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COMPARATIVE PROFILE OF ETOPOSIDE AND A PODOPHYLLOTOXIN DERIVATIVE ON TUMOR ANGIOGENESIS AND PERMEABILITY

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ABSTRACT: Objectives: A novel podophyllotoxin derivative OMe2-TH (*7,8-dimethoxy-5-phenyl-4,5-dihydro-naphtha[1,2-d] thiazol-2-yl amine*) was compared with Etoposide for its potential to inhibit VEGF-induced permeability and tumor angiogenesis in Ehrlich Ascites Tumor model. Methods: The anti-angiogenic potential *in vivo* was analyzed by the administration of podophyllotoxin derivative OMe2-TH intraperitoneally at a dose of 4mg/kg and compared with a known anti-tumor podophyllotoxin derivative Etoposide at a dose of 10mg/kg in Ehrlich Ascites Tumor model. The extravasation of micro vessels of the excised peritoneum was observed by Haematoxylin and Eosin stained sections of the tissue. The activity of these molecules as anti-permeability agents was assessed by Miles permeability assay in Swiss Albino mice. Key findings: OMe2-TH and Etoposide are potent molecules inhibiting Ehrlich Ascites tumor burden and angiogenesis with the reduction in body weight, cell number, ascites secretion and inhibit VEGF-induced vascular permeability. They inhibit the extravasation of micro vessels suggestive of their role in inhibiting tumor angiogenesis and invasion. Conclusions: OMe2-TH and Etoposide inhibit proliferation and tumor angiogenesis in Ehrlich Ascites Tumor model. OMe2-TH in comparison with Etoposide is a promising candidate with pronounced inhibition of VEGF-induced permeability and invasiveness indicating its therapeutic applications in breast cancer and other malignant ascites tumors.

KEYWORDS: Angiogenesis, Etoposide, Podophyllotoxin, Ehrlich Ascites Tumor, Vascular Endothelial Growth Factor.

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1.INTRODUCTION

Blood vessels are constructed by the processes of vasculogenesis and angiogenesis. Vasculogenesis is achieved by the in-situ differentiation of endothelial progenitor cells to endothelial cells (ECs), whereas angiogenesis is achieved by capillary sprouting from preexisting small vessels [1]. Angiogenesis is proved to be an essential component in the development, wound healing and tumor metastatic pathway [2]. The most prominent and commonly found angiogenic proteins like Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF), whose activities are known to be synergistic are released when there is an overexpression of positive regulators of angiogenesis in tumor cells leading to the mobilization of angiogenic proteins from the extracellular matrix [3]. Vasculature adopts a distinct subtype in pathological conditions, significantly deviant from that observed in normal physiological state [4-6]. Tumor angiogenesis is characterized by abnormal vasculature and altered ultrastructure of tumor vessels resulting in chaotic blood flow and leaky vessels [7]. Leaky vasculature has been primarily attributed to exposure to vascular permeability inducing agents, particularly VEGF [8]. Podophyllotoxin, a naturally occurring aryltetralin lignin obtained from a number of plants belonging to the family Podophyllum is a known anti-tumor agent that acts by binding to tubulin thus preventing these macromolecules to form microtubules thereby arresting mitotic cell division at metaphase. The development of different synthetic and semi-synthetic derivatives of podophyllotoxin and the composite pharmacophore model proposed by different research groups has provided insight regarding the mechanisms of action of podophyllotoxin [9][10]. Different semisynthetic derivatives like Etoposide (VP-16) and Teniposide (VM-26) that are predominantly DNA topoisomerase-II inhibitors are currently the widely used chemotherapeutic drugs for various cancers like small cell lung cancer, testicular carcinoma, lymphoma, Kaposi's sarcoma, neuroblastoma and soft tissue sarcoma. But the severe side effects and drug resistance associated with these drugs has led to the synthesis of novel derivatives of podophyllotoxin which are safer and with enhanced therapeutic efficacy [11][12]. Though many podophyllotoxin derivatives are known to be anti-neoplastic and pro-apoptotic in nature, there are very few derivatives which are known to be potential angiogenic inhibitors. Some of the anti-angiogenic podophyllotoxin derivatives include deoxypodophyllotoxin (DPT), novel quinozolino linked 4β-amidopodophyllotoxin conjugates and deoxypodophyllotoxin derivative (DPMA) [12-14]. Low dose of oral Etoposide inhibits endothelial and tumor cell proliferation and VEGF induced angiogenesis and permeability [15]. Identifying drug formulations that inhibit vessel hyperpermeability has increasingly emerged as a platform for therapeutic targets. The synthesis of OMe2-TH (*7,8-dimethoxy-5-phenyl-4,5-dihydro-naphtha[1,2-d] thiazol-2-yl amine)* has been previously reported [16]. Hence, we designed this study with an objective to compare the anti-tumor and anti-angiogenic potential of a podophyllotoxin derivative, OMe2-TH with Etoposide, the latter being a potential VEGF antagonist suppressing hyperpermeability and a promising candidate for

anti-tumor and anti-angiogenic therapy leading to important clinical implications in many diseased states. The aim of the study was not limited to unravel the angio inhibitory and anti-permeability potential of the novel molecule OMe2-TH, but also to understand the unexplored role of Etoposide in inhibiting tumor burden and angiogenesis in Ehrlich Ascites Tumor model, a mouse mammary carcinoma.

2. MATERIALS AND METHODS

In vivo maintenance of Ehrlich Ascites Tumor (EAT) cells

EAT cells were cultured in 8-weeks-old Swiss albino mice, a mouse mammary carcinoma model. The EAT cells were obtained from the peritoneal cavity of the tumor bearing mice. $5x10^6$ cells were transplanted into 8-weeks-old Swiss albino mice and allowed to grow for 12 days. At the end of the growth period, the mice were sacrificed either for further transplantation or experimental studies. EAT cells/mouse mammary carcinoma cells are being maintained in our laboratory by *in vivo* transplantation as an ascites tumor model. Swiss albino mice (6–8 weeks old) were obtained from the animal house, Department of Studies in Zoology, University of Mysore, Mysore, Karnataka, India. The animal experiments were approved by the Institutional Animal Care and Use Committee, University of Mysore, Mysore, Karnataka, India. "All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted." All experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India (REGD #: 122/99/CPCSEA). Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium were purchased from Gibco, Life Technologies, USA. All other reagents used were of analytical grade.

Test compound preparation

The test compound was dissolved in 0.1% Dimethyl Sulfoxide (DMSO) and filter sterilized using a 0.22 μm syringe filter for use in experiments. In control samples, similar concentration of DMSO was added as vehicle to rule out its cytotoxic effect. Etoposide was obtained as a sterile injectable formulation.

Peritoneal Angiogenesis

The EAT bearing mice were treated with podophyllotoxin derivative OMe2-TH and Etoposide for 6 days post transplantation at a concentration of 4mg/kg and 10 mg/kg of the body weight of mice respectively along with untreated EAT bearing mice as control. The mice were divided into three groups with five mice in each group and inoculated with 5×10^6 EAT cells, intra peritoneal. The first group was vehicle treated control, the second group was treated with OMe2-TH and the third group was treated with Etoposide. The animals were sacrificed on the thirteenth day, saline was injected (i.p), and a small incision was made in the abdominal region to collect the tumor cells along with ascites fluid. The pelleted cells were counted by trypan blue dye exclusion method using a

hemocytometer. The abdomen wall was extended and the exposed peritoneum was examined for neo-vascularization and photographed.

Evaluation of difference in body weight of mice

The body weight of the animals was recorded from day one to thirteenth day to assess the difference in tumor burden in different treatment groups. On the thirteenth day, the animals were sacrificed to assess the parameters of packed cell volume, viable cell number, volume of ascites secretion and peritoneal angiogenesis.

Assessment of packed cell volume and viable cell number

The cell suspension with ascites fluid from all the treatment groups were collected. The cell suspension was subject to centrifugation at 3,000 rpm for 5 minutes. Following centrifugation, the supernatant ascites fluid was separated and the difference in packed cell volume was documented. The harvested cells were washed, diluted in sterile PBS and diluted at a 1:6 ratio and were counted on a haemocytometer using trypan blue dye exclusion method in order to verify the cell viability. The number of viable cells in different treatment groups was documented.

Evaluation of ascites volume

The volume of ascites separated from the cell pellet obtained from different treatment groups was recorded. The difference in the volume of ascites secreted in different treatment groups was documented.

Microvasculature Density (MVD)

The abdominal wall of the animals was extended and photographed using a high-resolution camera. The difference in the blood vasculature corresponding to angiogenesis was observed. The peritoneums were excised, embedded in paraffin prior to staining with haematoxylin and eosin (H&E). They were further photographed using a bright field microscope fitted with a camera to observe the difference in the extravasation of micro vessels in different groups. The analysis of the number of blood vessels in the peritoneum was done using ImageJ. Ink.

Analysis of VEGF induced Vascular permeability using Miles Assay

The right and left dorsal flanks of Swiss albino mice were shaved 24 hours prior to treatment. Mice were anesthetized using ketamine/xylazine (100 mg/kg i.p. and 10 mg/kg i.p., respectively). Evans Blue Dye (100 µL of 1% w/v) was administered to the dorsal tail vein and allowed to circulate for 20 minutes. Intradermal injections of VEGF in increasing concentrations (5, 10, 20 ng), Etoposide (10 mg/kg) with and without VEGF (20 ng), OMe2-TH (4 mg/kg) with and without VEGF (20 ng) were administered along with Phosphate Buffered Saline (PBS), a negative control. ınjection volumes were normalized to 50 µL. After 20 minutes, the mice were sacrificed and the dermis exhibiting dye leakage was photographed. Similar sized regions of dermis containing Evans Blue dye were excised and incubated in 250 µL of formamide for 3 days at room temperature to extract the dye. The samples were centrifuged at 10,000 x g for 40 minutes and 100 µL of dye-containing

supernatant from each sample was transferred into a transparent, flat bottom 96-well plate. Evans blue absorbance was measured at 620 nm with reference reading of 740 nm using Varioskan™ Flash Multimode Reader, Thermo Scientific. A graph representing treatment groups vs absorbance (OD 620 nm/740 nm) was plotted. The graphical representation of the results of all the above-mentioned experiments has been done using GraphPad Prism 8.0.

Statistics

All experiments were performed in triplicates. Wherever appropriate, the data are expressed as the mean ± SD and means were compared using one-way analysis of variance. Statistical significance of differences between controls, compound treated cells were determined by Dunnet's test. For all tests, $P<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Effect of OMe2-TH and Etoposide on growth of EAT cells *in-vivo*:

The three groups of animals that were transplanted with 5 x 10^6 EAT cells among which two groups that received 10 mg/kg of Etoposide or 4 mg/kg of OMe2-TH showed reduction in the body weight corresponding to tumor burden as compared to untreated EAT bearing mice. The untreated EAT bearing mice showed an increase of body weight of 12.21g on the 13^{th} day of transplantation due to tumor burden. The group with Etoposide showed a decrease of 3.116 g and the group with OMe2-TH exhibited a decrease of 3.9 g in body weight (Fig 1A) respectively.

Effect of OMe2-TH and Etoposide on tumor cell proliferation *in vivo* in EAT model

The two groups of mice treated with OMe2-TH or Etoposide showed a considerable decrease in the packed cell volume, with OMe2-TH showing 1.28ml and Etoposide of 1.4 ml of packed cell volume compared to untreated EAT bearing mice with 5.3ml of packed cells. The untreated EAT bearing mice exhibited the viable cell number to be 49.2x10^6 as compared to mice treated with Etoposide with 23.75x10^6 and the group treated with OMe2-TH showing 22.6x10^6 cells (Fig 1B & C).

Effect of OMe2-TH and Etoposide on the secretion of ascites

The volume of ascites secreted in untreated EAT bearing mice was 7.625 ml as compared to ascites fluid secreted from OMe2-TH being 0.7 ml and Etoposide with 0.8 ml respectively (fig 1D).

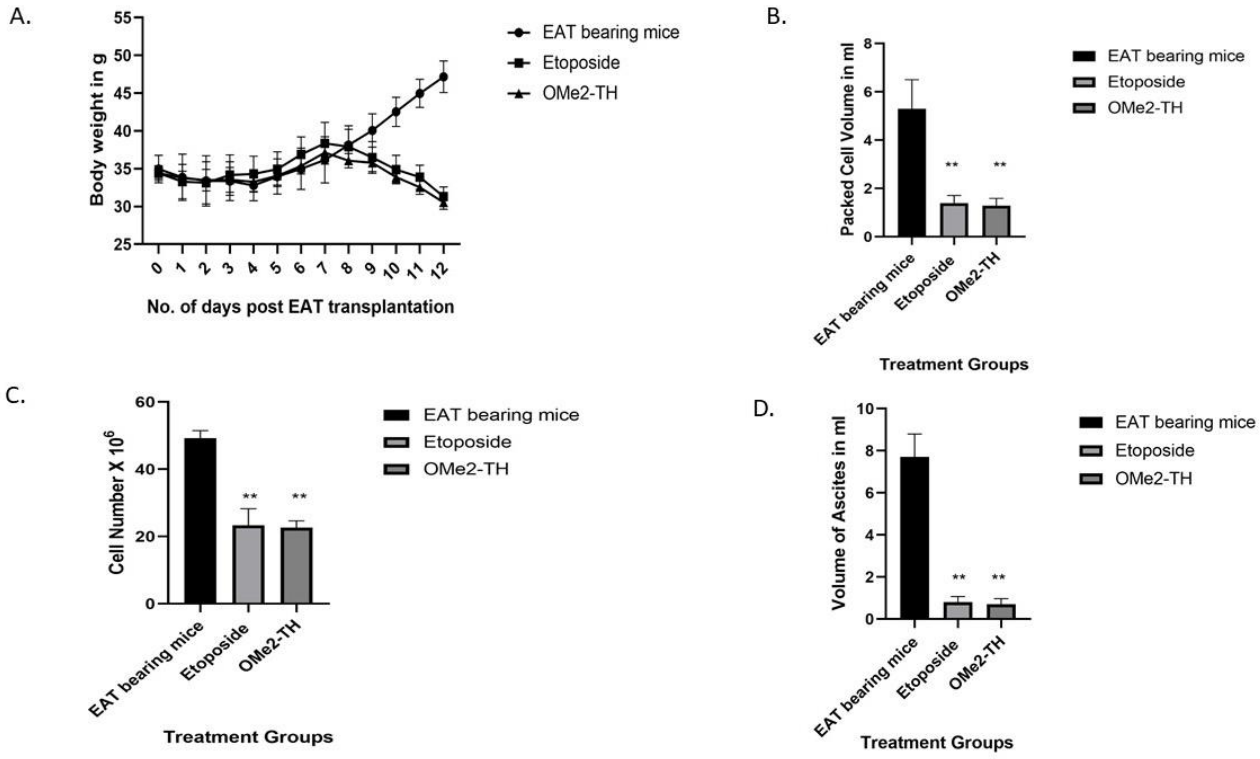

Fig 1: Anti-tumor activity of podophyllotoxin derivative OMe2-TH and Etoposide *in vivo* in EAT model: EAT bearing mice were administered a daily dose of 10 mg/kg of Etoposide and 4 mg/kg of OMe2-TH along with vehicle (0.1 % DMSO) treated control from sixth day of transplantation till the thirteenth day. A. Differential reduction of body weight of mice in untreated and treated EAT bearing mice. B. Quantification of packed cell volume and Enumeration of number of viable cells in untreated and treated groups of EAT bearing mice (C). D. Comparison in the volume of ascites secreted in mice of untreated and treated groups of Etoposide and OME2-TH. Data are presented as the mean ± SEM of three independent experiments; **P<0.01.

Effect of OMe2-TH and Etoposide on blood vessels and microvasculature

The photographed peritoneum of treated groups showed decreased blood vasculature as compared to untreated EAT bearing mice. On assessing the number of blood vessels using ImageJ analysis, the data in Fig 2A & C shows that OMe2-TH showed least number of blood vessels that is 13 as compared to an average of 14.2 in case of Etoposide. However, there was a significant increase in the blood vessels of untreated EAT bearing mice with an average of 48.8. The tumor cells as identified by dark nucleus, in the tissue sections of the H&E stained peritoneum showed that in OMe2-TH and Etoposide treated mice, a significant decrease in tumor cell infiltration is evident as compared with the untreated peritoneum from EAT bearing mice (Fig 2B).

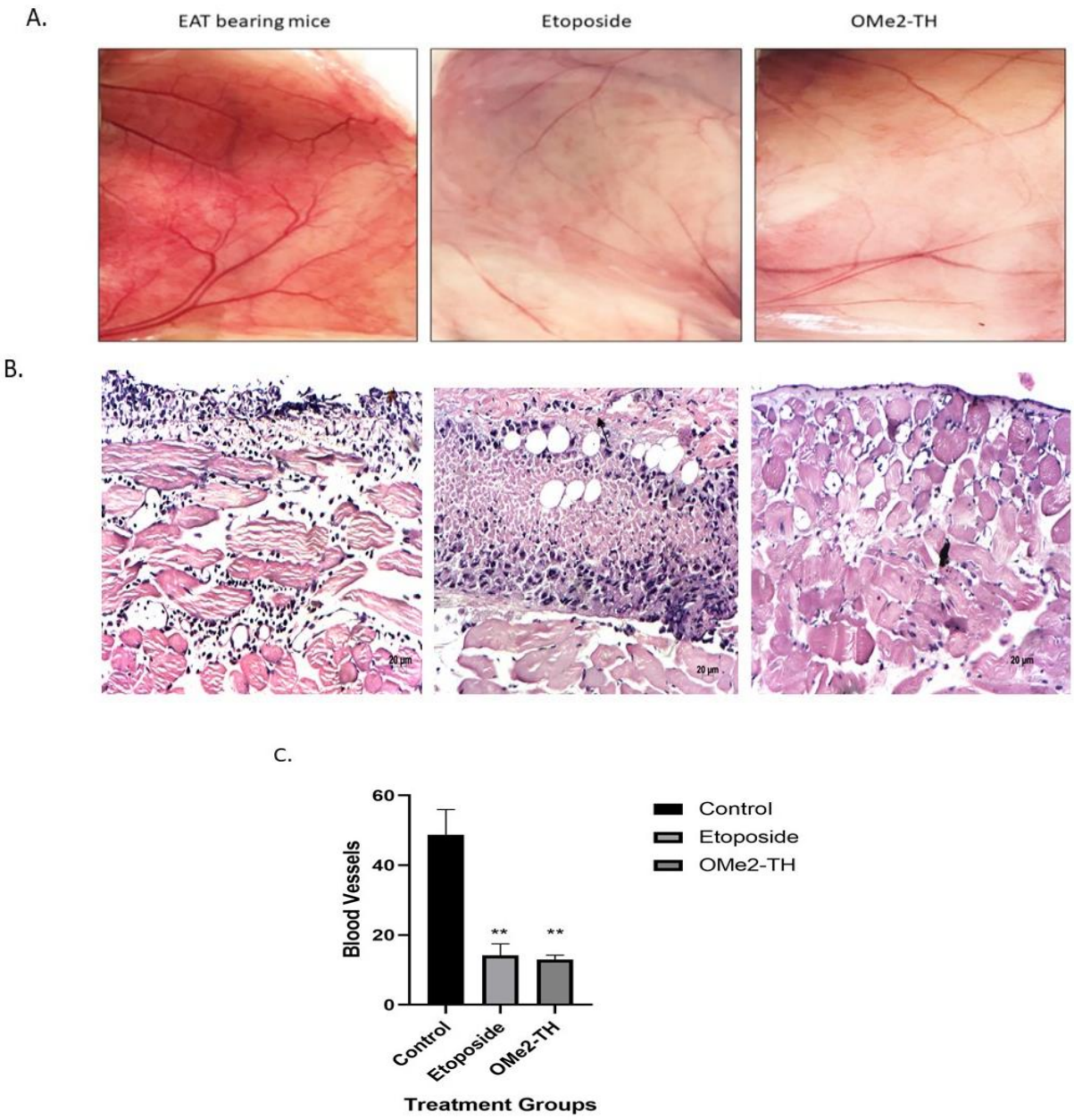

Fig 2: Anti-angiogenic activity of OMe2-TH and Etoposide in EAT model: Comparison in the blood vasculature and invasiveness of micro vessels in the peritoneum of mice in untreated and treated groups. EAT bearing mice were injected (i.p) with a daily dose of 4mg/kg of OMe2-TH and 10mg/Kg of Etoposide and compared with vehicle (0.1 % DMSO) treated control from sixth day of transplantation till the thirteenth day. A. Representative photographs of peritoneum of EAT bearing mice and treated groups. B. Haematoxylin and Eosin staining on the peritoneum to show differential invasiveness of micro vessels. C. Graphical representation of the difference in the blood vessels formed in the peritoneum of mice in untreated and treated groups. Data are presented as the mean ± SEM of three independent experiments; **P<0.01.

Anti-permeability activity of OMe2-TH and Etoposide

Miles permeability assay is a gold standard to assess the permeability potential of VEGF *in vivo*. The data in Fig 3A & B are suggestive of the dose response of VEGF, the optical density values recorded positively correlate with the increase in VEGF concentration. Maximal absorbance was observed with VEGF at a concentration of 20 ng. The data represented in Fig 3C & D is conclusive of the action of both compounds in inhibiting VEGF induced vascular permeability. Etoposide and OMe2-TH inhibit VEGF-induced permeability by 80.65% and 88.13% respectively, normalized to VEGF (20 ng), indicating that OMe2-TH is a superior inhibitor of VEGF induced permeability as compared to Etoposide.

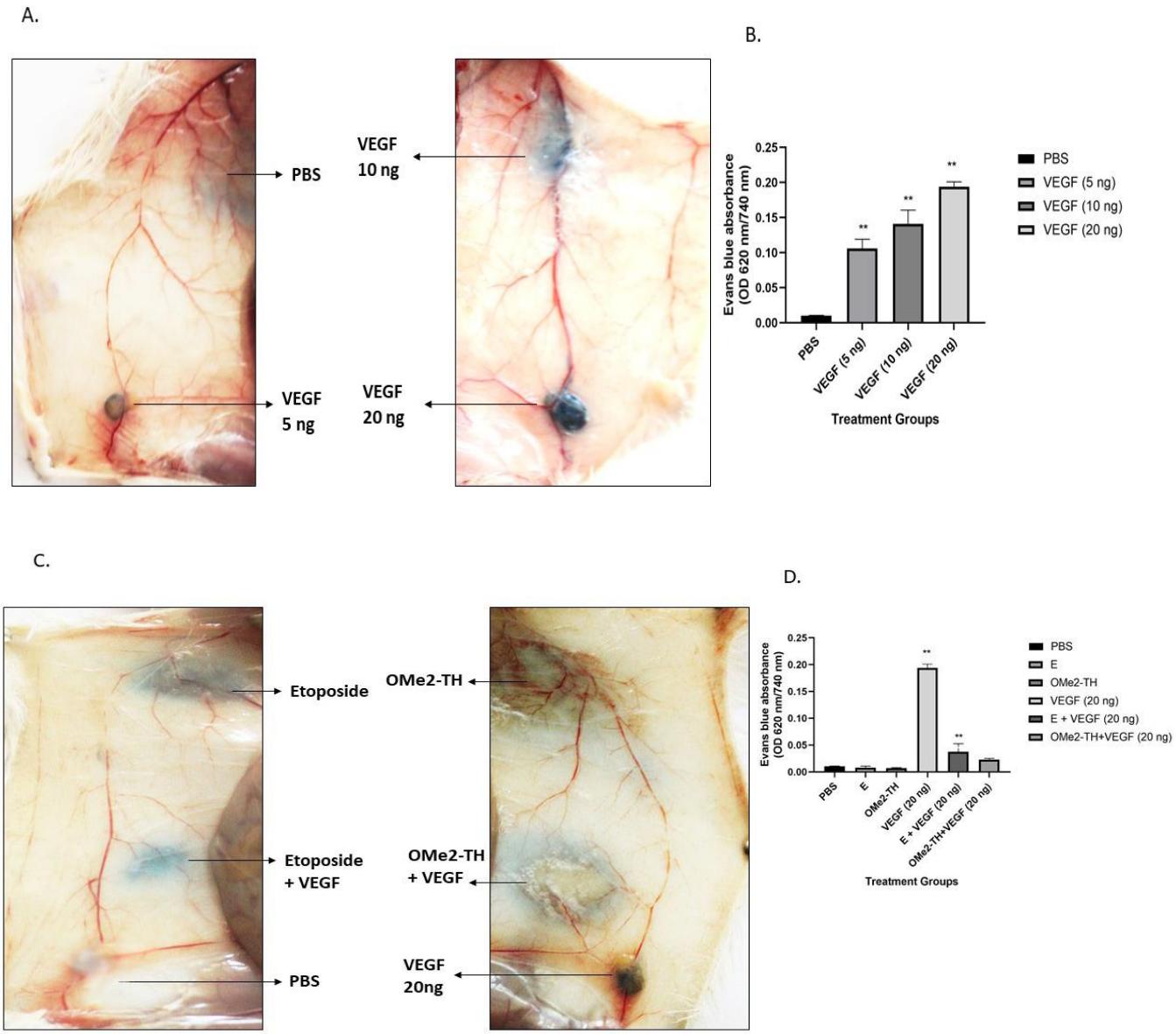

Fig 3: Analysis of inhibition of VEGF induced vascular permeability by Miles Permeability Assay: Representation of Evans Blue leakage in Swiss albino mice. Evans Blue dye (100 µL) was administered by tail vein injections. After 20 minutes, intradermal injections of test compounds were

given for 20 mins and mice were sacrificed and the skin was excised in formamide to perform spectrophotometric analysis of the dye extract at 620 nm with 740 nm as reference wavelength. A. VEGF in increasing concentrations (5 ng, 10 ng and 20 ng) in the dermis and corresponding spectrophotometric analysis (B.), C. Etoposide (10mg/kg) with and without VEGF (20ng) and OMe2-TH (4mg/kg) with and without VEGF (20ng) with PBS as negative control in the dermis and corresponding spectrophotometric analysis of Evans blue (D.) respectively.

Etoposide or VP-16 is an important chemotherapeutic drug that has clinical applications in treating a wide spectrum of cancers for decades and is known to be one of the highly prescribed drugs in the world [17]. Oral Etoposide, is an active agent in the treatment of various malignancies, including recurrent brain tumors, leukemia, lymphoma, hepatocellular carcinoma, Kaposi's sarcoma, ovarian and testicular cancer. Etoposide is known to inhibit angiogenesis *in vitro* and *in vivo* by decreasing micro vessel density and VEGF production by tumor cells [15] [18]. Etoposide and its analogues not only cause cell cycle arrest and apoptosis, but also exhibit strong anti-proliferative, anti-angiogenic and pro apoptotic activity by the modulation of microRNAs by targeting the genes like Bcl-2, STAT3 and VEGF that are regulating apoptosis and angiogenesis. Highly invasive tumors express VEGF which correlates with vascularity and cell proliferation [11][15]. Therefore, we undertook this study to understand and validate the effects of etoposide in the inhibition of tumor cell proliferation and angiogenesis in a mouse mammary carcinoma *in vivo* model and its role as inhibitor of VEGF induced permeability. Our results were consistent with the recent reports on anti-angiogenic effects of etoposide [12]. Furthermore, we intended to compare the efficacy of this well-established chemotherapeutic podophyllotoxin derived drug etoposide with a novel podophyllotoxin derivative OMe2-TH in order to understand the role of VEGF regulated pathways in its tumor regression ability. The *in vivo* results demonstrated a clear evidence of the superiority of OMe2-TH over etoposide at a low dose of 4 mg/kg (i.p.) of body weight of mice as compared to 10mg/kg (i.p.) of etoposide in inhibiting tumor burden due to ascites secretion, cell proliferation, peritoneal angiogenesis and tumor infiltration of muscle tissue which is an important hallmark of sustained angiogenesis and metastasis. Further, we performed Miles permeability assay in order to understand the role of these molecules in the inhibition of VEGF-induced permeability, as the role of VEGF as permeability inducer is critical for the accumulation of ascites in all malignant ascites tumors. Our results with Etoposide were consistent with previous reports indicating its inhibitory potential on VEGF-induced permeability [15] which competitively matched with the ability of OMe2-TH to inhibit VEGF-induced vascular permeability in a non-tumor context. The similar behavior of both the molecules could be accounted to the parent podophyllotoxin class they belong to and their structural conformation. Etoposide and its conjugates are known to regulate angiogenesis via VEGF-dependent pathway [12]. This serves as an indication to further the investigation of VEGF regulated pathways in anti-angiogenic activity of OMe2-TH.

4. CONCLUSION

Etoposide and a podophyllotoxin derivative OMe2-TH are potent anti-tumor and anti-angiogenic agents inhibiting cell proliferation, ascites volume corresponding to tumor burden and peritoneal angiogenesis in Ehrlich Ascites Tumor model. They have proved to be potential inhibitors of tumor induced invasion by the reduction of infiltration of micro vessels. OMe2-TH has shown superior inhibition of tumor angiogenesis and VEGF induced permeability as compared to Etoposide. The predominant role of VEGF in tumor angiogenesis and the inhibition of VEGF-induced permeability by OMe2-TH hints us of its anti-angiogenic action via VEGF-mediated angiogenic pathways. Further confirmatory assays are required to elucidate the exact mechanism of action of this molecule for its use in targeted therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

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