Original Article

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Evaluation of the Effect of *Enicostemma axillare* Extract on Migration of MCF-7 Cell Line

Biochemistry Section

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ABSTRACT

Introduction: Breast cancer is the second leading cause of death among women and majority of them are due to metastasis of cancerous cells. The use of plant based compounds as complementary or alternative treatment has gained much significance in the recent times due to their low toxicity levels. *Enicostemma axillare* is the perennial herb known to have anti-inflammatory, anti-allergic, anti-oxidant, anti-microbial, anti-cancer and hypoglycaemic properties.

Aim: To evaluate the effect of *E. axillare* extract on the migration of cancerous cells.

Materials and Methods: The secondary metabolites from the plant were extracted using ethanol. Wound healing assay and western blotting were used to study anti-migratory effect and expression of migration associated proteins in human breast cancer MCF-7 cell lines. Statistical analyses were conducted by one-way ANOVA and Tukey HSD post-hoc tests, the p-value <0.05 was considered as significant.

Results: The rate of migration of cells in control group was 100% accounting to a mean of 325 cells. In case of cell lines treated with *E. axillare* extract, the rate of migration was 32% accounting to a mean of 104 cells. There was a significant reduction (p-value <0.05) in the expression of Vascular Endothelial Growth Factor Receptors (VEGFR), phosphorylated Vascular Endothelial Growth Factor Receptor (pVEGFR), Insulin Like Growth Factor 1 Receptor (IGF1R), Matrix Metallopeptidase 9 (MMP-9), Phosphoinositide 3-kinases (PI3K) and Protein Kinase B (Akt) proteins in cell lines treated with *E. axillare* extract (12.5 µg) in comparison to the control. In addition, the extract also significantly upregulate the expression of Phosphatase and Tensin Homolog (PTEN) and protein (p-value <0.01).

Conclusion: The study indicates that *E. axillare* extract inhibit the migration of cancer cells by inhibiting the expression of Matrix Metalloproteinase (MMPs) and Epidermal Growth Factor Receptors (EGFRs).

Therefore, *E. axillare* extract can be used in formulations for breast cancer treatments.

Keywords: Antineoplastic agents, Breast carcinoma, Cell migration assay, Immunoblotting, Matrix metalloproteinase 9

INTRODUCTION

Cancer develops from transformation of normal cells into cancerous cells that divide uncontrollably and spread into surrounding tissues. World Health Organisation (WHO) 2018 report revealed that cancer was the leading cause of death worldwide with an estimated of 9.6 million deaths and every 1 in 6 deaths were from cancer. Amongst all the cancer, breast cancer stands second with 2.09 million cases per year [1].

Breast carcinoma is classified as either non-invasive or invasive, depending on the invasion of the basal membrane. Non-invasive cancers stay within the lobules of breast and do not invade surrounding normal tissues of the breast. In invasive carcinomas, the altered cells diffuse into surrounding connective tissues thus metastasising to distant organs of the body [2].

Metastasis of cancer is the major process that leads to nearly 90% cancer related mortality. Growth factors and their receptors which also includes EGFR commonly overexpressed by breast cancer cells. Epidermal growth factor receptor is a transmembrane tyrosine kinase receptor, involved in the regulation of proliferation and survival of epithelial cells [3]. Over-activation of EGFR in breast cancer is associated with lower rates of survival. Vascular endothelial growth factor mediated signalling is one of the most important regulatory signal pathways in cancer. These EGF-EGFR binding activates MMPs which causes proteolytic degradation of Extracellular Matrix (ECM) components. Thus, by disrupting the basement membrane, MMPs aid in tumour invasion and metastasis [3].

Treatments for breast cancer involve surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapy and immunotherapy. Though these lines of treatment do not decrease mortality, to a great extent they reduce morbidity [2].

The use of plant extracts to treat various ailments can be traced back to ancient times. With the increasing level of resistance to chemotherapy and radiotherapy and the associated toxicity of these therapies, the plant-based compounds have been in vogue in the recent times. It has been reported that 74.9% of new anticancer drugs between 1981 and 2000 were derived from natural products [4]. Anticarcinogenic effect of plant products have been effectively utilised in the management of the common malignancies [5-9].

Enicostemma axillare is a perennial herb found throughout India especially in the coastal areas [10]. In traditional medicine, this plant is used to treat rheumatism, diabetes mellitus, abdominal ulcers, swelling, hernia, insect bite and skin disorders [10]. Numerous studies reports anticancer, hypoglycaemic and anti-inflammatory properties of the plant products [11-15]. Although the anticancer effect of *E. axillare* extract has been extensively studied in various *in-vitro* models, but there are no reports on the action of the extract at molecular level. Hence, in the present study, the effect of *Enicostemma axillare* extract on the migration of MCF-7 cell lines using western blotting was evaluated.

MATERIALS AND METHODS

Plant Material and Extract Preparation

The plant materials were collected during July 2016 to September 2016 from Chengalpet, Kanchipuram District, Tamil Nadu, India and authenticated by Institute of Herbal Science-Plant Anatomy Research Centre, Tambaram, Chennai, Tamil Nadu, India. Ethical clearance is not required, since it is an in-vitro cell line based study.

The whole plant materials were shade dried and powdered. The plant extract was prepared by mixing 2 kg of dry powder and 6 litres of ethanol in an individual aspirator bottle, which were shaken in orbitory shaker 72 hours. The extract was filtered. The process was repeated thrice and the extracts were pooled, filtered using Whattman filter paper No. 2, solvent was removed using rotary evaporator at 40°C [16]. The total extract obtained was 10 gm. The concentrated extracts were used for further studies.

Cell Line Culture

Michigan Cancer Foundation-7 (MCF-7) cell line derived from the pleural effusion of invasive breast ductal carcinoma was used in the present study. MCF-7 cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, Maharasthra, India. The cells were cultured in T25 flask containing Dulbecco's Modified Eagle Medium (DMEM) with 10% Foetal Bovine Serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching 80% of confluency, the cells were trypsinized and passaged.

Wound Healing Assay

The concentration of E. axillare extract and Doxorubicin required for 50% inhibition of viability (IC50) was determined as 12.5 µg/mL and 1 µg/mL respectively by cell viability study. MCF-7 cells (1×105 cells/well) were seeded into six-well plates and after 72 hours, the cell monolayer was scraped with a sterile 200 µL micropipette tip to create a wound, washed with PBS and examined under an inverted microscope and photographed. Thereafter, the cells were treated with DMSO (0.01%) (Group-I), E. axillare extract (12.5 µg/mL) (Group-II) and Doxorubicin (1 µg/mL) (Group-III) and incubated at 37°C in 5% CO2 incubator. After 24 hours treatment, the plates were viewed under a microscope and the images were captured at 20x magnification. The number of migrated cells was calculated by comparing the photographs and counting the migrated cells. The Group I treated is considered negative control and Group III is considered as positive control. Group II involves the compound of our interest and its activity is compared with Group I and III.

Western Blot Analysis

MCF-7 cell lines were treated with DMSO (0.01%), *E. axillare* (12.5 µg/mL) and Doxorubicin (1 µg/mL). After the treatment period of 24 hours, the cells were lysed in Radioimmuno Precipitation Assay (RIPA) buffer containing 1X protease inhibitor. The protein concentrations were determined by Lowry's method followed by electrophoresis of 50 µg of protein in 12% polyacrylamide gel and transfer to Polyvinylidene Difluoride (PVDF) membranes. The membranes were incubated with primary antibodies against VEGFR, pVEGFR, PI3K, Akt, MMP9, IGF1R and PTEN in Tris-buffered saline at 4°C overnight. The company, catalog number and concentration of antibodies are mentioned in the [Table/Fig-1]. β-actin served as internal control. After washing, the membranes were incubated with HRP-conjucated anti-rabbit IgG secondary antibodies pro used from Genei Banglore, India and bands were visualised in Chemidoc BIO-RAD-Discovery series instrument using quantity-one 1-D analysis software version 4.6.8.

STATISTICAL ANALYSIS

Data were expressed as Mean±SEM. Statistical analyses were performed using one-way ANOVA analysis and comparison between treatments was made using Tukey HSD post-hoc tests of Statistical Package for the Social Sciences (SPSS) software version 16.0. The p-value <0.05 was considered as significant.

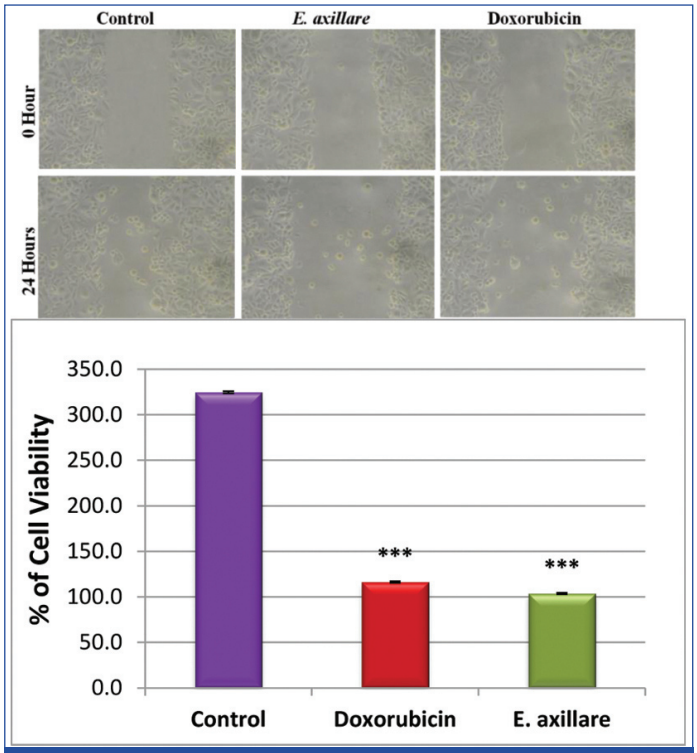
RESULTS

Effect of *E. axillare* Extract on Cell Migration

The effect of *E. axillare* extract on the migration of cancer cells was studied in comparison with control and doxorubicin by wound-healing or scratch assay. The images were taken before (0 hours) and after (24 hours) the treatment [Table/Fig-2]. In the control group, increased migration of cells was observed. In *E. axillare* extract treated group, there was significantly less number of migrated cells in comparison with control group. The rate of inhibition of migration by *E. axillare* extract was at par with the positive control, doxorubicin.

Target protein	Catalog No. of antibody	Company name	Antibody concentration
VEGFR	9698	Cell Signalling	1:2000
pVEGFR	9698	Cell Signalling	1:2000
PI3K	4249	Cell Signalling	1:2000
Akt	9272	Cell Signalling	1:2000
MMP9	2270	Cell Signalling	1:2000
IGF1R	8521	Cell Signalling	1:2000
PTEN	9188	Cell Signalling	1:2000
β-actin	sc-8432	Santa Cruz Biotechnology	1:1000

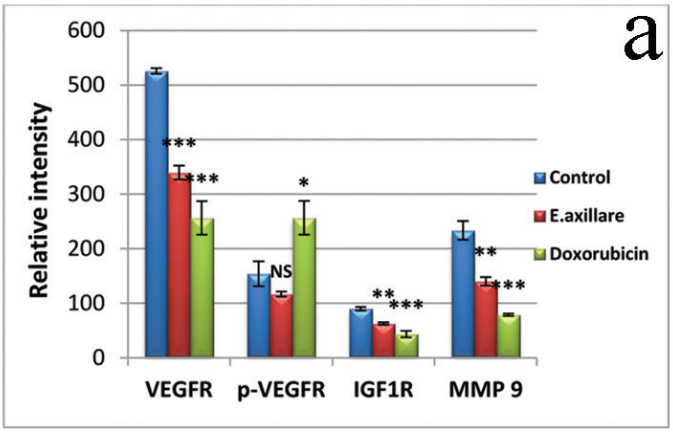
[Table/Fig-1]: The details of antibodies used for Western blot analysis.

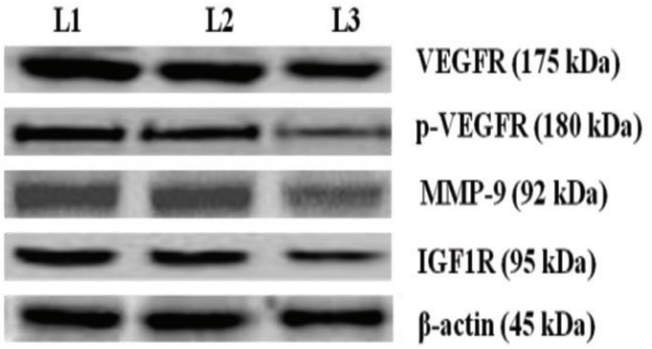


[Table/Fig-2]: Effect of *E.axillare* on migration of MCF7 cell lines. The rate of migration is less in cell lines treated with *E.axillare* extract compared to control. Doxorubicin is the standard drug at concentration of 1 µg and negative control is DMSO at concentration of 0.01%. The statistical differences were analysed using one way ANOVA and the treatments done were compared using Tukey HSD post-hoc analysis (***p-value <0.001=highly significant, **p-value <0.05=moderately significant, *p-value <0.05=significant, NS=Not significant).

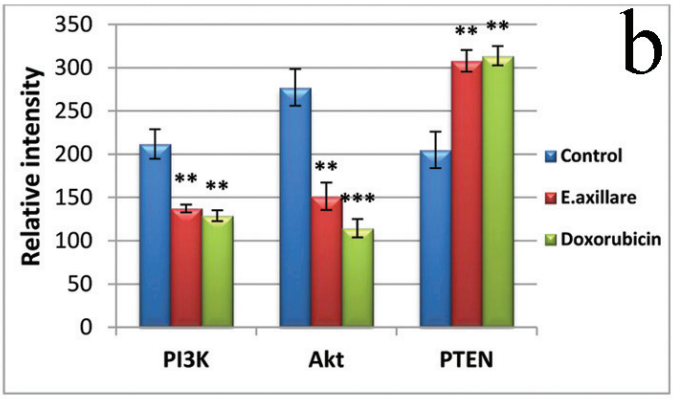
Effect of *E. Axillare* Extract on Migration Associated Proteins

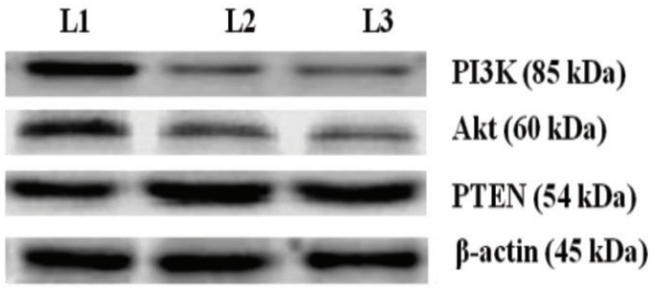
The effect of *E. axillare* extract on proteins associated with migration was analysed at the molecular level by western blotting with β-actin as internal control [Table/Fig-3]. The expression of VEGFR, p-VGEFR, IGF1R, MMP9, PI3K, Akt and PTEN proteins were studied. The expression of VEGFR was significantly downregulated (p-value: <0.001) in cells treated with *E. axillare* extract. Similarly, the expression of MMP 9, PI3K and AKt were also significantly downregulated in comparison with control at a level of 0.1%, 1% and 1%, respectively. The level of PTEN in cell lines treated with *E. axillare* extract was significantly higher than the control cell lines (p-value: <0.01). Overall it was observed that the treatment of MCF 7 cell lines with *E. axillare* extract significantly downregulated the expression of VEGFR (p-value <0.001), IGF1R (p-value <0.01), MMP9 (p-value <0.01), PI3K (p-value <0.01) and Akt (p-value <0.01) proteins and significantly upregulated the expression of PTEN protein (p-value <0.01).





L1 - Control, L2 - *E. axillare*, L3 - Doxorubicin





L1 - Control, L2 - *E. axillare*, L3 - Doxorubicin

[Table/Fig-3]: Effect of *E.axillare* on expression of migration associated proteins in MCF-7 cell lines analysed using western blotting and the level of expression depicted in graph (s). β-actin is the internal control.
A. Represent the expression of VEGFR, p-VEGFR, IGF-1R & MMP-9,
B. Represent the expression of PI3K, Akt, PTEN.
The statistical differences were analysed using one way ANOVA and the treatments using were compared using Tukey HSD post-hoc analysis (***p-value <0.001=highly significant, **p-value <0.05=moderately significant *p-value <0.05=significant, NS=Not significant)

DISCUSSION

Breast cancer is one of the most common cancers in women and is a multifactorial disease. The treatment options available for breast cancer like surgery, radiation, chemotherapy, hormone therapy are reported to offer fewer benefits with many adverse effects [17]. Medicinal herbs are important for cancer treatment due to their multiple chemical compounds composition that helps in management of cancer [3]. It is believed that anti-tumour effects of plants develop by suppressing cancer stimulating enzymes, repairing deoxyribonucleic acid, stimulating production of anti-neoplastic enzymes in cell, which ultimately increases the host's immunity and scavenge free radicals in the body [5,18]. The literature review shows the anti-proliferative activity of various secondary metabolites such as flavonoids, terpenoids, saponins, anthocyanins and phenolic compounds in MCF-7 cell lines [8,19-24]. Quercetin and quercetin-3-β-D-glucoside are the secondary metabolites of apple peels and they exhibit anti-proliferative activity against breast cancer [24,25]. Similarly, punicic acid from pomegranate exhibited anti-proliferative activity in MDA-ERα7 and MDA-MB-231 cells [26]. Thus, the present study had focused on the effect of ethanol extract of *Enicostemma axillare* on migration of MCF-7 cell lines. MMPs are a group of proteases that use zinc as a cofactor. They play an essential role in tissue remodelling by degrading collagen, elastin and fibronectin, the components of extracellular matrix and basal membrane. In this process degradation of other non-characteristic extracellular matrix proteins also happens such as growth factors, cytokines, chemokines, and cell surface receptors [27]. On the proliferation of the tumour cells, MMPs have both direct and indirect impact. The explosion of neoplastic cells and metastatic distribution via the degradation of the extracellular matrix and basal membrane has been directly promoted by it, while indirectly, it supports angiogenesis, distribution of the tumour and providing nutrition [28,29]. An association between the expression of MMP-9 and poor prognosis in breast tumours has been suggested by previous studies [30]. MMP 9 is reported to cause degradation of basement membrane leading to tumour invasion [31]. In the present study, use of *E. axillare* extract on MCF-7 cell lines reduced the expression of MMP-9. However, the exact mechanism of action of the extract on MMP-9 remains debatable.

Epidermal growth factor receptor is a central mediator of the development and progression of breast cancer that stimulates cancer cell growth, survival and resistance to conventional therapy [32]. Repression of VEGF and its receptors reduce tumour growth in multiple tumour models demonstrating its vital role in tumour progression [33]. In the current study, a significant reduction in the expression of VEGFR protein was observed in MCF-7 cell lines treated with *E. axillare* extract. This indicates that the plant extract targets VEGFR along with MMPs. VEGFR is also known to activate MMP-9 through series of pathways that involve PI3K/AKT pathway [2]. The activation of the PI3K/Akt signalling pathway is strongly implicated in the regulation and survival of cancer cells [34]. Downregulation of VEGFR resulted in the lower levels of PI3K and AKT proteins in cell lines treated with *E. axillare* extract. IGF-1 protein has both high mitogenic and anti-apoptotic effect on mammary epithelial cells and is strongly implicated in breast cancer progression. Walsh LA and Damjanovski S demonstrated that IGF-1 plays a cardinal role in the activation of MMPs and thereby increases invasive potential of breast cancer cells [35]. Binding of IGF1R to IGF stimulates cancer cell proliferation, growth and migration [36]. *E. axillare* extract downregulates the expression of IGF-1R protein in comparison with the control (DMSO 0.01%). Thus *E. axillare* inhibits the migration of cancer cells by downregulating the expression of proteins involved in cell proliferation, growth and invasion. Apart from downregulating the above mentioned proteins, *E. axillare* upregulates the expression of PTEN, the protein known to inhibit PI3K and AKT [37]. This, in turn, inhibits the activation of MMPs and prevents the metastasis of cancer.

LIMITATION

The expression of various proteins was only assessed in MCF-7 cell lines. For better understanding of different types of acquired resistance in breast cancer, further studies on different types of

human breast cancer cell lines are required. Furthermore, the effect of ethanolic extract of *E. axillare* on the activity of MMP9 should be studied by multiple techniques such as focal adhesion gelatine zymography and traction force microscopy. This may provide a clear understanding on the mechanism of action of *E. axillare* and ultimate use of the plant in breast cancer therapies.

CONCLUSION

E. axillare extract inhibits the metastasis of cancer and downregulate several proteins involved in epithelial mesenchymal transition. The extract also upregulates PTEN, that inhibits metastasis of cancer. Thus identifying the bioactive constituent(s) of the extract and formulating it into a drug will increase the efficiency and reduce the toxic side effects of breast cancer treatments.

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