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ANTI-NEOPLASTIC ACTIVITY OF *DIOSCOREA BULBIFERA* ROOT EXTRACTS BY TARGETING MTA1

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ABSTRACT: Objective: Activity-guided purification of *Dioscorea bulbifera* root extracts and anti-neoplastic activity of different solvent extracts were tested against MCF-7 and murine mammary carcinoma cells. Methods: Powder of roots of *D. bulbifera* was continuously extracted by solvents with increasing polarity, such as petroleum ether, hexane, benzene, chloroform, ethyl acetate, acetone, methanol, ethanol and hot water extracts by Soxhlet extraction method. The dried extracts were tested for its biological activity against the metastasis-associated protein (MTA1) induced cell proliferation and migration. Anti-angiogenic activity by corneal micropocket assay, *in vivo* anti-tumour activity and nuclear staining experiments were done. Results: Among the series, only ethyl acetate, acetone and methanol extracts were found to be most promising. These three active extracts were obtained in the earlier steps much prior to the ethanolic fractionation and showed on an average of 60-70% cell death on mouse mammary carcinoma cells. Also, showed 70-80% inhibition on a recombinant MTA1 induced proliferation and cell migration in MCF-7 cells. The anti-angiogenic and anti-tumour activity on MTA1 induced angiogenesis inferred the regression of vasculature. And the evoking apoptotic process was visualized by cell membrane blebbing. These findings show that the active extracts of *D. bulbifera* roots, are the potentially rich source of bioactive molecules and it is also an effective beginning to explore and purify the novel molecules and delineate their mechanism for cancer therapeutics by targeting MTA1, which is the need for the hour.

KEYWORDS: *Dioscorea bulbifera,* Soxhlet extraction, Anti-proliferative, Anti-angiogenesis.

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1. INTRODUCTION

Cancer also is known as malignant neoplasm is characterized by unregulated cell growth with the potential to invade and spread to distant organs [1]. Breast cancer is the most common malignancy among women and it is the second most leading cause of death worldwide accounting for 9.6 million deaths globally in 2018. More cases are expected to rise in the near future [2]. Natural products from plants either as pure compounds or as plants extract provide unlimited availability of chemically diverse compounds. In Asia and other parts of the world, people still rely on traditional medicine as the primary source of medical treatment [3]. Plants contain a wide range of substances which find its uses in treating various diseases. Thus, research has developed in investigating the potential anti-cancer compounds in various plants that have been used in traditional herbal medicine. Phytochemicals of plants have a good history of use in the treatment of cancer. According to a report more than 50% of available anticancer drugs are derived from plant sources [4]. Currently, chemotherapeutic agents for breast cancer such as doxorubicin, paclitaxel, docetaxel, vincristine, vinblastine, vinorelbine, podophyllotoxin and its derived compounds are in use [5]. However, they are associated with adverse side effects and in most cases, the effect is not long lasting because resistance to chemotherapy is a major obstacle for effective treatment. Therefore, there is a significant need for new anticancer agents with minimal side effects to lower the health burden of this devastating disease. *Dioscorea bulbifera* also named as air yam is a traditional medicinal plant taxonomically belonging to family *Dioscoraeceae*. It is made up of polyphenols along with organic acids, some of them are potential antioxidants, and it also contains the steroidal saponin diosgenin. Investigations showed several phytochemicals extracted from D. *bulbifera* which does include many active molecules for cancer therapy [6]. Recent studies showed that metastasis-associated protein (MTA1) enhances angiogenesis and its overexpression is closely related with many malignant tumours including breast cancer metastasis [7]. Many plant-derived compounds like Pterostilbene, Curcumin and Resveratrol have shown to inhibit MTA1 expression in cancer cells [8–10]. Also, earlier studies from our laboratory have shown many naturally derived plant compounds from *Glycyrrhiza glabra* and *Tinospora cordifolia* to inhibit MTA1 induced angiogenesis and migration [11,12]. Hence, MTA1 can be a possible target molecule for anti-angiogenic drugs in breast cancer treatment. Also, previously we have reported the anticancer activity from the crude ethanolic extract of *D. bulbifera* [13]. In continuation of this work, the present study involves activity guided purification of *D. bulbifera* by solvents with increasing polarity, such as petroleum ether, hexane, benzene, chloroform, ethyl acetate, acetone, methanol, ethanol and hot water extracts by soxhlet extraction method. And the extracts were evaluated for their anticancer potency, whether the extracts can inhibit MTA1 induced cancer cell migration, proliferation and angiogenesis using human breast cancer cell line MCF7 and murine mammary carcinoma cells.

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2. MATERIALS AND METHODS

2.1. Cell lines and chemicals

The human breast cancer cell line MCF-7 was obtained from National Centre for Cell Science (NCCS) Pune, India. It was cultured in DMEM medium containing 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (Invitrogen, USA). Ehrlich Ascites Tumor (EAT) cells/mouse mammary carcinoma cells were maintained in Swiss albino mice (6-8 weeks old). Rats (Wister strain) were obtained from the animal house facility, Department of Studies in Zoology, University of Mysore, India. The animal experiments were approved by the animal ethical committee and institutional animal care and use committee. Department of Studies in Zoology, University of Mysore (UOM/IAEC/27/2016). pHEMA-[Poly(2-hydroxyethyl methacry late)] from (Sigma Aldrich, USA). Recombinant (MTA1) Metastasis-Associated Protein-1 was expressed in E-coli and purified by GST affinity columns in our laboratory, using plasmid pGEX-5X-2-MTA1 which was a kind gift from Dr. Rakesh Kumar, Department of Biochemistry and Molecular Biology, The George Washington Medical centre, Washington, USA. All other chemicals and reagents used were of analytical grade.

2.2. Collection and identification of plant materials.

The rhizomes of *D. bulbifera* were collected from Western Ghats, Shimoga, India. Identification of the plant material was confirmed by depositing the voucher specimens in the Herbarium of the Department of Studies in Botany, University of Mysore, Mysore.

2.3. Fractionation and purification of the active compound from *Dioscorea bulbifera*

Dried powdered roots of *D. bulbifera* (1kg) was subjected to polarity based soxhlet fractionation using solvents such as petroleum ether, hexane, benzene, chloroform, ethyl acetate, acetone, methanol, and ethanol. All the solvent extracts were subjected to evaporation to retain the residue. Dried extracts were dissolved 1mg/ml and assayed for its activity guided purification [11,14].

2.4. Trypan blue dye exclusion assay for different solvent extracts

1 x10^4 cell per well was seeded in 24 well culture plates containing complete DMEM medium. After 12 h, the media was replaced with complete medium containing 100µg/ml of different solvent extracts along with respective controls and incubated for 3h at 37^0C with 5% CO_2 in a humidified incubator. After incubation cells were harvested by centrifugation at 3000 RPM for 5 min. The cell pellet was then re-suspended in 100 µl of PBS; 10 µl of the cell suspension was stained with an equal volume of 0.4% trypan blue in 980 µl of PBS and incubated for 2 min at 37^0C. The total number of cells was counted using a Neubauer's Chamber and the percentage of viability was calculated [15] using the formula;

% viability = No. of viable cells/Total No.of cells (Viable + dead) x 100.

2.5. Proliferation assay

In vitro cell proliferation assay was carried out using ^{3}[H] thymidine incorporation into DNA in

exponentially growing cells as described earlier [12] with minor modifications. 3×10^4 cell/well was plated in a 12-well plate and maintained overnight in complete medium. Subsequently, cells were treated with different solvent extracts of *D. bulbifera* (100 µg/ml) alone or with MTA1 protein (10ng). ^{3}[H]-thymidine (1µC/ml) was added and incubated for 48h. Cells were then washed with PBS and trypsinized. 1ml ice cold 10% (w/v) trichloroacetic acid was added to the cell pellet. Radionucleotides incorporated into DNA were harvested with 0.5% (w/v) SDS and 0.2N NaOH and quantified by a liquid scintillation counter (Perkin Elmer Tri-Carb 2900 TR model). Scintillation cocktail fluid (5 ml) was added to all the samples and radioactivity was measured. Data expressed as the percentage control mean ± SEM of triplicate values.

2.6. Wound healing assay

MCF-7 cells were seeded in 6 well plates and grown to confluency. The cells were treated with mitomycin-C (10ng/ml) for 3h prior to addition of compounds. When cells reached 80-90% confluency, a small scratch was made using a fresh tip. Cells were washed with PBS and media was added to wells containing different solvent extracts of *D. bulbifera* (100µg/ml) alone or with MTA1 protein (10ng/ml) were incubated up to 24h. Photographs were captured after incubation with Zeiss phase-contrast inverted microscope at 20x magnification. The distance and number of cells migrated in the wound area were enumerated using Image J software [12].

2.7. Corneal micro pocket assay

In rat corneal micropocket assay [13], hydron polymer; poly-2-hydroxyethyl methacrylate (pHEMA) was dissolved in absolute ethanol to a final concentration of 12%. An aliquot of this mixture was then spread on a teflon sheet and allowed to dry for 2h. The pellets were divided into 4 groups, control (group I), MTA1 10ng (group II), ethyl acetate and acetone extract alone 50µg each (group III) and MTA1 + extracts (group IV) were placed on a teflon surface and allowed to dry overnight at 4^0C. All the above steps were performed under aseptic conditions. Male Wister rats were anesthetized with xylazine (6mg/kg,IM) and ketamine (20mg/kg,IM). A corneal pocket was made by inserting a 27- gauge needle and single pellet were inserted into the corneal pocket of the rat eye. Gentamycin antibiotic was applied to the anterior surface of the eye daily. On the 7th day, the rats were anaesthetized and the corneas were photographed using Research Stereo Zoom Microscope (Carl Zeiss, Germany) and analyzed by AXIO Imager A2 M software.

2.8. *In-vivo* peritoneal angiogenesis assay

To know the effect of different solvent extracts towards angiogenesis we performed *in vivo* peritoneal angiogenesis assay in EAT-bearing mice model. EAT cells was transplanted into female Swiss albino mice. EAT cells (5×10^6) were injected intraperitoneally (i.p) into all 40 mice (8 groups, 5 in each group), after 6th day of transplantation the animals were administered with different solvent extracts (25mg/kg body weight (b.w)) every day and growth was recorded every day from the day of transplantation. On the 16th day, the peritoneum of the respective animals was

cut open and the inner lining of the peritoneal cavity was examined for extent of neovasculature and photographed. Also, the volume of ascites was measured and packed cell number was counted [11].

2.9. Nuclear staining for apoptosis

The compounds which can modify programmed cell death are likely to be useful in therapeutics. Hence, to analyze the morphological changes of EAT cells upon treatment with different solvent extracts of *D. bulbifera*, Giemsa staining was performed. Briefly, as mentioned above, control and treated cells from the *in vivo* assay which had received different solvent extracts (25mg/kg b.w) were harvested and washed with PBS. Cells were fixed with methanol and stained with 10% Giemsa solution and examined by an optical light microscope at 40x magnification and photographed (Zeiss AxioVert).

3. RESULTS AND DISCUSSION

3.1. Cytotoxic effects of *D. bulbifera* root extracts

In our previous study, we have reported that *D. bulbifera* crude ethanolic extract exhibits anti-angiogenic property [13]. The results on further purification with activity-guided fractionation of *D. bulbifera* root extracts revealed that ethyl acetate and acetone extracts showed the cytotoxic effect on mouse mammary carcinoma cells. The cytotoxicity of different solvent extracts was assessed by trypan blue dye exclusion assay. The data from Fig.1 shows, chloroform extract was moderately cytotoxic whereas, ethyl acetate, acetone and methanol extracts showed 60-70% of cell death when compared with normalized respective vehicle control. These fractions were obtained by earlier steps before the ethanol fractionation.

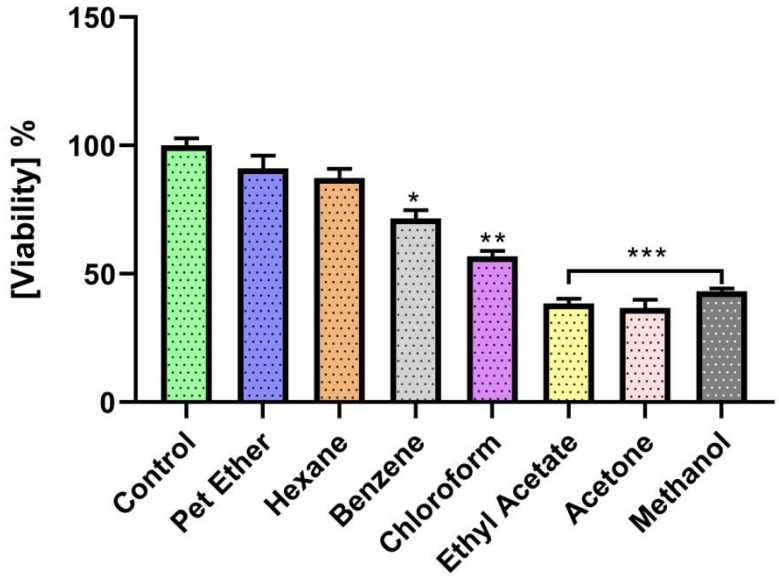


Fig1: Effect of *D. bulbifera* solvent extracts on the viability of EAT cells

Cells ($1x10^5$) were treated with 100 µg of different solvent extracts along with respective controls for 6h. The cells were harvested, washed with PBS and counted after adding trypan blue dye. The viable and dead cells were counted and the percentage viability was calculated. Data is represented as the mean ±SEM of three independent experiments; **P < 0.01 and *p<0.05.

3.2. Effect of *D. bulbifera* root extracts on MTA 1 induced cell proliferation

To gain insights into the anti-proliferative effect of different extracts on MTA1 induced proliferation, we performed a ^{3}[H] thymidine incorporation using MCF-7 cell line. Cells were treated with different extracts (100µg/ml) either alone or with MTA1 protein (10ng/ml). The rate of proliferation of cells was measured as the percentage of incorporation of ^{3}[H]thymidine. The data from Fig.2 indicates that ethyl acetate and acetone extracts significantly decreased the proliferation of MCF-7 cells. There was 70-75% decrease in cell proliferation in comparison to vehicle-treated control at 48 h.

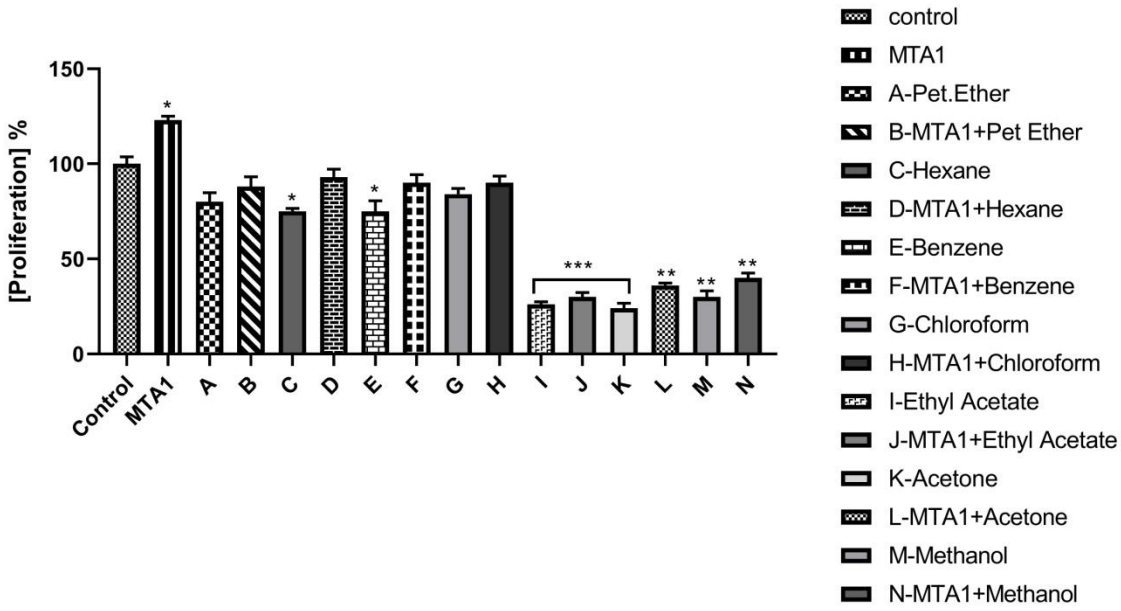


Fig.2: *D. bulbifera* extracts inhibit MTA1-induced proliferation of MCF-7 cells

Cells were treated with different solvent extracts (100µg/ml) in presence of ^{3}[H] thymidine (1µCi/ml). After 48h of incubation, the incorporated ^{3}[H] thymidine into the cells was quantified by scintillation counting. The data represents the mean ±SEM of three independent experiments. Ethyl acetate and acetone extracts showed significant antiproliferative activity in comparison with respective control; **P < 0.01 and *p<0.05.

3.3. Wound healing activity of different extracts

The wound healing assay was used to investigate which extracts could influence MTA1 induced migration of MCF-7 cells. Wound gap of similar size was made on the monolayer of vehicle-treated cells (control group), MTA1 alone as a positive control and cells were treated with

different solvent extracts (100µg/ml) either with or without MTA1 and were incubated up to 24h. In control, the gap was gradually narrowed down and in presence of MTA1 alone; the gap was nearly closed after 24h. However, cells which were exposed to chloroform, ethyl acetate, acetone and methanol extracts along with MTA1 inhibited the migrating cells and kept the wound open as shown in Fig.3.

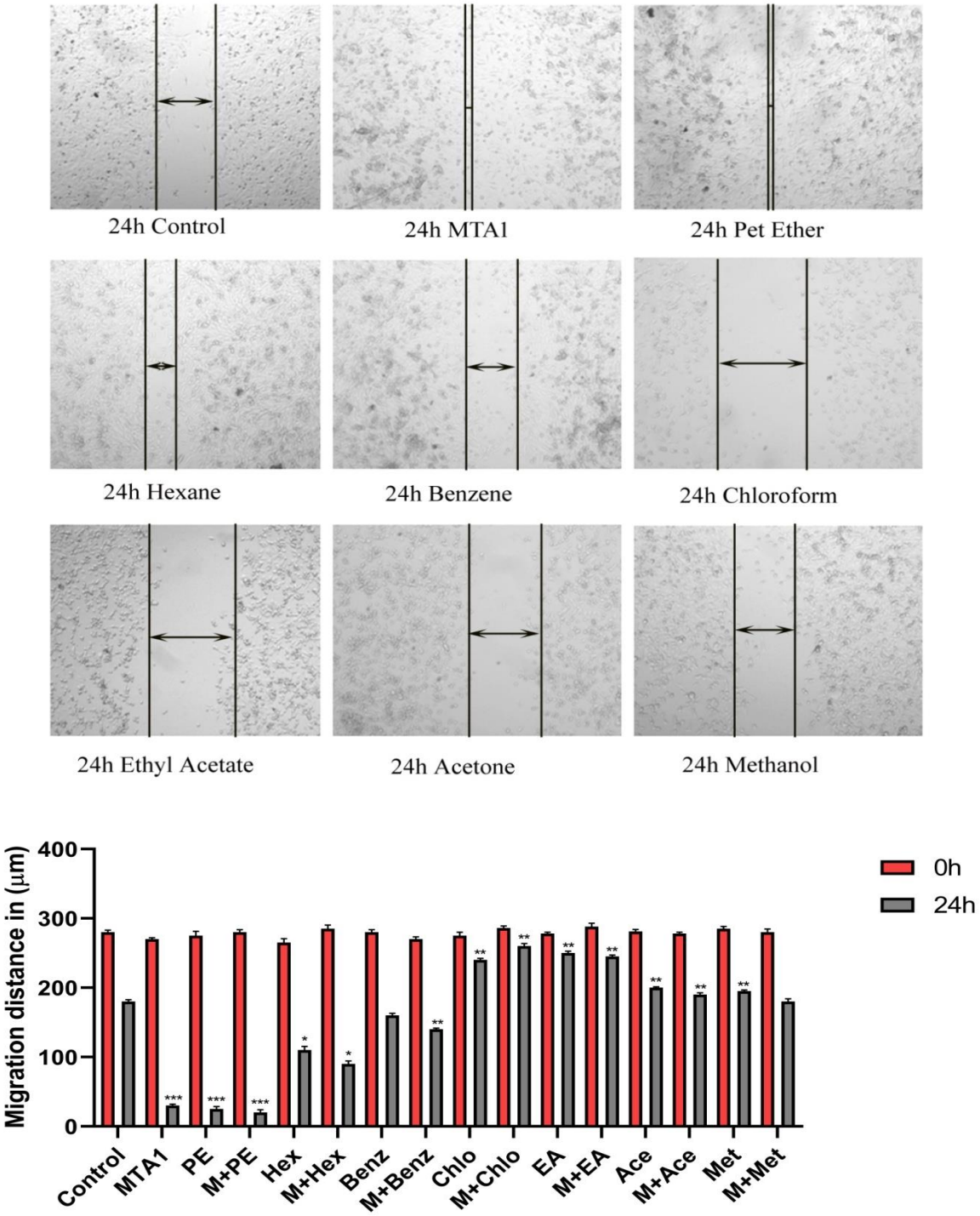
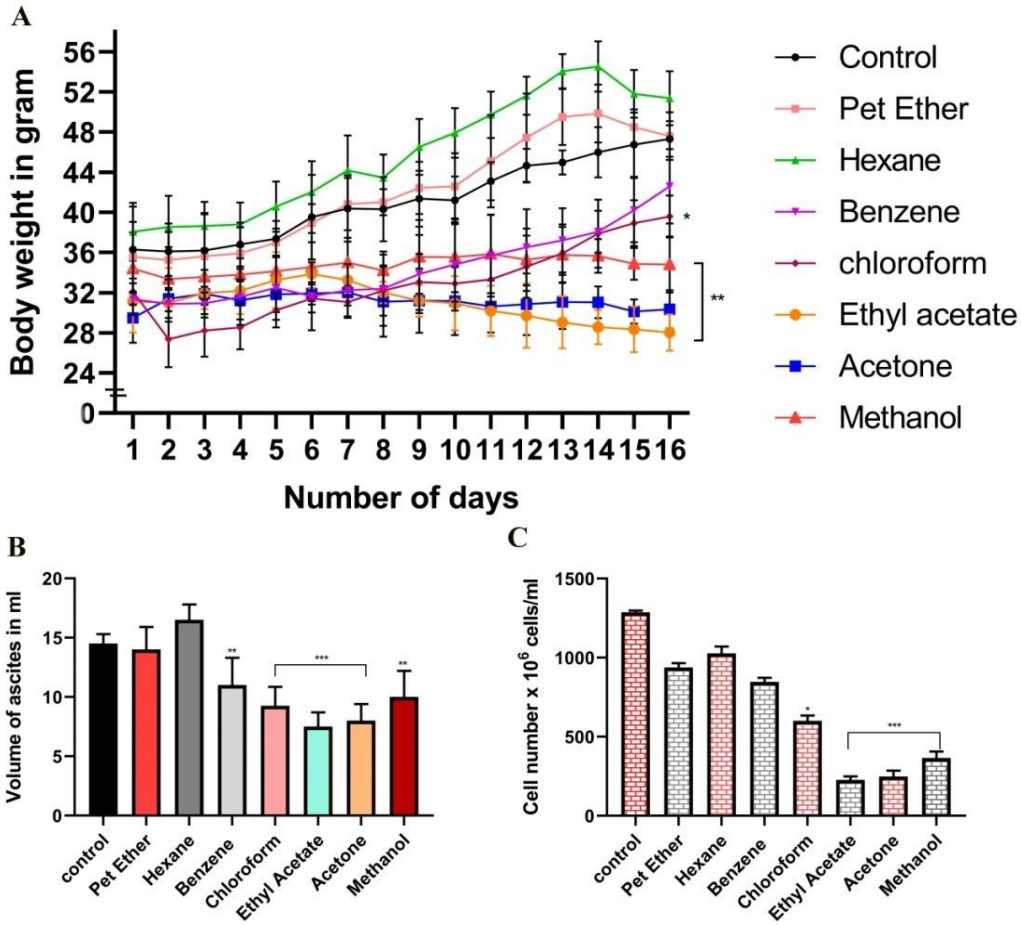


Fig.3: Effect of *D. bulbifera* extracts on MTA1-induced cell migration: 3(A)-Representative pictures of wound healing assay from three repetitive experiments. Wounds were made using a

sterile tip on confluent monolayer of MCF-7 cells. Wounded cultures were incubated with different extracts along with MTA1 for 24h. Phase contrast microphotographs were taken after 24 h incubation. **5(B)** - The distance between the two edges of each wound were measured and the graph was plotted respectively. The data represents the mean ±SEM of three independent experiments; **P < 0.01 and *p<0.05.

3.4. Angio-inhibitory effect of different extracts was evaluated by peritoneal angiogenesis assay in EAT-bearing mice *in vivo*

To study the effect of different extracts on the angio-inhibitory activity we performed an *in vivo* peritoneal angiogenesis assay. There was a nearly exponential increase in body weight of EAT-bearing mice during a growth period of 16 days (**Fig 4A**). Further, upon treatment with different extracts (25mg/kg body weight) injected intraperitoneally (i.p), there was a reduction in body weight. Also, there was an inhibition on growth of EAT cells and formation of ascites fluid **(Fig B and C)** when compared to that of untreated tumour-bearing control mice. **Fig D**-The peritoneal lining on dissection revealed regression of neovascularization in the ethyl acetate and acetone extracts treated mice when compared to that of tumour-bearing mice. The data shown in **(Fig.4)** clearly indicates that ethyl acetate and acetone extracts have potent angio-inhibitory activity.



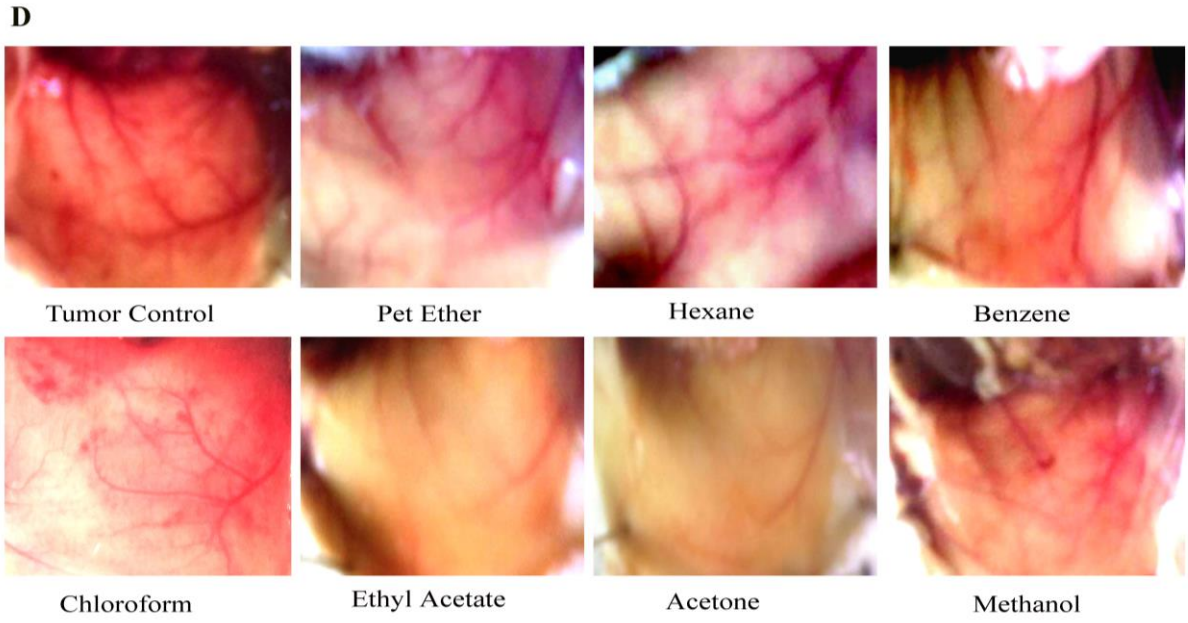


Fig.4: *In vivo* cytotoxicity of all extracts evaluated by peritoneal angiogenesis assay in EAT-bearing mice

EAT-bearing mice treated with different extracts (25mg/kg b.w). **4(A)**-Body weights were recorded for 16 days. **4(B)**- Ascites volume and **4(C)**- Cell number were also counted. **7(D)** – Peritoneum of a tumour-bearing control and treated mice were observed for the extent of angiogenesis. Data represented as the mean ± SEM of three different observations (five animals per treatment group).

3.5. Apoptosis induced by different solvent extracts of *D. bulbifera*.

Apoptotic cells are morphologically distinct which shows nuclear condensation, cytoplasmic shrinkage and membrane blebbing. In order to know whether the compound induced cytotoxicity was due to apoptosis, EAT cells obtained from an *in vivo* assay were subjected to morphological examination by staining the treated and control cells with 10% Giemsa solution. Control cells were observed with intact nuclei. Also, petroleum ether and hexane extracts did not influence any morphological changes. Whereas ethyl acetate and acetone extracts treated cells showed late apoptotic morphology, membrane blebbing and fragmented nuclei of cells undergoing apoptosis was clearly visible. From the data in **Fig 5**, it is clear that ethyl acetate and acetone extracts induce apoptosis in EAT cells which corroborate with the previous results.

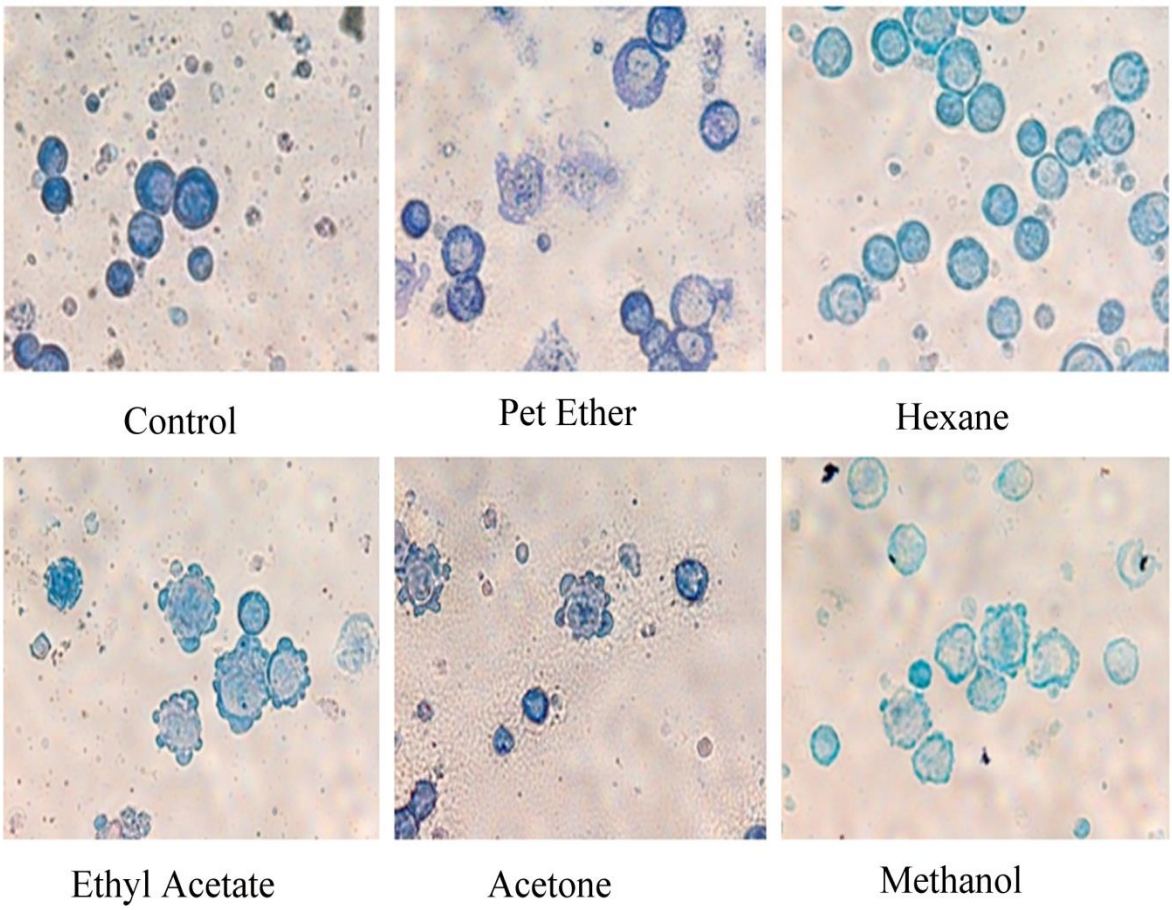
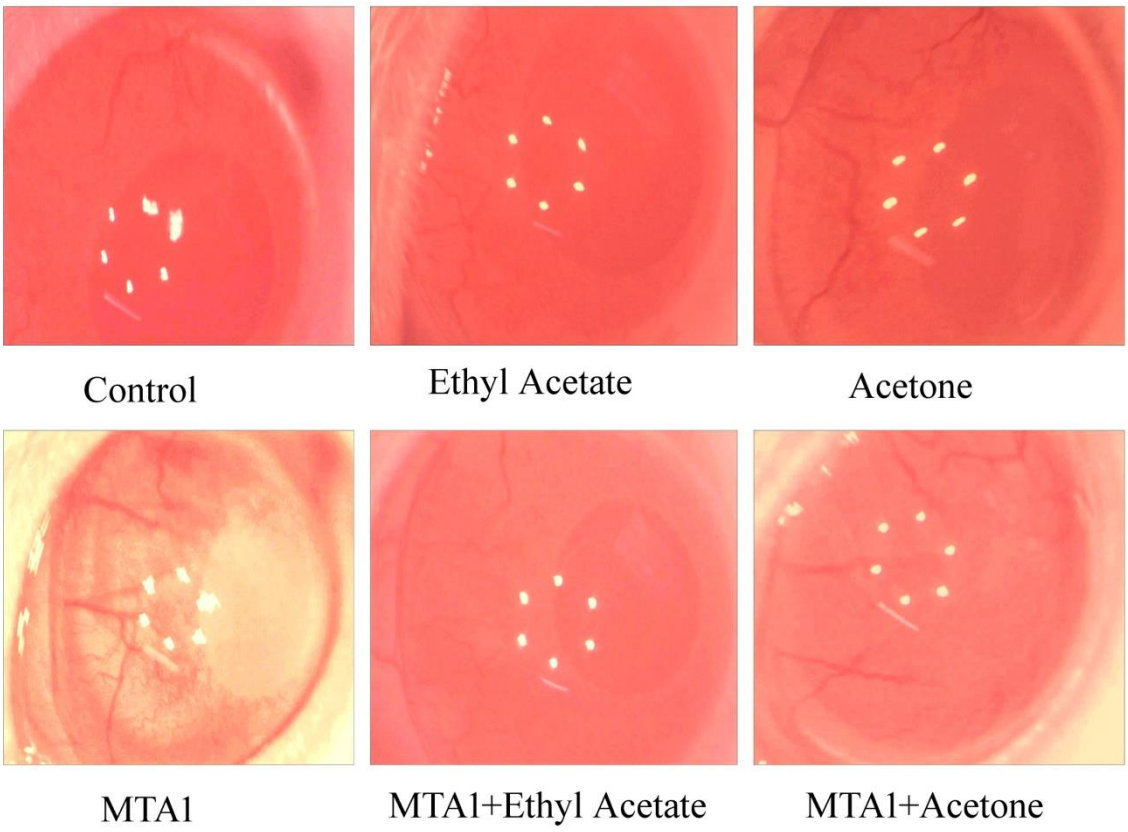


Fig.5: Ethyl acetate and acetone extracts induced apoptosis in EAT cells, as assessed by Giemsa staining: The EAT cells harvested from the *in vivo* assay were stained with Giemsa and photographed. Data are representative of three independent experiments.

3.6. Inhibition of corneal neovascularization by *D. bulbifera* root extracts

To further investigate the anti-angiogenic activity of different extracts in non-tumour context, we studied the inhibitory effect on neovascularization induced by MTA1 protein in corneal micro pockets of Wister rats. When compared to the avascular cornea in the control MTA1 induced extensive neovascularization. MTA1-induced formation of new blood vessels could be inhibited by ethyl acetate and acetone extracts effectively. This data (Fig.6) clearly indicates that ethyl acetate and acetone extracts contain potent anti-angiogenic molecules.



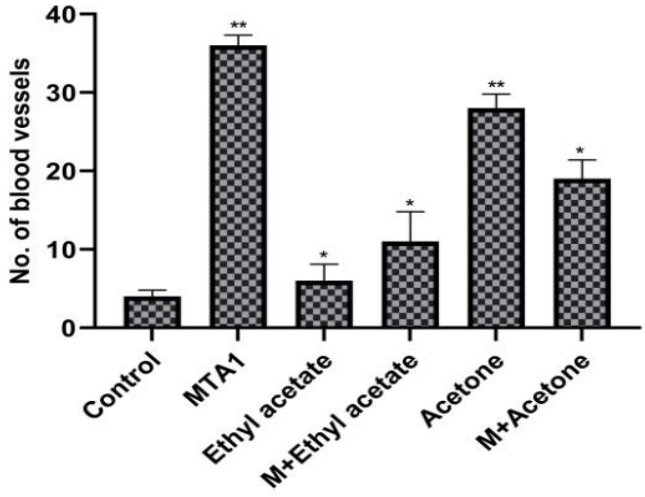


Fig.6: The extracts exhibiting suppression of MTA1 induced neovascularization in rat cornea

Pellets containing Group I- Control, Group II- MTA1, Group III and IV- ethyl acetate and Acetone extract 100µg w/wo MTA1 were inserted into the rat's corneal region of the eye. Photographs showing inhibition of neovascularization by ethyl acetate and acetone extract is evident. **(F)-** The number of blood vessels was counted and represented graphically; Data represented as the mean ± SEM of three independent experiments; **P < 0.01 and *p<0.05.

3.7. DISCUSSION

Medicinal plants have a special place in the management of cancer. *D. bulbifera* is used more often in traditional Chinese medicine; it had long been used to treat a sore throat and struma. People from Bangladesh are known to use this plant to treat leprosy. Japanese have even patented the active molecule from the rhizome that helped in treating diabetes [16]. Apart from this, in comparison of the polyphenol oxidase activity of various species of yam tubers, the greatest enzyme activity was found in *D. bulbifera* [17]. H. Cui et al. have shown that *Dioscorea bulbifera* polysaccharides could attenuate cyclophosphamide treatment-induced immunosuppression and oxidation, and to improve the anti-tumour effect of cyclophosphamide [18]. Our previous investigation revealed that *D. bulbifera* crude extract had an anti-proliferative and anti-angiogenic property [13]. Therefore, we continued to investigate the anti-neoplastic activity of different extracts from *D. bulbifera.* Bioassay-guided fractionation has been the best-known method for identifying bioactive natural compounds for many years. This approach involves continuous extraction by solvents with increasing polarity for preparative-scale fractionation and assessment of selected biological activity [19]. Following soxhlet extraction method seven extracts were obtained from *D. bulbifera* root and their biological properties were evaluated. Our earlier report and also recent studies show that MTA1 overexpression is closely related with angiogenesis and metastasis [7,11,12]. Hence, it can be a possible target molecule for anti-angiogenic drugs in breast cancer treatment. Our investigation in this regard demonstrated inhibition of MTA1 induced proliferation and migration of MCF-7 cells on treatment with ethyl acetate, acetone and methanol fractions of *D. bulbifera* root extracts. The difference in activity may be due to different solvent polarity fractions. This observation is in agreement with other reports where the diterpenoids have been identified in the *D. bulbifera* which have shown similar effects [6,20]. Tumour growth is dependent on the formation of neovascularization. Investigation of this correlation was carried out for the first time by Folkman [21]. After our observation of potent *in vitro* effects against breast cancer cells, we further investigated the *in vivo* antitumor effects of all 7 extracts using EAT bearing tumour model. Consistent with our *in vitro* results, mice administered with ethyl acetate or acetone extracts at a dose (25mg/kg b.w) significantly inhibited tumour growth compared with the control group. A significant decrease in peritoneal angiogenesis on peritoneal wall confirmed the angio-inhibitory activity of ethyl acetate and acetone extracts. A decrease in ascites formation in EAT-bearing mice has a clinical correlation with regression of tumour size [22]. Kuroyanagi et.al has purified 8 phytochemicals from the EtOAc fraction of this rhizome and their structures identified include diosbulbin B [16]. Wang et.al has also reported anti-tumour activity of Diosbulbin B in S180 or H22 ascites tumour cells *in vivo* [23]. Apart from angio-inhibitory activity the cells treated with the active extracts also exhibited apoptosis which was confirmed by membrane blebbing, nuclear condensation and degradation as visualized by Giemsa staining.

Inhibitors of angiogenesis block any of the steps in the angiogenic process, including proliferation, migration and invasion of cancer cells through the matrix, which is required for the metastatic activity of tumour cell [24–26]. In agreement with this, our results from rat cornea neovascularisation show significant inhibition on capillary sprouting on treatment with ethyl acetate and acetone extracts. The active extracts (ethyl acetate and acetone extracts) were obtained in the earlier steps much before the ethanolic fractionation. Hence, there may be several other potent phytochemicals present in these two active extracts of *D. bulbifera* which need to be further purified and characterized. In spite of many alternative therapies available for cancer treatment, there is a wide scope to investigate plant-derived phytochemicals for novel therapeutic agents [27–30]. Additional research into isolation and characterization of the phytochemicals from the three active extracts of *D. bulbifera* can yield novel compounds for cancer therapeutics. However, detailed investigations are required at the molecular level to validate the mechanism of action.

4. CONCLUSION

In conclusion, our results have demonstrated that ethyl acetate and acetone extracts of *D. bulbifera* show inhibition on MTA1 induced cell proliferation and migration of MCF-7 cells. Both fractions have also known to induce apoptosis. Meanwhile, we also investigated the antitumor effect of all extracts *in vivo*, and the two active extracts exhibited significant regression of tumour growth *in vivo*. Also, the angio-inhibitory activity was confirmed by rat cornea neovascularisation assay. However, additional investigations on purification of the novel compounds are required to delineate the molecular mechanism of the active ingredient. Our preliminary findings further support the development of *D. bulbifera* as an alternative therapeutic agent against breast cancer.

4.1. Statistical analysis

All experiments were performed in triplicates. Wherever appropriate, the data are expressed as the mean ± SEM. Statistical significance was evaluated by one-way analysis of variance followed by Dunnet's test. For all tests, *P<0.05 and **p < 0.01 was considered statistically significant.

4.2. Human and Animal Rights

The animal experiments were approved by the institutional animal care and use committee. Department of Studies in Zoology, the University of Mysore (Ref. No. UOM/IAEC/27/2016).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENT

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