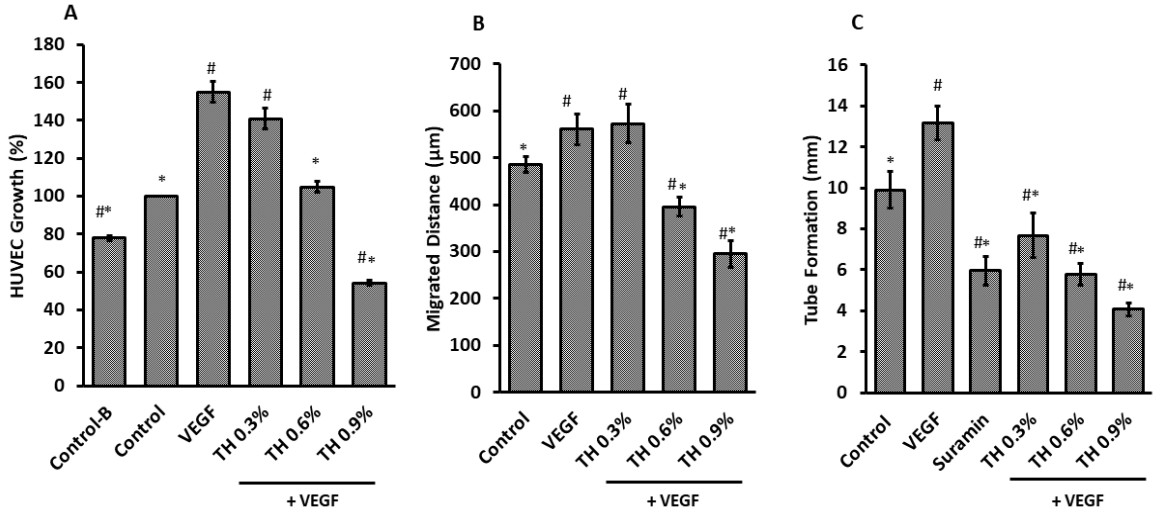

FIGURE 1. Effect of MTH on VEGF-induced angiogenesis. HUVEC was co-cultured with 0.3 - 0.9% MTH and 10 ng/mL VEGF for 72 h. (A) HUVEC proliferation, (B) HUVEC migration, the migration inhibition is presented as distances migrated between the two edges of the scratch and (1C) capillary tube formation induced by VEGF. The value expressed are the mean ± SEM of three independent experiments and compared against control group. # $p < 0.001$ were considered significant when compared to untreated group (Control). * $p < 0.001$ were considered significant when compared to VEGF alone group. Control-B = Control baseline (0 h); VEGF = VEGF 10 ng/mL; MTH = Malaysian *Tualang* Honey

MTH INHIBITS VEGF-INDUCED MATRIX METALLOPROTEINASE-2 (MMP-2) SECRETION

VEGF significantly (p<0.001) increased the HUVEC MMP-2 production to 59.3 ± 0.5 ng/mL from 43.16 ± 1.3 ng/mL in the untreated control, as expected (Figure 2). This effect was reversed by MTH at all tested concentrations (p<0.001), of which the effect was similar to suramin treated group (p<0.001).

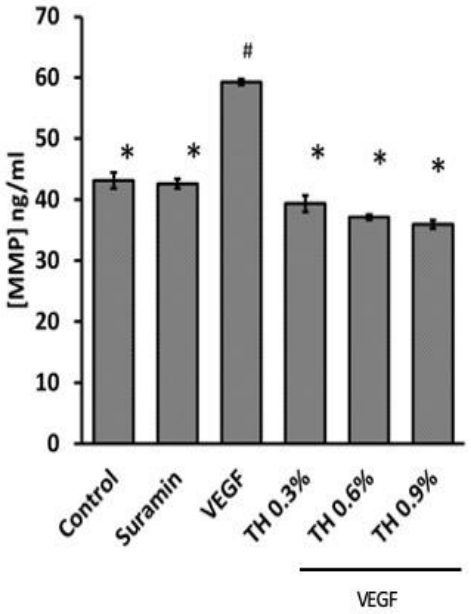

FIGURE 2. Effect of MTH on VEGF-induced MMP-2 production in HUVEC. The values presented are the mean ± SEM of three independent experiments and compared against untreated group (VEGF group). # p < 0.001 were considered significant when compared to untreated group (Ctrl). *p < 0.001 were considered significant when compared to VEGF alone group. Control = untreated group; VEGF = VEGF 10 ng/mL; Suramin = Suramin 50 µM; MTH = Malaysian *Tualang* Honey

MTH MARGINALLY REDUCED VEGF PRODUCTION FROM MCF7 BREAST CANCER CELLS

Data demonstrated MTH at concentration of 0.3% did not affect the production of VEGF production by MCF7 cancer cells (Figure 3). Although significant reduction of VEGF level was observed in MCF-7 cells treated with 0.6% (851.7 ± 25.2 pg/mL, p<0.001) and 0.9% (797.3±44.3 pg/mL, p<0.001) as compared to the control (994.6 ± 14.5 pg/mL), the magnitude was significantly lesser than Suramin which reduced 40% of VEGF from MCF-7 cancer cells to 591.2±27.5 pg/mL (p<0.001).

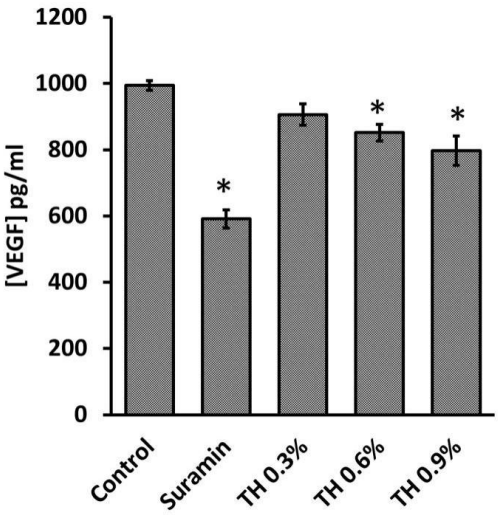

FIGURE 3. Effect of MTH on VEGF levels in MCF7 breast cancer cells. The values presented are the mean ± SEM of three independent experiments and compared against untreated group (Ctrl). *p < 0.001 were considered significant when compared to control group. Control = untreated group; Suramin = Suramin 50 µM; MTH = Malaysian *Tualang* Honey

DISCUSSION

Angiogenesis is necessary for tumour growth and its malignancy. Increase in blood supply could further support tumour growth and promote metastasis. On the other hand, by reducing the number of blood vessels in milieu could potentially turn tumour to dormancy. Thus, anti-angiogenic therapy is one of the promising strategies to slow cancer progression. Increased vascular permeability is one of the cardinal features of pathogenic angiogenesis (Venkatraman & Claesson-Welsh 2019). Prolonged, uncontrolled increase in permeability could promote tumour cell invasion and spreading in addition to fluid accumulation in the stroma and the elevated interstitial pressure, which are commonly observed in solid tumour (Weis & Cheresh 2005). Previous data showed that MTH is capable of suppressing oxidative stress-induced endothelial hyperpermeability (Devasvaran et al. 2019). Hence, this study aimed to examine if the observed inhibitory effect of endothelial permeability can be translated to promote anti-angiogenic effects using an *in vitro* model.

Angiogenesis is a complex process which involves secretion of matrix-degrading enzymes such as metalloproteases, increased endothelial permeability, cell migration, proliferation, vascular tube formation, and maturation (Lugano et al. 2019). In this study MTH was found to be cytotoxic when tested on HUVECs at concentrations 0.9%, as assessed by MTT assay. Our data showed that HUVEC treated with MTH at 0.6% exhibited reduced proliferative capability in the presence of pro-angiogenic mitogen, the VEGF. This suggests that the bioactive constituents of MTH subdue the VEGF effect on endothelial cells and reversed the growth rate similar to the rate at baseline without VEGF treatment. Similarly, HUVEC became less migratory in the presence of MTH at 0.6%, recorded at a distance shorter than the VEGF group ($p<0.001$). Collectively this supports the conclusion that MTH inhibits VEGF-induced HUVEC proliferation and migration.

Increased expression of matrix degrading enzymes such as matrix metalloproteinases (MMPs) is part of the angiogenic event especially in the process of cancer invasion and metastasis (Lv et al. 2018). MMPs are responsible for degradation of various extracellular matrix (ECM) components which could lead to endothelial hyperpermeability and promote tumour metastasis. Among all MMPs, MMP-2, and MMP-9 are known as the key molecules in the regulation of endothelial cell migration and matrix remodelling during angiogenesis (Liu et al. 2018). In this study, suppression of MMP-2-derived from endothelial cells was evident in the presence of MTH. This suggests that MTH, at least in part, is capable of decreasing MMP-2 secretion in the endothelial cells, thus, resulting in anti-angiogenesis and could potentially hinder the cancer cell migration and invasion.

Although our data supports that MTH can mitigate the effect of VEGF on endothelial cells, it would be better if MTH could act on the source of VEGF where it derives from. In this instance, cancer cells are known to secrete VEGF partly, if not completely, via hypoxia pathway (Bao et al. 2012; Ferrara 2019). Therefore, VEGF secretion by MCF7 breast cancer cells was investigated in response to MTH. Although there was some decrease in VEGF secretion by MCF7 cells, MTH failed to provide the degree of inhibition similar to Suramin. Suramin, was used as an anti-trypanosomal agent, but also proved to exhibits potent VEGF inhibition and anti-angiogenic effect. This suggests that MTH could suppress VEGF secretion from MCF7 breast cancer cells, but the effect was marginal. Previously we have shown that MTH contains phytochemical active compounds were identified, including 5-hydroxy-methyl-2-furancarboxaldehyde, 3-furaldehyde, 4H-pyran-4-one-2,3, -dihydro-3,5-dihydroxy-6-methyl-, phenylacetaldehyde, levoglucosenone, 2-furanmethanol and malto (Tan et al. 2014). Thus, future study will focus on examining these phytochemical active compounds of which are responsible for the observed anti-angiogenic activities.

CONCLUSION

In conclusions, our results demonstrated that MTH was able to inhibit endothelial cell proliferation, migration, and capillary tube formation and the effects could be attributed to its inhibitory effects on MMP-2 secretion by the endothelial cells. However, minimal effect was observed in VEGF secretion MCF7 breast cancer cells. These findings suggest that MTH has more profound effects on endothelial cells than the breast cancer cells to exert its anti-angiogenic potential, albeit more study is needed to elucidate MTH-mediated signalling mechanism involving the endothelial cells and the bioactive constituent that is responsible for the observed effects. Natural honey consisted of more than 200 constituents, to search the only active compound which exhibits the anti-angiogenic effect, variation of constituent from batch to batch of the honey, and possibility of synergistic/agonist and antagonist effect among the constituents might contribute to the limitation for the current study. Taken together, this study demonstrates

the fundamental insight and scientific ground to warrant further examination on MTH and its potential to be the candidate for treating pathologic angiogenesis.

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