Sains Malaysiana 51(5)(2022): 1437-1447
http://doi.org/10.17576/jsm-2022-5105-14

In vitro Anti-Angiogenic Properties of Ethanolic Crude Extract of *Vernonia amygdalina*

(Sifat Anti-Angiogenik *in vitro* daripada Ekstrak Kasar Etanol *Vernonia amygdalina*)

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Received: 21 April 2021/Accepted: 16 September 2021

ABSTRACT

Angiogenesis is the process of generating new blood vessels that deliver tumor cells with oxygen and essential nutrients for growth and metastasis. This study examined the *in vitro* antiangiogenic properties of the ethanolic crude extract from *Vernonia amygdalina* (VA) grown in Malaysia. The direct antiangiogenic activity of VA was evaluated on EA.hy926 cells using *in vitro* assessments: Cell proliferation, colony formation, migration, and cell invasion assays. VA ethanolic crude extract cytotoxic activity was evident in the antiproliferative and colony formation assays. The growth inhibition (IC_{50}) of 50% against EA.hy926 endothelial cells was achieved after 72 h treatment at a concentration of 85.43±3.57 µg/mL. Upon 48 h treatment, colony formation was inhibited completely at 100 µg/mL while 51.94% inhibition was achieved at 50 µg/mL. Moreover, the extract showed 54.72% and 31.99% inhibitory effects against migration of cells when treated for 24 h treatment at two different concentrations, 25 µg/mL and 12.5 µg/mL, respectively. The use of 100 µg/mL VA ethanolic extract inhibited cell invasion by 35.43%, which was lower than that of 57.81% inhibition achieved by the vinblastine as a positive control. All in all, the present work clearly demonstrated the antiangiogenic properties of VA ethanolic extract that may reflect a chemotherapeutic and/or chemoprevention potential for biomedical applications.

Keywords: Angiogenesis; EA.hy926; ethanolic extract; *Vernonia amygdalina*

ABSTRAK

Angiogenesis adalah generasi saluran darah baru yang menyampaikan sel-sel tumor dengan oksigen dan nutrien utama yang penting untuk pertumbuhan dan metastasis. Dalam penyelidikan ini kami mengkaji sifat anti-angiogenik *in vitro* ekstrak kasar etanol *Vernonia amygdalina* (VA) yang tumbuh di Malaysia. Aktiviti anti-angiogenik VA dinilai pada sel EA.hy926 menggunakan penilaian *in vitro* untuk percambahan sel, pembentukan koloni, migrasi dan ujian pencerobohan sel. Kegiatan sitotoksik ekstrak mentah etanol adalah jelas dalam ujian anti-proliferatif dan pembentukan koloni. Perencatan pertumbuhan 50% (IC_{50}) setelah 72 jam rawatan dicapai pada kepekatan 85.43±3.57 µg/mL terhadap sel endotel EA.hy926. Pembentukan koloni dihambat sepenuhnya pada 100 µg/mL berbanding 51.94% pada 50 µg/mL setelah 48 jam rawatan. Selain itu, ekstrak menunjukkan kesan penghambatan 54.72% dan 31.99% terhadap penghijrahan sel dalam ujian calar selepas rawatan 24 jam pada dua kepekatan yang berbeza, masing-masing 25 µg/mL dan 12.5 µg/mL. 100 µg/mL ekstrak etanol VA menghalang pencerobohan sel sebanyak 35.43% berbanding perencatan 57.81% oleh vinblastine kawalan positif. Karya ini dengan jelas menunjukkan sifat anti-angiogenik ekstrak etanol VA yang mungkin mencerminkan potensi kemoterapi dan/atau kemoprevensi.

Kata kunci: Angiogenesis; EA.hy926; ekstrak etanol; *Vernonia amygdalina*

Introduction

Angiogenesis is a complex and highly regulated process of generating new blood vessels. The new vessels branch off from pre-existing vessels under normal physiological homeostasis or tumorigenesis, which involves partly extracellular matrix rebuilding, endothelial migration, proliferation, and tube formation (Blood & Zetter 1990). Tumor angiogenesis delivers essential nutrients and oxygenation for tumorigenesis. Besides, it also acts as a conduit for the migration of tumor cells to adjacent and distant tissues and the formation of secondary tumors known as metastasis (Ferrara 2010). The importance of angiogenesis was first discussed upon the observation by Folkman (1971) that the tumor was unable to grow beyond 1 mm^3 without angiogenesis. Hence, the ability of screening for angiogenesis inhibitors has become an attractive therapeutic choice for treating cancer patients.

Medicinal plants are considered to have a rich source of active phytochemicals with pharmacological properties such as anti-inflammatory, antioxidant, and anticancer effects (Duthie et al. 2006). To date, several anticancer agents originating from plant sources have been developed as commercially available anticancer agents, such as paclitaxel, vinblastine, and vincristine (Shoeb 2006). In addition, many studies have screened medicinal plants for their antiangiogenic actives. For example, apigenin, a flavone found in medicinal plants, has anticancer characteristics that are associated with its anti-inflammatory, antioxidant, and antiangiogenic properties (Anand et al. 2008). To cite another example, abisilin, a natural terpenoid with *in vitro* and *in vivo* antiangiogenic effects, was found to inhibit neovascularization by inhibiting endothelial cell migration, proliferation, and invasion (Kudryavtseva et al. 2016).

Vernonia amygdalina (VA), which is commonly known as 'bitter leaf', is a tropical shrub native to Nigeria (Ogidi et al. 2019). The leaves of VA have been recognized as traditional African medicine with various health benefits. Traditionally, the leaves are used as a remedy to treat stomach discomfort, vomiting, diarrhea, and intestinal illnesses (Farombi & Owoeye 2011). As nutritional supplement rich in antioxidants and phytochemical contents, it has been deemed useful for maintaining human health and disease prevention (Yedjou et al. 2019). The crude extracts of VA have also been reported to possess immune-boosting, antioxidant, anti-bacterial, anti-inflammatory, and anticancer activities (Farombi & Owoeye 2011; Yedjou et al. 2019). These activities could be attributed to the various phytochemicals found in VA, such as vernolide, vernodalol, anthraquinones, flavonoids, lignans, terpenes, phenolic acids, and xanthones (Farombi & Owoeye 2011). The anticancer properties of VA were first reported by Kupchan et al. (1969) when a leaf chloroform extract was tested against human carcinoma of the nasopharynx. To date, studies have shown that VA decreases cell viability and induces apoptosis in numerous cancer cell lines in both concentration and time-dependent manner (Yedjou et al. 2018).

The importance of this study arises from the fact that available clinically approved antiangiogenic therapies normalize the tumor vasculature by blocking the VEGF/VEGFR2 pathway (such as bevacizumab). Thus, on one side, the therapies enhance tumor perfusion and the delivery of chemotherapies, while on the other hand, resistance was developed by tumor adaptation and production of other proangiogenic factors such as fibroblast growth factor (FGF). Other clinically approved antiangiogenic therapies are small molecules targeting receptor tyrosine kinase-mediated signal transduction mechanisms such as sunitinib. Although these agents target different receptors involved in the angiogenesis process, they may have potentially life-threatening side effects.

The present study assumed that plants with anti-inflammatory properties could have good antiangiogenic effects. This is because inflammation and angiogenesis are two interlinked processes that support tumor growth and progression (Albini et al. 2016). Therefore, the study aimed to evaluate *in vitro* antiangiogenic properties of *Vernonia amygdalina* shrub with previously reported anti-inflammatory effects. The direct effect of the extract was evaluated on endothelial cells EA.hy926 using antiproliferative, colony formation, migration, and cell invasion assays. Subsequently, the VA extract was further screened for its antiproliferative properties against MCF7 and HT29 human cancer cell lines.

This study is valuable to introduce an extract that possesses antiangiogenic properties in addition to its previously reported anti-inflammatory effects. These bioactive properties are mediated by various active phytochemicals, which could be subsequently evaluated for *in vivo* prevention of tumor progress and adjuvant therapy to treat solid tumors.

Materials and Methods

Plant Collection and Extraction

Dried leaves of VA were purchased from Herbagus Sdn Bhd (Malaysia). A voucher specimen (11798) of VA

has been deposited in the School of Biology, Universiti Sains Malaysia (USM) Herbarium. To obtain plant extract, dried leaves of VA were first ground into a fine powder using pestle and mortar. The powder was then dissolved in absolute ethanol (1:10 w/v), sonicated for 30 min, and centrifuged for 15 min at 6000 RPM. Next, the supernatant was collected, filtered, and dried with a rotavapor to remove residual ethanol. The resulting paste was dissolved in dimethyl sulphoxide (DMSO) to make a final stock concentration of 20 mg/mL. The final extract was kept at -20 °C until the further experiment.

CELL LINES AND CELL CULTURE

Human breast cancer cell line MCF7, human colon cancer HT29, and endothelial cell line EA.hy926 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). MCF 7 and EA.hy926 were maintained in Dulbecco's Modified Eagle Medium (DMEM), while HT29 was maintained in Roswell Park Memorial Institute (RPMI 1640). Medium were supplemented with 1% (v/v) penicillin-streptomycin and 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS). Cells were incubated at 37 °C under 5% CO_2 using a humidified incubator.

ANTIPROLIFERATIVE AND *in vitro* CYTOTOXICITY ASSAY

MCF7 and HT29 cells were seeded in 96-well plates at a density of 4×10^3 cells/well, while EA.hy926 cells were seeded at a density of 5×10^3 cells/well. Cells were left to adhere overnight at 37 °C and 5% CO_2 humidified incubator. The cells were then treated for 72 h with a complete culture medium containing VA extract (200 µg/mL). The final concentration of DMSO (v/v) was 1%. Blank and negative controls were included in each plate consisting of cell culture medium and cells treated with 1% DMSO (v/v), respectively. After 72 h treatment, the MTT colorimeter assay was performed as previously described by Mosmann with minor modifications (Mosmann 1983). Briefly, the supernatant was removed and replaced with 200 µL of cell culture media containing 0.5 mg/mL MTT and incubated for 4 h. Next, the insoluble formazan was dissolved with 150 µL DMSO per well, and the absorbance was measured at 570 nm. The average absorbance in the control wells reflected 100% survival from which the IC_{50} of VA extract was calculated.

COLONY FORMATION ASSAY

The colony formation assay measures the ability of VA extract to inhibit cell reproduction and colony formation behavior (Franken et al. 2006). Briefly, 2 mL of EA.hy926 endothelial cells suspension containing 500 cells/well were seeded in 6-well plates and left to adhere overnight. The cells were then treated with different concentrations of VA extracts (i.e. 100, 50, 25, and 12.5 µg/mL) in DMEM and incubated for 48 h at 37 °C with 5% CO_2. A total of 0.5% DMSO was used as a negative control. After 48 h, treatments were discarded and replaced with the fresh medium before incubating up to 10 days until at least 25 cells/colony were formed in the negative control well. Following incubation, the cells were fixed using 500 µL/well of 4% paraformaldehyde for 1 h, stained with 500 µL/well of 0.2% crystal violet, and left for 30 min in the incubator. Finally, crystal violet was removed gently by washing the plates under tap water and left to dry overnight. The percentage of plating efficiency (PE%) and percentage of surviving fraction (SF) were calculated.

THE SCRATCH ASSAY

The Scratch assay was performed according to Liang et al. (2007). Briefly, EA.hy926 cells were seeded at 5×10^5/well in 6-well plates and incubated at 37 °C in 5% CO_2 in a humidified incubator until it reached full confluence as a monolayer. After that, the layer of cells was scratched with a sterile 200 µL micropipette tip to create a wound of ±1 mm width. The cells were then washed twice with phosphate buffer solution (PBS) and treated with 2 mL of 2% FBS fresh medium containing the plant extract at concentrations of 100, 50, 25, and 12.5 µg/mL. Five images for each scratch at 0, 12, and 24 h were taken and observed under an inverted microscope at ×4 magnification. The mean distance between scratch borders of the five images at a specific time was analyzed using the ImageJ 1.51n® software (National Institute of Health, Bethesda, MA, USA). Next, the rate of cells migrating/h at a specific time was calculated by subtracting the mean distance between scratch borders at zero time and that at the specific time (i.e. 12 and 24 h). Finally, the percentage of inhibiting migration by VA extract was calculated by the following formula:

$$\%\ \text{inhibiting migration of cells} = 1 - \left(\frac{\text{RT}}{\text{RC}}\right) * 100\% \quad (1)$$

where RT is the rate of cell migrating/h that exposed to treatment at a specific time; and RC is the rate of cell migrating/h that exposed to DMSO (0.5%) at a specific time.

CELL INVASION ASSAY

The cell invasion potential of VA extract on EA.hy926 cells was performed using Matrigel Matrix (Corning) as an artificial basement membrane matrix (Moutasim et al. 2011). Briefly, 50 μL matrigel in DMEM (at a ratio of 1:1) was added to each well of 96-well plate and left for one hour to solidify. After that, 5×10^3 cells/well were transferred gently to each well and exposed to either 100 μg/mL of VA extract, 0.5 % DMSO (as a negative control) and 10 μM vinblastine (as positive control) for 24 h at 37 °C and 5% CO_2 humidified incubator. Next, the upper layer was removed gently and washed with PBS to remove any non-invaded cells and examined under an inverted microscope at ×10 magnification to observe the invaded cells. Mean cell number was calculated from different pictures, and the percentage of inhibiting cell invasion was calculated using the following formula:

$$\% \text{ inhibiting cell invasion} = 1 - \left(\frac{NT}{NC}\right) * 100\% \quad (2)$$

where NT is the mean cell number invading Matrigel after treatment; and NC is the mean cell number invading Matrigel without treatment (control).

RESULTS

ANTI-PROLIFERATIVE AND *in vitro* CYTOTOXICITY ASSAY

VA extract was evaluated for its antiproliferative activity against human cancer cells, MCF7 and HT29, as well as EA.hy926 endothelial cells. After 72 h of treatment with different concentrations, IC_{50} was calculated and presented in Table 1. This experiment aims to investigate if there is a direct cytotoxic effect for the plant extract. The results in Table 1 and Figure 1 showed that IC_{50} for VA extract was over 100 μg/mL against selected cells.

TABLE 1. Results of IC_{50} values for VA extract

Cell Lines	IC50 ± SD (μg/mL)
EA.hy926	85.43 ± 3.57
MCF7	87.31 ± 0.27
HT29	89.70 ± 5.17

COLONY FORMATION ASSAY ON ENDOTHELIAL EA.hy926 CELLS

In this assay, EA.hy926 endothelial cells were treated with different concentrations of VA extract (i.e. 100, 50, 25, and 12.5 μg/mL). The study indicated that the extract was cytotoxic on cells in a dose-dependent manner. The Plate Efficiency percentage (PE%) was 28.3±2.12%. Surviving fractions at 100, 50, 25, and 12.5 μg/mL were 0, 51.94±1.4, 85.51±3.4, and 90.81±4.3%, respectively. These results were comparable to the negative control with 0.5% DMSO (Figures 2 & 3).

THE SCRATCH ASSAY ON ENDOTHELIAL EA.HY926 CELLS

Migration endothelial cells is considered an important step in angiogenesis. Therefore, the results are represented as the percentage of migration inhibition by VA extract relative to untreated cells (Figures 4 & 5). A significant reduction in EA.hy926 cell migration was achieved at sub-cytotoxicity concentrations of 25 and 12.5 μg/mL with 36.28±13.67 and 24.63±6.06%, respectively, at 12 h treatment exposure. At 24 h, the percentages of inhibition were 54.72±17.73 and 31.99±10.77%, respectively.

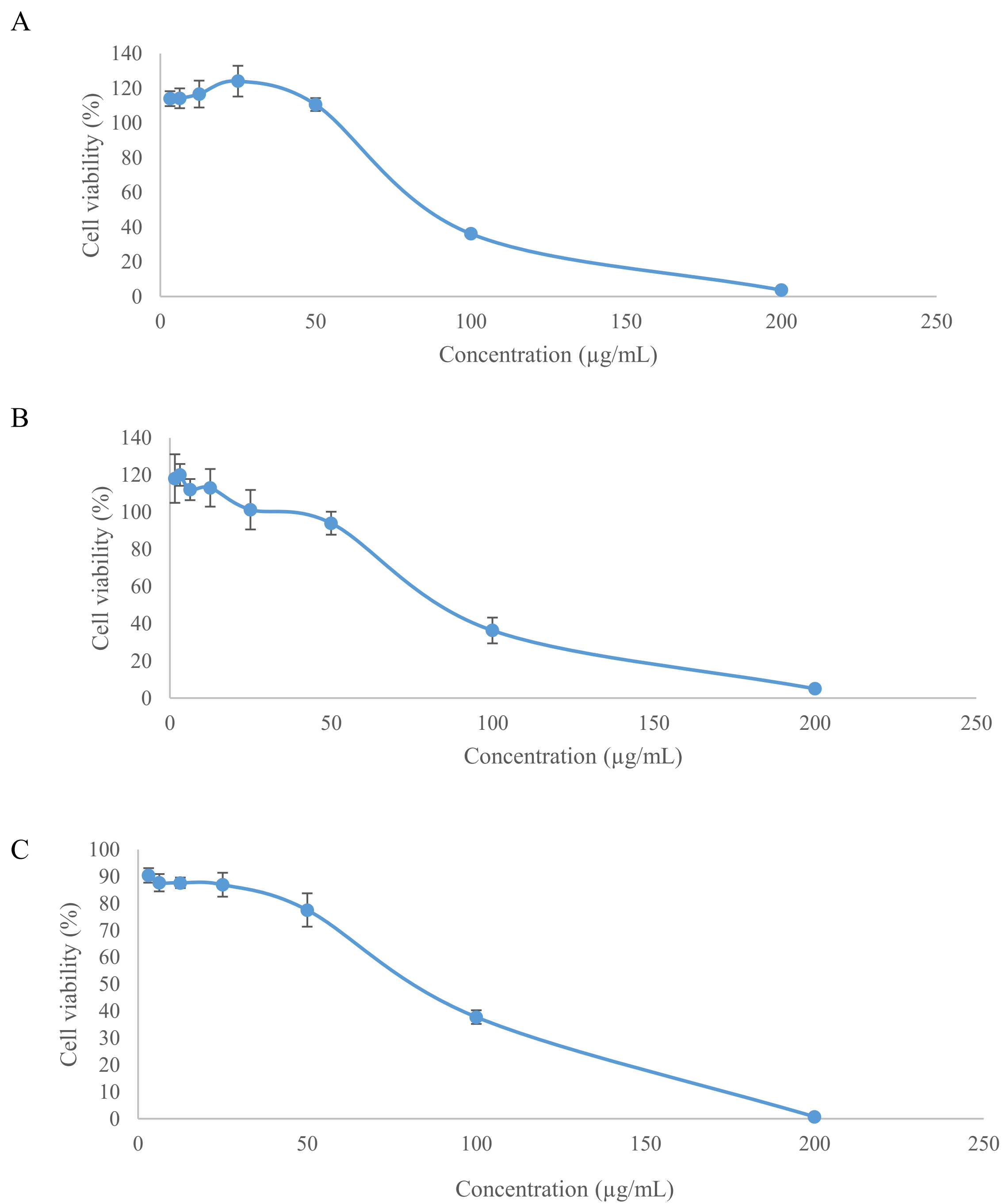

FIGURE 1. Effect of VA extracts on cell viability in MCF-7. (A), (B), and (C) shows VA extract treatment for 24, 48, and 72 h, respectively

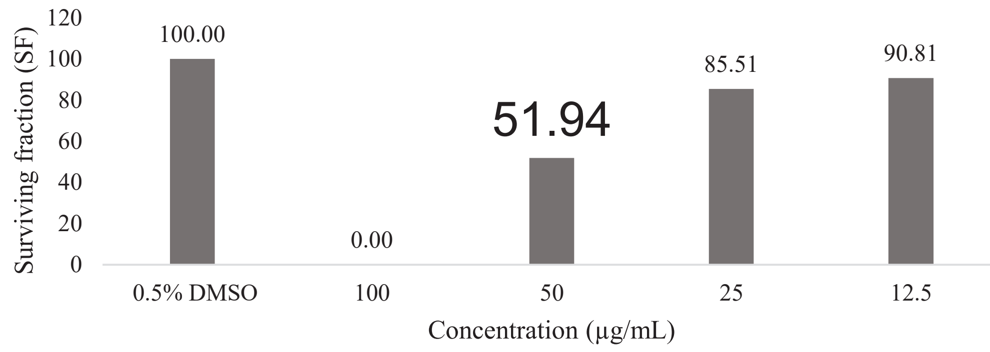

FIGURE 2. Effect of VA extract on colony formation in EA.hy926 cells

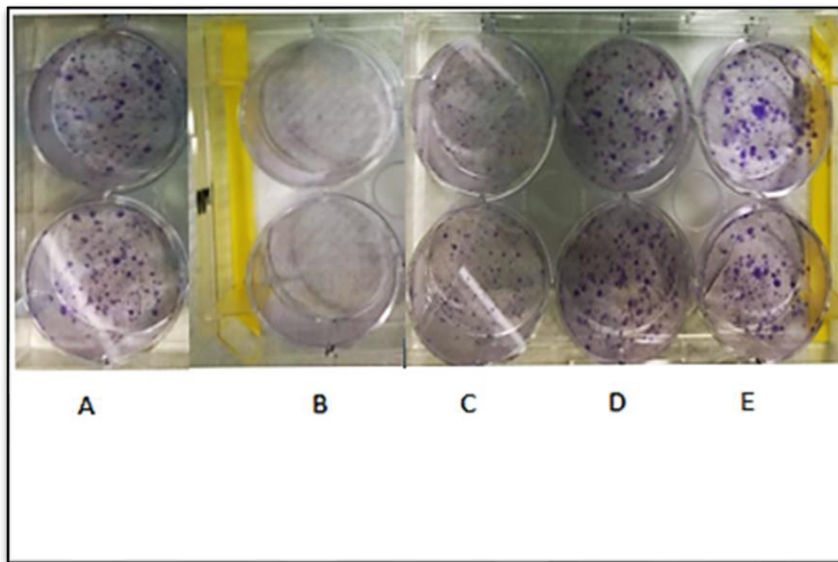

FIGURE 3. Effect of VA extract on colony formation in EA.hy926 cells with crystal violet staining

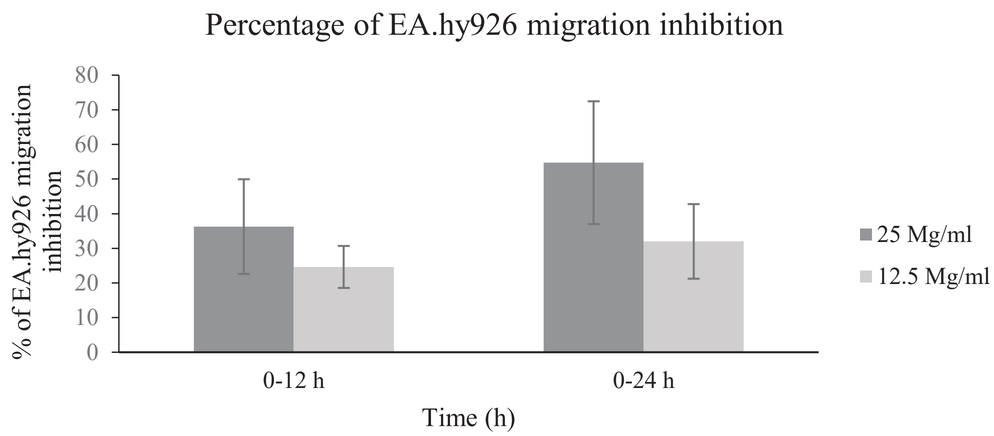

FIGURE 4. Effect of VA extract on cell migration in EA.hy926 cells

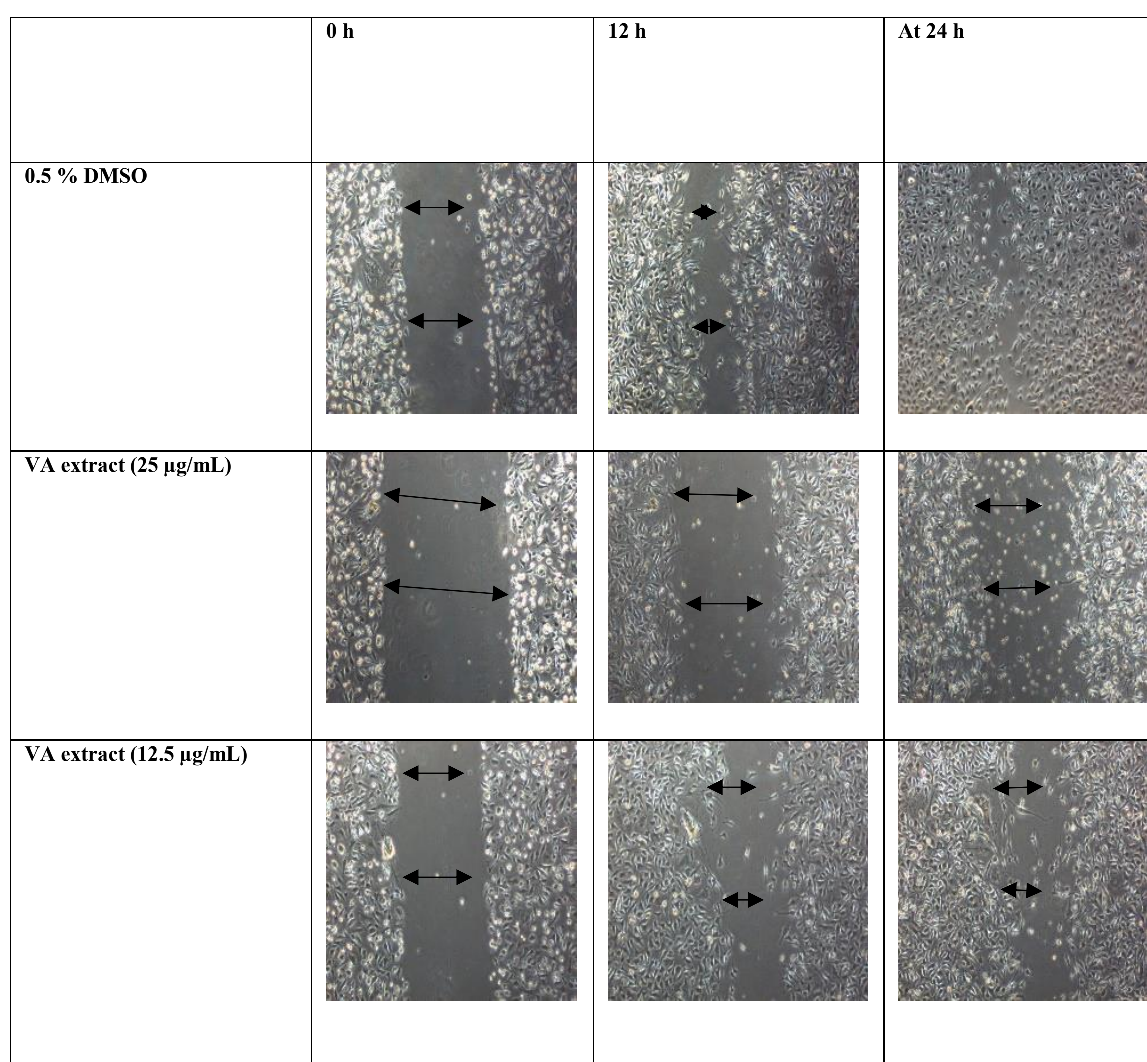

FIGURE 5. Effect of VA extract on cell migration in EA.hy926 cells using scratch assay

CELL INVASION ON ENDOTHELIAL EA.HY926 CELLS

Cell invasion refers to the ability of cells to degrade Matrigel matrix®. The results are represented as the number of cells that invaded Matrigel/mm^2 and percentage of inhibition by VA extract compared to untreated wells as well as vinblastine which was used as a positive control (Figure 6). After 24 h, the number of cells were 55.72±9.14, 86.29±2.71, and 36.41±2.35% after exposure to 100 µg/mL VA extract, 0.5% DMSO and 10 µM vinblastine, respectively. On the other hand, the percentage inhibition of cell invasion was 35.43 and 57.81% for VA extract and vinblastine, respectively.

DISCUSSION

Degree of tumor angiogenesis is linked to poor prognosis and metastasis (Kerbel 2008). Since anti-angiogenesis therapy has beneficial outcomes for some cancer treatments, many synthetic or natural compounds are being screened for their antiangiogenic properties. In this study, we focused on the antiangiogenic activity of VA ethanolic extract against endothelial growth, migration, or invasion pathways. To the best of our knowledge, this is the first study that has presented the *in vitro* antiangiogenic properties of VA.

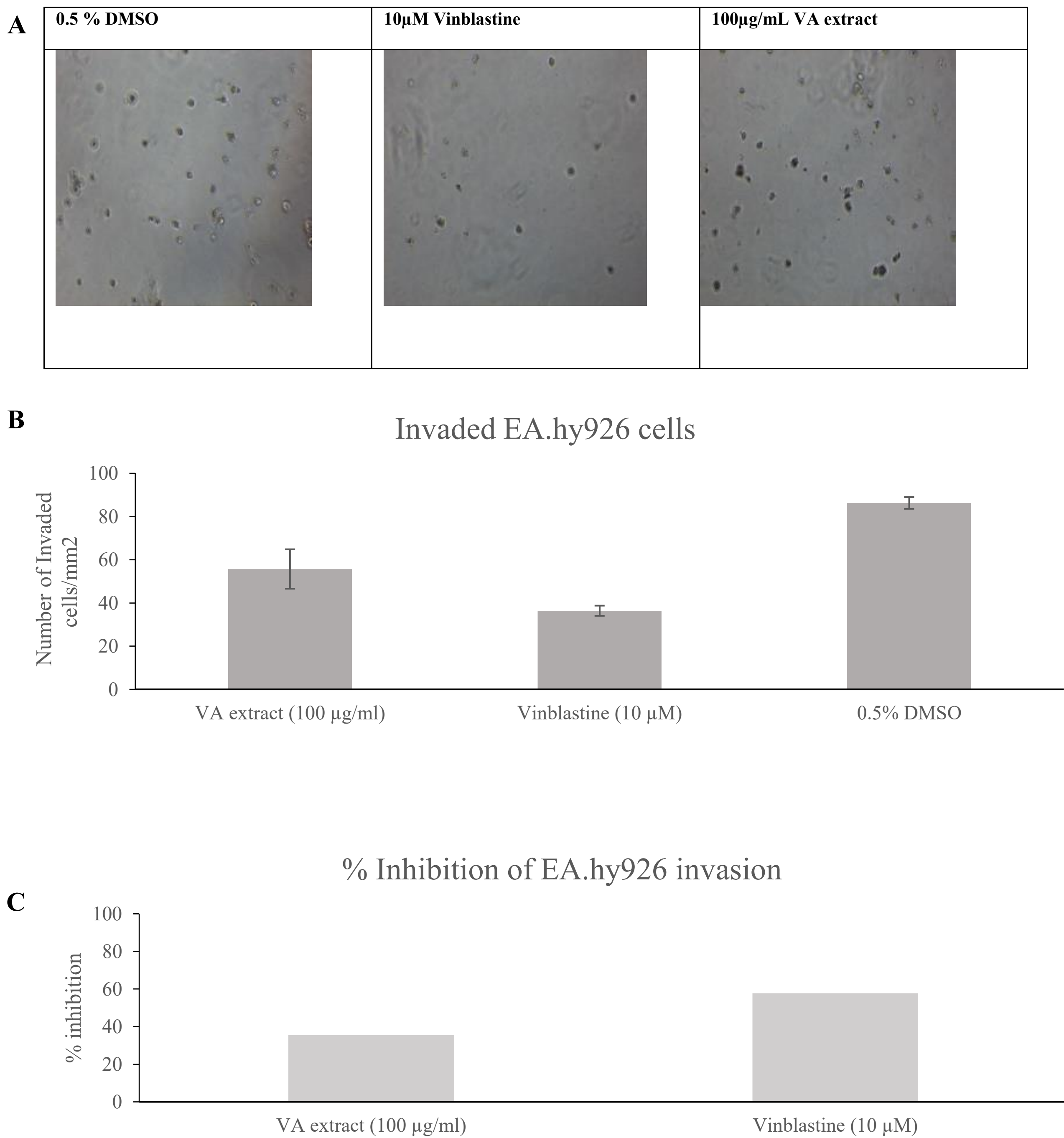

FIGURE 6. Effect of VA extract on cell invasion in EA.hy926 cells. (A) shows the microscopic image of cells that invaded the Matrigel matrix®, (B) shows the number of invaded cells, and (C) shows the percentage of cell invasion inhibition after treatment for 24 h

The findings showed that ethanolic extract of VA leaves had IC50 around 90 µg/mL against cancer cells MCF7 and HT29, as well as normal endothelial cells EA.hy926 using MTT assay. These results are comparable to that showed by Wong et al. (2013), indicating that VA had inhibited the growth of MCF7 in a dose and time-

dependent manner through induction of cell cycle arrest and apoptotic pathways. Other studies demonstrated wide discrepancy on MCF7 with IC50's ranged from 5.6 to 1000 μg/mL (Yedjou et al. 2008). The inconsistency of VA potencies could be related to the discrepancies in the plant extraction method, time of harvesting, and treatment time.

To evaluate the direct cytotoxicity potentials of VA extract against EA.hy926 cells, a colony formation assay was performed. The results showed that no colonies were formed after 48 h exposure to 100 μg/mL of extract, while about 50% of colony formation was observed at 50 μg/mL compared to that in control. This observation suggested that VA extract has a direct cytotoxic effect on endothelial cells, possibly through the interference of growth or survival pathways of endothelial cells. These findings have been confirmed by other *in vitro* and *in vivo* studies, which indicate that VA extract induces apoptosis through caspase 3 and 9, and results in cell death (Howard et al. 2016). This cytotoxic effect may be due to vernodalin and vernolide-A, which are cytotoxic sesquiterpene lactones that showed anticancer properties through activation of the JNK pathway, targeting extracellular signal-regulated kinase 1 (ERK-1), extracellular signal-regulated kinase 2 (ERK-2), nuclear factor-κB (NF-κB), signal transducer, and activator of transcription 3 (STAT3), matrix metalloproteinase 2 (MMP-2), and matrix metalloproteinase 9 (MMP9) (Mohebali et al. 2020; Nguyen et al. 2020a). Other studies had showed that vernodalol had shown proapoptotic properties via the cleavage of caspase9 and the release of cytochrome c into the cytosol (Wu et al. 2018).

To ensure that VA extract could inhibit angiogenesis at a sub-cytotoxic concentration, two different concentrations (i.e. 25 and 12.5 μg/mL) were studied against the migration of EA.hy926 cells. The results showed about 54% vs. 31% inhibition for migration of endothelial cells after 24 h exposure at 25 and 12.5 μg/mL, respectively. The response was lower at 12 h exposure for both concentrations. This observation indicated that the effects had followed a dose and time-dependent profile. Therefore, VA extract properties are likely a consequence of active ingredients which interfere with the mobility of endothelial cells. Although there are no previous studies to confirm the VA mechanism of inhibiting endothelial migration, the anti-inflammatory properties of VA have been demonstrated in migration of leukocytes studies, implicating that the inhibition of prostaglandins synthesis is a plausible explanation (Onasanwo et al. 2017). Both antimigration and anti-inflammatory properties might be due to vernonioside v, which has anti-inflammatory properties through inhibition of TNFα, IL-6, and IL-8 production (Nguyen et al. 2020b). IL-8 was found to drive angiogenesis and induce chemotaxis in endothelial cells (Brocker et al. 2010; Ferrara 2010). Additionally, targeting TNFα will prevent the adhesion of monocytes to the endothelial cells monolayer leading to inhibition of inflammatory angiogenesis (Baci et al. 2018). Since angiogenesis and inflammation are two linked processes controlled by different mediators such as prostaglandins, VA action on the mediators of these two processes may potentially be involved in the antiangiogenic mechanism (Albini et al. 2016, 2012; Onasanwo et al. 2017). Furthermore, other studies showed that VA is a novel inhibitor for a vascular endothelial growth factor (VEGF) and extracellular-signal-regulated kinase (ERK), whether the latter is a protein regulator for the master key regulator of angiogenesis. Both VEGF and ERK are responsible for the proliferation and migration of endothelial cells (Dvorak et al. 1995; Howard et al. 2016; Tabana et al. 2016).

On the other hand, the potential of VA extract against endothelial cell invasion was studied in our investigation using 100 μg/mL and was compared to vinblastine which has known antiangiogenic effects. The results indicated that the extract at 100 μg/mL inhibited endothelial cell escaping or invading through the Matrigel matrix after 24 h treatment by 35% inhibition when compared to 57% inhibition for vinblastine. The cell invasion process indicates the ability of endothelial cells to degrade the basement membrane to escape and migrate. Therefore, it can be concluded that VA extract could have components that inhibit metalloproteinases (MMPs) activity. MMPs are enzymes implicated in the degradation basement membrane and extracellular matrix. These effects allow for new blood vessels to sprout from exciting ones. As the release of MMPs is also enhanced during the inflammation process, the anti-inflammatory properties of VA will suppress the ability of cells to invade the basement membrane (Shishodia & Aggarwal 2002). Moreover, vinblastine has antiangiogenic properties by inhibiting vascular endothelial growth factor receptor (VEGFR) expression. This may reflect that VA produces antiangiogenic properties by regulating either VEGF/VEGFR or ERK signaling pathways (Meissner et al. 2008).

Overall, VA extract had shown a potential antiangiogenic effect through direct cytotoxicity, anti-migration, and anti-invasion effects towards

EAhy926 cell lines, in addition to previously reported anti-inflammatory properties. These results, along with the antiproliferative effects against the HT29 cell line, indicate the clinical importance of VA extract to prevent tumor development, most probably in the premalignant colorectal polyps or initial stages of the tumor where both the inflammatory and angiogenesis processes support tumor progress. Thus, our results indicated that VA extract exerts antiangiogenic properties through the inhibition of cell proliferation, cell migration, and cell invasion of endothelial cells EA.hy926.

Conclusions

VA has unequivocally shown to be a potent antiangiogenic agent. The antiangiogenic effect of VA is related to cytotoxicity properties against endothelial cells, while other antiangiogenic effects of VA are through colony formation, cell invasion, and cell migration inhibition. The activity could also be indirectly through interference and modulation of growth factors or their receptors on endothelial cells, such as vascular endothelial growth factor (VEGF) or its receptor (VEGFR), which has regulatory effects on the endothelial migratory behavior. A majority of tumors could not be effectively treated by single modality therapy. The role of polymolecular botanical drugs is undeniably showing importance as potential adjuvant therapies. Therefore, future studies on VA in *ex-vivo* and *in-vivo* anti-angiogenesis models are thus worthy of investigation.

Study Limitations

This study treated the immortalized endothelial cell line EA.hy926, a hybridoma of the epithelial cell line A549 and HUVEC. Normal endothelial cells such as human umbilical vascular endothelial cells (HUVEC) are not being investigated in this study. However, it is well known that there are functional differences among them regarding interaction with cytokines and growth factors. This study employed angiogenesis properties of EA.hy926 but not tumor endothelial cells. Recent studies showed that there are molecular differences between normal and tumor endothelial cells. Moreover, tumor endothelial cells had shown several cytogenic abnormalities such as aneuploidy and abnormal centrosomes that make them more resistant to therapies. HT29 and MCF7 cancer cell lines were used as a model for epithelial colorectal and breast cancer cells, respectively. However, other epithelial cancerous cell lines such as DLD-1, HCT116, MDA-MB-231, and Caco2 may respond differently to the treatment. *Vernonia amygdalina* leaves were collected from Malaysia. Therefore, the results presented in this study are limited to the extract from the plant of a specific locality.

Acknowledgements

We would like to thank the Ministry of Higher Education, Malaysia for the Fundamental Research Grant Scheme with project code: FRGS/1/2020/STG01/USM/03/2.

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