IJPSR (2021), Volume 12, Issue 5 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 14 May 2020; received in revised form, 19 September 2020; accepted, 23 September 2020; published 01 May 2021

AN *IN-VITRO* STUDY ON THE ANTI-ANGIOGENIC EFFECT OF *MAJORANA HORTENSIS* LEAVES

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Keywords:

Angiogenesis, Majorana hortensis, CAM assay, MDA-MB-231 cells, Chick embryo fibroblasts

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ABSTRACT: Angiogenesis is an inevitable process of tumour growth, invasion, and metastasis. Understanding the process of new blood vessel formation has gained prime importance recently, and anti-angiogenic therapy has now been considered as an important strategy for cancer therapy. Many bioactive compounds, especially of plant origin, have been explored for their anti-angiogenic potential. The present study was formulated to evaluate the effect of *Majorana hortensis* leaf extract on angiogenesis, in an effort to examine its influence against cancer cell proliferation, invasion, and metastasis. Cell viability analysis in non-transformed chick embryo fibroblasts showed that *Majorana hortensis* leaf extract alone exhibited slight cytotoxicity as assessed by MTT and SRB assay. However, in combination with etoposide, the leaf extract exhibited a synergistic effect as evident from the improved cell viability in chick embryo fibroblasts. Using CAM assay, the anti-angiogenic potential of the *Majorana hortensis* leaf extract alone was inferred from the significant reduction in blood vessel formation. The anti-invasive potential of *M. hortensis* leaf extract was further substantiated by a scratch assay using breast cancer (MDA-MB-231) cells, wherein the leaf extract inhibited the proliferation of tumour cells. Cell cycle analysis using flow cytometry showed that the proportion of MDA-MB-231 cells under-going early stage (G0-G1 phase) of apoptosis was significantly increased whereas cells in G2-M phase decreased after co-treatment with the plant extract and etoposide.

INTRODUCTION: Cancer, a multifactorial disease, is one of the leading causes of death in most well-developed countries. The spread of cancer is dependent on angiogenesis. Cancer cell proliferation and its metastatic spread depend on an adequate supply of oxygen and nutrients and the removal of waste products.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(5).2939-49

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(5).2939-49

This requires a new growth in the vascular network called cancer neovascularization, a complicated process involving both angiogenesis and vasculogenesis.

An increase in tumour size is usually in correlation with an increase in the blood supply or an increase in angiogenesis. Hence, cancer neovascularization is a key event in the process of tumourigenesis, invasion, and metastasis [1]. Under physiological conditions, angiogenesis is an important process involved in embryonic development, tissue regeneration, the female reproduction cycle, and wound healing. On the contrary, under pathophysiological conditions, angiogenesis leads

to abnormal and massive vascular growth, resulting in many ailments, including diabetic retinopathy, rheumatoid arthritis, juvenile hemangioma, psoriasis and, especially, in tumour growth and metastasis [2]. Cancer without angiogenesis will become an inactivated and dormant tumour disease [3].This implicates that effective inhibition of tumour angiogenesis might arrest or prevent tumour progression. In the premalignant stage, inhibition of angiogenesis at the initial stage called angio prevention has the potential to block the expansion of hyperplastic foci and subsequent tumour development.

Since angiogenesis plays a vital role in tumour growth and metastasis, angio prevention is considered an important strategy for cancer therapy [4]. Anti-angiogenic therapies can result in transitory improvement, in the form of tumour standstill or constriction, and in some cases, increase the survival of patients [5.] Anti-angiogenic therapy can normalize the tumour vasculature and reduce vessel growth for a period. Hence, anti-angiogenesis has become one of the standard-of-care therapies for several types of solid tumours [6].

Recently, significant progress has been achieved in understanding the mechanisms involved in the pathophysiology of neovascularization, to identify and develop new compounds that can influence angiogenesis. At present, several anti-angiogenesis drugs have already been approved by FDA and are in use for cancer treatment, while a few other agents are in different stages of preclinical and clinical evaluation. In an effort to identify novel anti-angiogenic drugs, many bioactive compounds, plant extracts, and dietary products are being tested for their anti-angiogenic potential [7].

Many anti-angiogenic agents that inhibit the different stages of angiogenesis in tumour growth processes have been identified and developed from plant sources. Such anti-angiogenic agents are more effective when used in combination with chemotherapy. Anti-angiogenic therapy, thus, has become an imperative strategy to treat cancer 8. Many research studies have been carried out to identify plants with significant anti-angiogenic potential by analyzing their effect on blood vessel formation, cell motility, and proliferation using both *in-vitro* and *in-vivo* systems.

The candidate plant of the present study was *Mojorana hortensis*. This plant has already been studied extensively in our laboratory and has been found to possess a strong antioxidant potential and biomolecular protective effect against oxidative damage [9, 10]. It has also been found to exhibit significant anticancer activity against cancer cell lines by inducing apoptosis and inhibiting cell viability [11]. In accordance with this, the present study was formulated to evaluate the effect of *Majorana hortensis* leaf extract on angiogenesis using both non-transformed (primary chick embryo fibroblasts) and transformed (breast cancer MDA-MB-231) cells, in an effort to examine its influence against cell proliferation, invasion, and metastasis.

MATERIALS AND METHODS:

Preparation of Leaf Extract: The plant sample was collected from the local areas of Coimbatore and was authenticated by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore. The voucher specimen was collected and maintained. The methanolic extract of the leaves was prepared as described below for each assay. About 5 g of fresh leaves were collected and cleaned to remove adhering dust particles, washed under running tap water, and gently blotted dry between folds of tissue paper. The leaves were chopped into fine pieces and were ground using mortar and pestle by adding 50 ml of methanol. The extracts were then filtered and evaporated below 50 ºC. The residue obtained was dissolved in dimethyl sulfoxide (DMSO) to get a final concentration of 0.2 mg/5µl.

Culturing of Chick Embryo Fibroblasts (CEF): Primary cultures, comprised of cells that are derived from embryonic tissue, play a major role in the preclinical drug development process. A primary culture is that stage of the culture after isolation of the cells but before the first subculture. The culture of primary CEF was established as described by Hernandez and Brown, 2010 [12]. Fibroblast cells isolated from 8^{th} or 9^{th} day-old chick embryo were cultured by resuspending it in fresh DMEM supplemented with 10% FBS and Penicillin-streptomycin (1X). The cells were incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere. The cells were treated with etoposide (200 µM), both in the presence and the

absence of the leaf extract. The exposure of etoposide was given for 24 h at 37 ºC.

Culturing of Breast Cancer (MDA-MB-231) Cells: The triple-negative breast cancer (MDA-MB-231) cells were cultured under aseptic conditions. The cell line was procured from National Centre for Cell Science, Pune, India. The cells were maintained in CO_2 incubator with 5% CO_2 and 95% humidity, supplemented with DMEM and 10% FBS. Penicillin and streptomycin (Himedia) were also added to the medium to 1X final concentration from a 100X stock. Once the cells had attained confluent growth, the cells were trypsinized using Trypsin–EDTA (Himedia), and 105 cells needed for carrying out various assays were seeded into sterile 6-well plates. The plates were then incubated in a CO_2 incubator with 5% CO_2 and 95% humidity atmosphere. The cells were treated with etoposide (200 µM) both in the presence and the absence of the leaf extract. The exposure of etoposide was given for 24 h at 37 ºC.

Cell Viability Analysis: The effect of leaf extract on cell viability of primary chick fibroblasts was quantified by MTT and SRB assays.

i) MTT Dye Reduction Assay: The MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay was used to evaluate the cell viability as described by Igarashi and Miyazawa (2001) [13]. Accurately 100 µl of the treated cells were taken, and 50 µl of MTT (3 mg/ml in PBS) was added to it and incubated at 37 ºC for 3 hours with mild shaking. After centrifugation, the cells were washed thrice with 20 µl of PBS followed by centrifugation at 10,000 rpm for 3 min. The supernatant was aspirated, and 200 µl of 2-propanol containing 0.04N HCl was added to the cells and kept overnight in the dark. The absorbance was read at 650 nm in a microtitre plate reader (Biorad, USA).

ii) Sulphorhodamine B (SRB) Assay: The SRB assay explained by Skehan *et al.*, (1990) [14] was employed to determine the cell viability in the presence and the absence of leaf extract in etoposide-treated cells. After the treatment, the medium was completely removed and washed with 200 µl PBS to remove any traces of medium and serum. The treated cells were layered with 350 µl of ice-cold 40% TCA and were gently suspended in it followed by incubation at 4 ºC for 1 h. After incubation, the pellets were collected and washed 5 times with cold water. The excess water was drained off. SRB stain (350µl) was added to each tube and incubated for 30 min at room temperature. The cells were washed 4 times with 1 ml portions of 1% acetic acid. Then 350 µl of 10mM tris (pH 10.5) was added to each tube to solubilize the dye. The pellets were shaken gently for 20 min on a gyratory shaker. The debris was spun down, and the absorbance of the tris layer in each group was measured in a 96-well plate in a microtitre plate reader (Biorad, USA) at 492 nm. Cell survival was measured as the percentage absorbance compared to the control (untreated cells).

Angiogenic and Cell Proliferation Analyses: The effect of the leaf extract on angiogenic progression was analyzed using chick chorioallantoic membrane (CAM) assay. The effect of leaf extract on cell proliferation was analyzed using a scratch assay in breast cancer (MDA-MB-231) cells.

i) Chorioallantoic Membrane (CAM) Assay: CAM assay was carried out as described by Ponce and Kleinmann (2003) [15]. The angiogenesis progression on the eggs was observed. Viable eggs with the developing embryo were selected and incubated. On the sixth day of incubation, the eggs were candled and checked for blood vessels. The eggs were cleaned with 70% ethanol, and a window on the broad end of the egg was cut in the shell using a sterile needle and forceps. Whatman's sterile filter paper discs containing the test compounds were implanted inside the eggs, and the window made was closed with a porous plaster. These windows were then reopened after 48 h of incubation on the 8th day and were examined for angiogenesis.

ii) Cell proliferation Assay: The influence of methanolic extract of *Majorana hortensis* leaves on cell motility was studied under *in-vitro* conditions using scratch assay (Liang *et al.*, 2007) [16]. A scratch was made on the confluent breast cancer (MDA-MB-231) cells in a straight line with a sterile micropipette tip. The treatment was given as described earlier. The debris was removed, and the edge of the scratch was made smooth by washing the culture with 1.0 ml of DMEM.

A volume of 5 ml of DMEM supplemented with serum and antibiotics was then added. The methanolic extract was then added to the medium and dispersed gently. Markings were made close to the scratch using an ultrafine tip marker to use a reference point to obtain the same field during image acquisition. Images of the scratch were taken by placing the culture dish under a phase-contrast microscope, followed by incubation of the dish in the CO_2 incubator at 37 °C for 24 h.

After the treatment period, the culture dish was taken out, and the images were captured for the control and the treated groups under a phase-contrast microscope matching the reference points.

Cell Cycle Analysis by Flow Cytometry: Cell cycle analysis was performed using flow cytometry as described by Krishan (1975) [17]. MDA-MB-231 cells treated with methanolic extract of *M. hortensis* leaves were harvested and washed with PBS. The cells were then fixed in 70% ethanol and kept at -20 °C until analysis. Cells were then stained with propidium iodide (50 µg/ml) and incubated for 30 minutes at room temperature in the dark. The DNA content of the stained cells (1×10^6 cells/ml) was analyzed by flow cytometry. The population of cells in sub-G0, G0/G1, S, and G2/M were quantitated using CellQuest Software with the FACSVerse flow cytometer (Becton Dickinson, USA) and expressed as a percentage of the cells in each phase.

Statistical Analysis: Statistical significance was determined by one-way analysis of variance using Sigma Stat (Version 3.1) statistical software, and the values with $P<0.05$ were considered to be significantly different.

RESULTS:

Effects of *Majorana hortensis* Leaves on Cell Viability in Chick Embryo Fibroblasts: The chick embryo has served a workhorse for experimental embryological studies designed to elucidate various mechanisms. Early chick embryos developing in whole-embryo culture can be readily manipulated, which makes it a good model system [18]. The influence of the methanolic leaf extract of the plant *Majorana hortensis* on chick embryo fibroblasts survival was determined by quantifying the percent cell viability using MTT and SRB assays. The extent of viability in the different treatment groups are presented in the **Fig. 1A** and **B**.

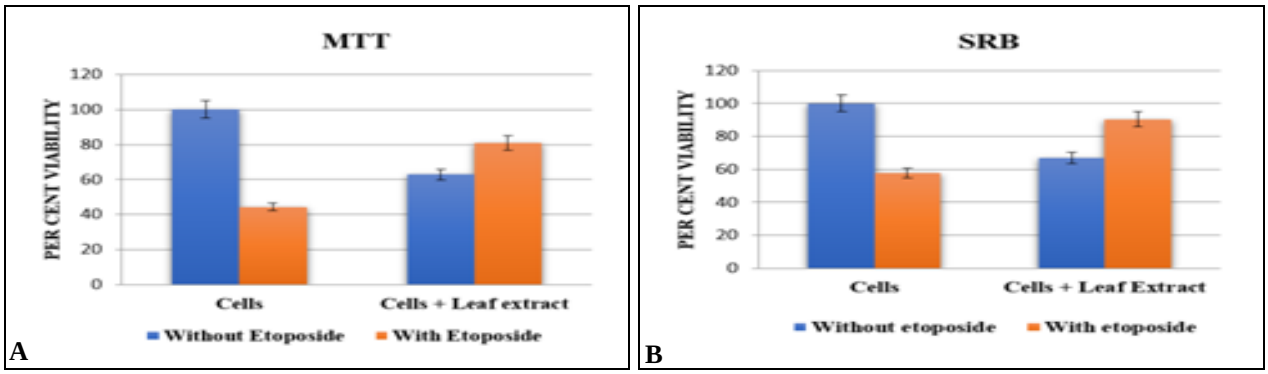

FIG. 1: EFFECT OF *MAJORANA HORTENSIS* LEAF EXTRACT ON THE VIABILITY OF CEFS AS DETERMINED BY MTT AND SRB ASSAY Values are expressed as Mean ± S.D of triplicates. The values of the untreated control group were fixed as 100%, and the percent viability in the other groups was calculated relative to this.

In both MTT and SRB assays, the results obtained showed that the exposure to the plant extract and etoposide exhibited a significant effect on the cell viability of CEFs. In the presence of etoposide alone, the cell viability was significantly decreased, which improved on co-treatment with the methanolic extract of *Majorana hortensis*. The results thus infer that *M. hortensis* works satisfactorily in combination with etoposide, and the cytotoxicity was markedly reduced.

Effect of *Majorana hortensis* Leaf Extract on Angiogenesis as Determined by Chorioallantoic Membrane (CAM) Assay: Using CAM assay, the extent of blood vessel formation in chick embryo was assessed by counting the number of primary vessels and vessels formed from the primary branches manually and compared between the treatment groups. The results obtained are depicted in **Fig. 2A** to **2D**. The extent of blood vessel formation in untreated control eggs exhibited a

normal pattern of angiogenesis, whereas the blood vessel formation in chick embryo treated with etoposide or *Majorana hortensis* leaf extract alone was significantly reduced, indicating their anti-angiogenic potential. On the contrary, eggs treated with etoposide in the presence of *Majorana hortensis* leaf extract promoted the development of capillary networks in CAM. These observations were further confirmed by measuring the angiogenesis-associated parameters, such as vessel density, total vessel network length, total branching points, and segments using a web-based Wimasisimage analysis system. The corresponding values are presented in **Table 1**.

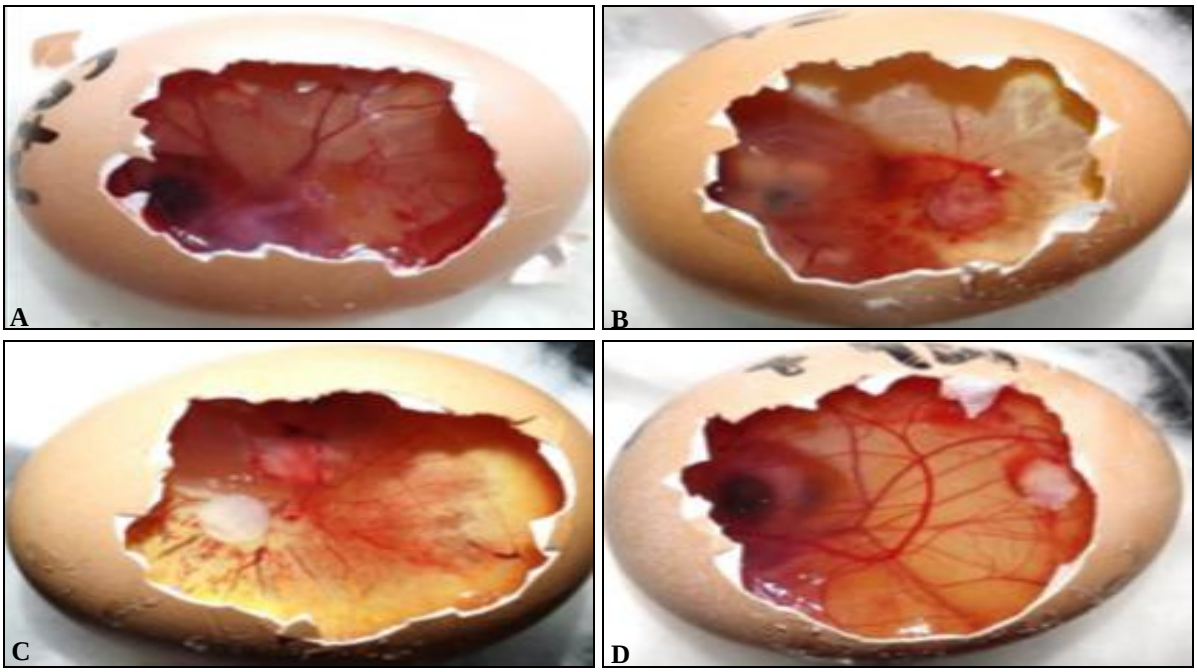

FIG. 2: EFFECT OF *MAJORANA HORTENSIS* LEAF EXTRACT ON ANGIOGENESIS AS DETERMINED BY CHORIOALLANTOIC MEMBRANE (CAM) ASSAY. A: BLOOD VESSELS IN CHICK EMBRYO IN CONTROL GROUP, B: BLOOD VESSELS IN CHICK EMBRYO TREATED WITH ETOPOSIDE, C: BLOOD VESSELS IN CHICK TREATED WITH *M. HORTENSIS* LEAF EXTRACT, D: BLOOD VESSELS IN CHICK EMBRYO TREATED WITH *M. HORTENSIS* LEAF EXTRACT + ETOPOSIDE

TABLE 1: PERCENT VASCULAR DENSITY AND TOTAL VESSELS NETWORK LENGTH AS DETERMINED BY CAM ASSAY

Treatment Group	Percent Vessel Density	Total Vessels Network Length (Analysed pixel px)	Total Branching Points	Total Segments
Untreated control	28.5	8445.9	223	427
Embryo treated with etoposide alone	18.6	2301.3	31	69
Embryo treated with leaf extract alone	18.1	4552.9	70	155
Embryo treated with leaf extract and etoposide	24.5	8217.5	165	304

Effect of *Majorana hortensis* on Cell Proliferation as determined by Scratch Assay using Breast Cancer (MDA-MB-231) Cells: The highly metastatictriple negative breast cancer (MDA-MB-231) cells were used to determine the effect of *Majorana hortensis* leaf extract on cell migration using scratch assay. Using a microscopic image analyzer, the extent of closure of the gap in the confluent cell monolayer was studied after a 24-hour treatment period with *M. horensis* leaf extract both in the presence and absence of etoposide. The experimental results obtained showed that in an untreated control group, a significant mobilization of breast cancer cells was observed as evident from the closure of the gap **Fig. 3A** and **3B**, whereas in *Majorana hortensis* treated group, significant inhibition of cell proliferation was found both in the presence and absence of etoposide **Fig. 3C** and **3D** as evident from the reduced cell number in the wound area of the cell line.

These findings were further confirmed by the percent decrease in the scratch area **Table 2** after treatment as measured using a web-based Wimasis image analysis system.

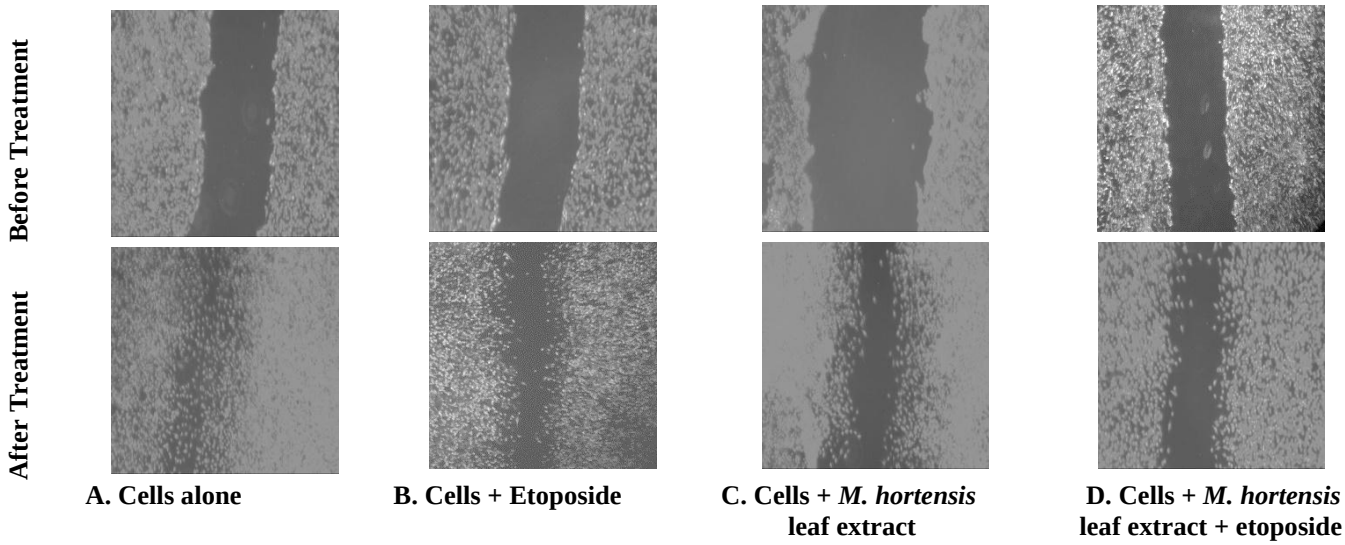

FIG. 3: PHOTOMICROGRAPH OF MOBILIZATION OF BREAST CANCER (MDA-MB-231) CELLS BEFORE AND AFTER TREATMENT

TABLE 2: PERCENT SCRATCH AREA BEFORE AND AFTER TREATMENT

Treatment Group	% Scratch area		% Decrease in Scratch Area
	Before Treatment	After Treatment	
Untreated control	29.8	7.1	22.7
Cells treated with etoposide alone	29.7	15	14.7
Cells treated with leaf extract alone	51.4	39.8	11.6
Cells treated with leaf extract and etoposide	23.6	17.9	5.7

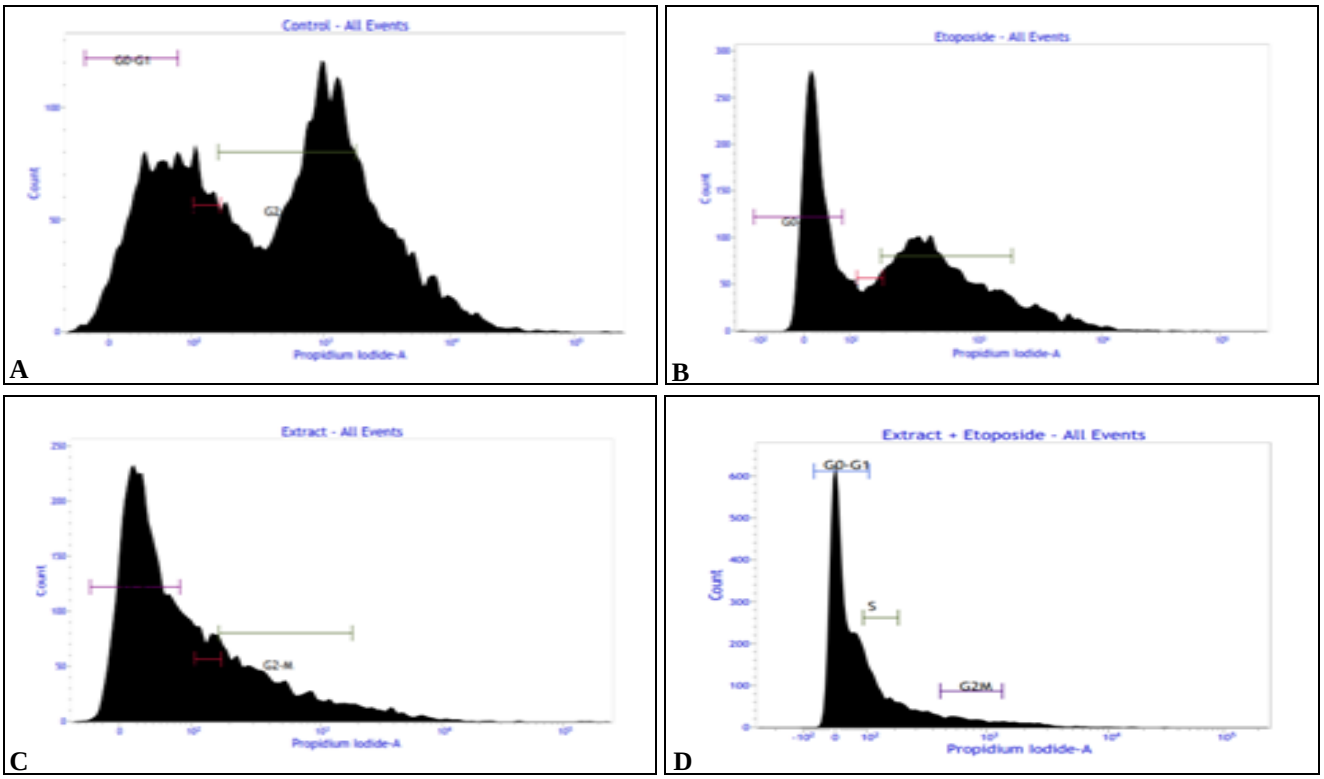

FIG. 4: HISTOGRAMS SHOWING THE PATTERNS OF CELLS STAINED WITH PROPIDIUM IODIDE A: CELLS ALONE, B: CELLS + ETOPOSIDE, C: CELLS + *M. HORENSIS* LEAF EXTRACT, D: CELLS + *M. HORENSIS* LEAF EXTRACT + ETOPOSIDE

TABLE 3: EFFECT OF *M. HORTENSIS* LEAF EXTRACT ON CELL CYCLE DISTRIBUTION OF MDA-MB-231 CELLS

Sample	Percent MDA-MB-231 cells in G0/G1 phase	
	Without Etoposide	With Etoposide
No extract	21.25 ± 0.53	35.89 ± 4.14 [a,b]
Leaf Extract	44.82 ± 4.53	67 ± 0.05

Values are expressed as Mean ± S.D of triplicates. a – statistically significant ($p<0.05$) compared to untreated control, b – statistically significant ($p<0.05$) compared to etoposide alone treated group

Effects of *Majorana hortensis* Leaves on Cell Cycle Distribution of Breast Cancer (MDA-MB-231) Cells: The effect of the leaf extract of *M. hortensis* on cell cycle progression of breast cancer (MDA-MB-231) cells was accessed using flow cytometry both in the presence and the absence of etoposide. The relative percentage of MDA-MB cells in each phase of the cell cycle was determined.

Treatment with etoposide or leaf extract alone and **Fig. 4B** and **4C** showed an increase in the percentage of cells in the G0/G1 fraction as compared to untreated cells, whereas no significant change in the proportion of cells in S phase was observed. Interestingly, co-treatment of MDA-MB-231 cells with both *M. hortensis* and etoposide **Fig. 4D** showed a significant decrease in the percentage of cells in S and G2-M phases, while G0/G1 phase cell populations increased significantly versus untreated control **Fig. 4A**. The average percentage of MDA-MB-231 cells in the G0/G1 phase was calculated and the results obtained was shown in **Table 3**. Thus, *M. hortensis* leaf extract caused G0/G1 phase cell cycle arrest in human breast cancer cells, clearly demonstrating the synergistic anticancer potential of the leaf extract in combination with etoposide.

DISCUSSION: Tumour cells can penetrate blood or lymphatic vessels and circulate through the intravascular stream and proliferate to another site, which is termed metastasis, which makes it life threatening19.The formation of neovasculature from pre-existing cells is called angiogenesis which is a crucial event during malignant tumour progression. Anti-angiogenesis is one of the standard-of-care therapies for several types of solid tumours [20].

Many researchers have uncovered the fact that various traditional medicinal plants have helped people in acting upon uncontrolled angiogenesis. Plants with anti-angiogenic properties are of considerable significance for diseases like cancer and diabetic retinopathy. Angiogenic and anti-angiogenic activities of plants can be evaluated using both *in-vitro* and *in-vivo* assays [21]. As a part of this search for natural product-based anti-angiogenic agents, in the present study, we sought to explore the influence of *Majorana hortensis* leaves on angiogenesis and cell invasion using chick embryo fibroblasts and breast cancer (MDA-MB-231) cell lines in the presence and absence of the standard chemotherapeutic agent etoposide.

Cell viability analyses showed that the etoposide-induced cytotoxicity of the non-transformed chick embryo fibroblasts was remarkably reduced, and the viability was improved in the presence of *Majorana hortensis* leaf extract. Similar findings have been reported by Badgujar *et al.* (2018), wherein the methanolic extract of *Butea monosperma* leaves exhibited a lesser cytotoxic effect on chick embryo fibroblasts as accessed by MTT assay [22]. In another study, Kiruthika *et al.* (2013) confirmed the protective effect of the methanolic extract of *Zea mays* leaves against H2O2-induced oxidative stress in primary cultured chick embryo fibroblasts [23]. Similarly, Suprapto (2012) confirmed the non-toxic effect of betel leaf extract (BLE) on the primary culture of chick embryo fibroblasts [24]. In addition, this observation has confirmed the finding of the previous study wherein different extracts of *Majorana hortensis* leaves rendered good protection against H_2O_2-induced apoptosis in Saccharomyces cerevisiae cells, among which methanolic extract showed maximum protective effect [25].

CAM assay is a robust technique that can be used to monitor the invasion of cancer cell lines and to assess the role of novel molecules and potential therapeutic targets [26]. It provides an overall highly efficient and cost-effective method to study the phenomenon of angiogenesis to identify compounds and develop drugs for angiogenesis-based therapies [27]. The CAM model is recognized as an intermediate model that can bridge the gap between cell-based and animal-based assays, other than showing similar patterns of cellular toxicity as

in-vitro models [28]. Many studies have used CAM assay to study angiogenesis. Bashir and Qadir (2017), reported that different doses of ginger extract exhibited anti-angiogenic effects on eggs with developing embryos as indicated by the reduction in primary and secondary blood vessels [29]. Tertiary blood vessels were also found to be decreased in the CAM area. Using the chick CAM model, Kota *et al.* (2018) reported new pharmacological effects of Nigella sativa by the proven inhibition of angiogenesis30. Eva *et al.* (2014) had found that ethanolic leaf extract of Antidesmabunis has anti-angiogenic activity when tested on duck embryos using CAM assay [31].

Using CAM assay, the anti-angiogenic potential of the *Majorana hortensis* leaf extract alone was inferred from the significant reduction in blood vessels formation. On the contrary, normal vascularization of CAM was observed on co-treatment with etoposide and *M. hortensis* leaf extract. Impaired perfusion of blood in tumours induces hypoxia which in turn promotes tumour progression and enhances the invasive and metastatic potential of cancer cells. Hence, therapeutic angiogenesis is one of the promising strategies to improve tumor perfusion and delivery of drugs [32].

This implies that the improved vascularization of CAM after combinatorial treatment of etoposide with leaf extract may contribute to tumour microenvironment and vasculature normalization, which in turn improve its efficiency for drug delivery and alleviating hypoxia.

Vascular, vascular, and metastatic stages are the three central stages of cancer. Tumour metastasis, the main characteristic feature of advanced-stage cancers, is the major cause of cancer-related mortality. In addition to angiogenesis, migration and invasion of cancer cells are the critical steps involved in tumour metastasis [33]. During metastasis, cancer cells migrate through the basement membrane and vasculature to get to target organs. Hence, anticancer agents that inhibit tumour cell migration have gained importance and are highly desirable for cancer therapy. Scratch wound assay has been extensively used to assess cell migration, a critical process in the establishment of metastatic foci [34].

Breast cancer, a highly heterogeneous disease, has been classified based on the presence and the absence of biological markers, namely estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [35]. Among these, the triple-negative breast cancers (TNBC) do not express any of these three receptors, and hence there are no targeted therapies available for these tumours. TNBC is highly invasive and metastatic, and hence women diagnosed with TNBC have been reported to exhibit a very short-term prognosis. Above all, the heterogeneity of breast cancer, lack of targeted therapy for TNBC, and highly toxic adverse effects of chemotherapy have made it imperative to develop alternative treatments with minimal side effects that could increase the life expectancy of breast cancer patients [36].

Scratch assay has been applied in many research studies involving the analysis of tumour metastasis. Using scratch assay, Wu *et al.* (2018) reported the inhibitory effect of *Ganoderma lucidum* on migration of MDA-MB-231 cells [37]. Domínguez *et al.* (2018) confirmed the anticancer potential of *Bursera copallifera* leaf extracts from their inhibitory effect of cell migration against triple-negative MDA-MB-231 breast cancer cells [38]. Similarly, Chowdhury *et al.* (2017) indicated that the ethanol and acetone extract of colocynth fruit pulp reduced cell viability and inhibited the migration of breast cancer (MCF-7, MDA-MB-231) cells and cervical cancer (SiHa) cells39. Ranjbarnejad *et al.* (2015) also reported the cytotoxic and anti-angiogenic potential of the methanolic extract of *Boswellia serrate* in human colon cancer (HT-29) cells using MTT and scratched assays40. The literature quoted above supports our findings in the present study, where scratch assay substantiated the anti-invasive potential of the *Majorana hortensis* leaf extract on breast cancer (MDA-MB-231) cells as evident from the inhibition of cell migration.

Flow cytometry is highly sophisticated and has great potential as evident from its extensive usage in diverse fields of biological sciences such as clinical diagnostics, biotechnology, healthcare, and in basic and applied research [41]. It has become essential to conduct a multi-parameter analysis of apoptosis in relation to cell cycle position in order

to explore the mechanism of action of anticancer drugs that target specific molecular targets of the cell cycle [42].

Using flow cytometric analysis, many studies have reported the cell cycle modulatory effects of herbal extracts on cancer cells. Kumar *et al.* (2017) reported that black turtle bean extract induces cell cycle arrest in S and G2/M phase in breast cancer MCF-7 and MDA-MB-231 cells [43]. Cell cycle analysis of MCF-7 cells treated with *Allium atroviolaceum* flower extract using flow cytometry revealed that the flower extract induced S and G2/M phase cell cycle arrest at 24 and 48 h of treatment with a substantial decrease in G1 phase and dose-dependent increase of sub-G0 phase [44]. Similarly, the apoptosis-inducing activity of *Momordica cochinchinensis* Aril extracts in human MCF-7 breast cancer cells was evident from the increased percentage of cells in an early apoptosis stage as analyzed by flow cytometry [45]. Co-treatment using Huaier extract and paclitaxel inhibited breast cancer cell proliferation by inducing cycle arrest in the G0/G1 and G2/M phases, which implicated that combinational therapy are more effective than monotherapy [46].

These observations, thus, corroborate with the present study, wherein the proportion of breast cancer (MDA-MB-231) cells arrested in G0/G1 phase increased after co-treatment with the methanolic extract of the *M. hortensis* and etoposide. Further studies on the cell cycle events associated with the leaf extract and/or etoposide need to be carried out on non-transformed cells to have more insight into the influence on cell division and death.

CONCLUSION: Angiogenesis is an inevitable process of tumour growth, invasion, and metastasis. Anti-angiogenic therapy has now been considered an important strategy for cancer therapy. In recent years, various bioactive compounds, especially of plant origin, have been explored for their anti-angiogenic potential. The observations of this study thus signify that the leaves of *Majorana hortensis* possess significant anticancer and anti-angiogenic potential. Further research needs to be carried out to validate the anti-angiogenic effect of the leaves by studying their effect on cell-tube formation, cell proliferation, and invasion in transformed cells.

ACKNOWLEDGEMENT: Authors would like to express their sincere thanks to the Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore -641043 for funding and successful completion of this study under the UGC block grant. The authors also would like to express their special thanks to Dr. P. R. Padma, Professor, Department of Biochemistry, Biotechnology and Bioinformatics for providing insights and expertise to complete this study.

CONFLICTS OF INTEREST: The authors declare that there is no conflict of interests.

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How to cite this article:
Yamuna ST, Sowndharyalatha B, Poornima A and Sumathi S: An *in-vitro* study on the anti-angiogenic effect of *Majorana hortensis* leaves. Int J Pharm Sci & Res 2021; 12(5): 2939-49. doi: 10.13040/IJPSR.0975-8232.12(5).2939-49.