ORIGINAL ARTICLE

Stimulation of Apoptosis in Michigan Cancer Foundation-7 Human Breast Cancer Cell Line by Whole Plant Ethanolic Extract of *Enicostemma axillare* through Extrinsic and Intrinsic Pathway

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Abstract

Introduction: With the increasing level of resistance to chemotherapy and radiotherapy and the associated toxicity of these therapies, the uses of plant-based compounds are gaining importance. This study was done to prove the apoptotic potential of *Enicostemma axillare* in breast cancer cells. **Materials and Methods:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was performed involving the treatment of Michigan Cancer Foundation-7 cells for 24 h with ethanolic extract of *E. axillare* (EEEA) and doxorubicin to assess the cell viability, lactate dehydrogenase (LDH) was done to assess cytotoxicity, ethidium bromide (EtBr) and acridine orange (AO) assay was done to assess nuclear morphology of apoptotic cells and the expression of proteins associated with apoptosis – Bad, Bcl2, Bax, CytoC, Caspase 3, 8, and 9 were analyzed by western blotting. The data are expressed as mean±SEM. **Results and Discussion:** IC_{50} value for EEEA is 12.5 µg/ml, whereas for doxorubicin is 1 µg/ml. A significant increased level of LDH release is seen in treated groups. EEEA and doxorubicin decrease cell viability. In AO/EtBr staining, the live cells of the EEEA treatment were similar to that of the doxorubicin. Cells stained green represent viable cells, whereas bright red staining represents late apoptotic cells. EEEA down-regulated the expression of Bcl2 (antiapoptotic protein) and up-regulated the expression of Bad and Bax (proapoptotic proteins) in MCF-7 cell line compared to control cells. In this study, EEEA treatment significantly increased the protein expression of caspase-3, 8, and 9 compared to control. **Conclusions:** Our finding showed that EEEA induces extrinsic and intrinsic pathway mediated apoptosis. Thus, *E. axillare* raises new hope for its use in breast cancer therapy.

Key words: Apoptosis, cytochrome C, doxorubicin, *Enicostemma axillare*

INTRODUCTION

Breast carcinoma is the most common malignancy diagnosed in women and the incidence rate is escalating alarmingly.[1,2] It is one of the frequent causes of death in underdeveloped countries and the second leading cause of cancer death in developed countries after lung cancer. In the US, 268,600 new breast cancer cases were diagnosed and the death rate is 42,260 in 2019.[3] Thus, research in this field is highly pertinent to overcome both economical and psychological burden of the disorder.[4] Breast cancer patients have shown higher resistance and increased vulnerability to chemotherapeutic toxicity.[5] Induction of apoptosis is the main criterion in cancer treatment as it destroys cells without causing inflammation.[6] Many studies established that apoptosis is induced by various stresses such as oxidative stress, endoplasmic reticulum stress, or chromatin damage.[7] Oxidative stress

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created by the excessive generation of reactive oxygen species plays a critical role in the induction of apoptosis.[8]

Due to faster setback of various diseases and severe side effects, most of the surgical resection, radiation therapy, and conventional chemotherapy have limited treatment options for breast cancer. Hence, there is a growing need and incessant urge to find new chemopreventive agents with minimum or no side effects that may be effective in preventing and/or managing breast cancer and that also improve the quality of life in patients. Alternative therapeutic methods which are less toxic, eco-friendly, and inexpensive are widely being investigated.[9] Herbal medicine is one of the best approaches to prevent, manage, and treat cancer that focuses on the naturally occurring chemical substances for improving health conditions, with fewer side effects in comparison with chemotherapy. Antiproliferation property is efficient by many herbs.[6] Many studies reveal that the possible mechanism underlying the anticancer effects of herbal medicine,[7] inducing apoptosis of cancer cells.[9]

About 80,000 species of plants are utilized for treating various diseases in different systems of Indian medicine. Recent studies have shown that bioactive peptides extracted from natural products usually possess low toxicity and anticancer activity.[10] As per the World Health Organization (WHO) report, 80% of the world population presently uses herbal medicine for some aspect of primary health care.[11] The previous study shows the anti-migratory effect of *Enicostemma axillare* on Michigan Cancer Foundation-7 (MCF-7) cell line.[12] *E. axillare* (synonym – *Enicostemma littorale*) is a perennial herb. It has been used traditionally for many diseases.[13]

The medicinal plant used in the present study was *E. axillare* belongs to the family *Gentianaceae*. It is also called as Vellarugu in Tamil, Chota chirayata in Hindi and Whitehead in English. It grows up to 1.5 feet height and it is seen near the sea in India. In the Indian system of medicine, it has been used to treat various skin diseases, helminthiasis,[14] and tumors.[15] The anticancer potential of the plant has been examined in rodent and cellular models.[16] The methanolic extract has been proven to possess a significant inhibitory effect on the growth of cancer cells.[17] Doxorubicin reduces breast cancer cell viability and stimulates apoptosis;[18] thus, it is used as a standard drug in this study. In the present study, we demonstrated the effect of ethanolic extract of *E. axillare* (EEEA) on cell viability and cytotoxicity against MCF-7 cell line and we further investigate the effect of EEEA on the apoptosis of MCF-7 breast cancer cell line through extrinsic and intrinsic apoptotic signaling pathway.

MATERIALS AND METHODS

Plant material and extract preparation

The plant materials were collected from July 2016 to September 2016 from Chengalpattu, Kanchipuram District, Tamil Nadu, India, and authenticated by Prof. P. Jayaraman, Institute of Herbal Science – Plant Anatomy Research Centre, Tambaram, Chennai.

The whole plant materials were shade dried and powdered. Two kilograms of dry powders were taken in individual aspirator bottles; 6 L of ethanol was used, and the mixture was shaken occasionally for 72 h. Then, the extract was filtered. This procedure was repeated 3 times and all extracts were decanted and pooled. The extracts were filtered before drying using Whatman filter paper no.2 on a Buchner funnel and the solvent was removed by vacuum distillation in a rotary evaporator at 40°C; the extracts were placed in pre-weighed flasks before drying. The concentrated extracts were used for further studies.

Cell line culture

MCF7 cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, Maharashtra, India. The cells were grown in T25 flask containing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal 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% confluence, the cells were trypsinized and passaged.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay

MCF7 cell viability was assessed by the MTT assay, cells (1×10^4/well) were plated in 96-well plates with DMEM medium. This was incubated at 5% CO_2 incubator for 24 h. Then, the cells were exposed to various concentrations of the EEEA 5–17.5 µg/ml and doxorubicin 0.5–1.5 µg/ml for 24 h at 5% CO_2 incubator. At the end of the treatment, 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated at 37°C in 5% CO_2 incubator for 1 h. Then, the formazan crystals formed were dissolved in 100 µl of dimethyl sulfoxide and incubated in the dark for 1 h. The intensity of the color developed was measured by a micro ELISA plate reader at 570 nm. Cell viability in the control medium was represented as 100%. The cell viability was calculated using the formula: % cell viability = (A570 of treated cells/A570 of control cells) × 100.

Cytotoxicity assay

The growth inhibitive effect of EEEA was further investigated through measuring lactate dehydrogenase (LDH) leakage into the culture medium. LDH is a stable cytosolic enzyme that is released in the culture medium upon cell lysis and the released LDH is measured using standard kits in Beckman Coulter AutoAnalyzer. LDH

catalyzes the oxidation of lactate to pyruvate, coupled with the reduction of NAD^+ to NADH. The increase of NADH is measured at 340nm and is directly proportional to the enzyme activity in the sample.

The treatment protocol was mentioned for MTT assay and the conditioned medium alone was taken for LDH leakage assay. Doxorubicin was used as the standard drug.

Apoptosis assay – acridine orange (AO) and ethidium bromide (EtBr) staining

The effect of EEEA in MCF-7 cell death was also determined by AO/EtBr dual staining. The Group I treated was considered as negative control and Group III was considered as positive control. Group II involved the compound of our interest and its activity was compared with Group I and III. The cells were treated with EEEA (12.5 µg) and doxorubicin (1 µg) for 24 h and the cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). The pellets were resuspended in 5 µl of acridine orange (1 mg/ml) and 5 µl of ethidium bromide (1 mg/ml). The cells were incubated at 37°C in 5% CO_2 incubator for 30 min. The stained cells were washed by 200 µl of warm PBS. The apoptotic changes of the stained cells were observed under a fluorescence microscope.

Western blot analysis

MCF-7 cell lines were treated with DMSO (0.01%), EEEA (12.5 µg/mL), and doxorubicin (1 µg/mL). After the treatment period of 24 h, the cells were lysed in radioimmunoprecipitation assay buffer containing ×1 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 membranes. The membranes were incubated with primary antibodies against Bcl2, Bad, Bax, Cyto C, caspase-3, 8, and 9 in Tris-buffered saline at 4°C overnight. The company, catalog number, and concentration of antibodies are mentioned in Table 1. β-actin served as an internal control. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies procured from Genei Bangalore, India and bands were visualized 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 comparisons between treatment groups were made using Tukey HSD *post-hoc* tests of Statistical Package for the Social Sciences (SPSS) software version 21.0. $P < 0.05$ was considered as statistically significant.

Table 1: The details of antibodies used for Western blot analysis

Target protein	Catalog No. of antibody	Company name	Antibody concentration
Bcl2	2872	Cell signalling	1:2000
Bad	9292	Cell signalling	1:2000
Bax	2774	Cell signalling	1:2000
Cyto C	1940	Cell signalling	1:2000
Caspase-3	9662	Cell signalling	1:2000
Caspase-8	4790	Cell signalling	1:2000
Caspase-9	sc-56076	Santa Cruz Biotechnology	1:1000
β-actin	sc-8432	Santa Cruz Biotechnology	1:1000

RESULTS

Effect of EEEA on the viability of MCF-7 cells

The effect of EEEA on MTT assay for cancer cells was studied in comparison with control and doxorubicin. In this study, cell viability was significantly less in EEEA treated group in comparison with control group. The rate of viability by EEEA was on par with the positive control, doxorubicin [Figure 1]. EEEA and doxorubicin decrease cell viability. The half-maximal inhibitory concentration (IC_{50}) was determined graphically. IC_{50} value for EEEA was 12.5 µg/ml, whereas for doxorubicin was 1 µg/ml. Therefore, this concentration was used further for MCF-7 cells in this study.

Cytotoxicity assay

The permeability and integrity of the cell membrane of MCF-7 cell lines after treatment with EEEA or doxorubicin were determined by LDH release assay. As shown in Figure 1, the group exposed to EEEA or doxorubicin alone presented a significant increase in LDH release when compared to the control.

Effect of EEEA on apoptosis by EtBr and AO staining

The breast cancer cell death induced by EEEA was further investigated using the AO/EtBr dual staining. AO/EtBr was used in evaluating the nuclear morphology of apoptotic cells. Acridine orange is a vital dye that will stain both live and dead cells, whereas EtBr will stain only those cells that have lost their membrane integrity. Cells stained green represent viable cells, whereas bright red staining represents late apoptotic cells. In control, uniformly green live cells with normal and large nucleus were observed, whereas, in EEEA and doxorubicin treated cells, bright red staining was observed

[Figure 2]. These results confirmed that EEEA significantly induced apoptosis in breast cancer cells.

Effect of EEEA on the expression of Bcl-2 family members

To examine the status of the intracellular signaling molecules responsible for the growth-inhibiting activity of EEEA in MCF-7 cells, western blot analysis was performed for Bcl-2 family members. The Bcl-2 protein family consists of both proapoptotic (Bax and Bad) and antiapoptotic (Bcl-2) proteins that regulate mitochondrial outer membrane integrity, cytochrome c release, and caspase activation. Therefore, we measured the protein expression of Bcl-2 family proteins by western blot analysis. EEEA significantly up-regulated the expression of proapoptotic proteins – Bax and Bad and

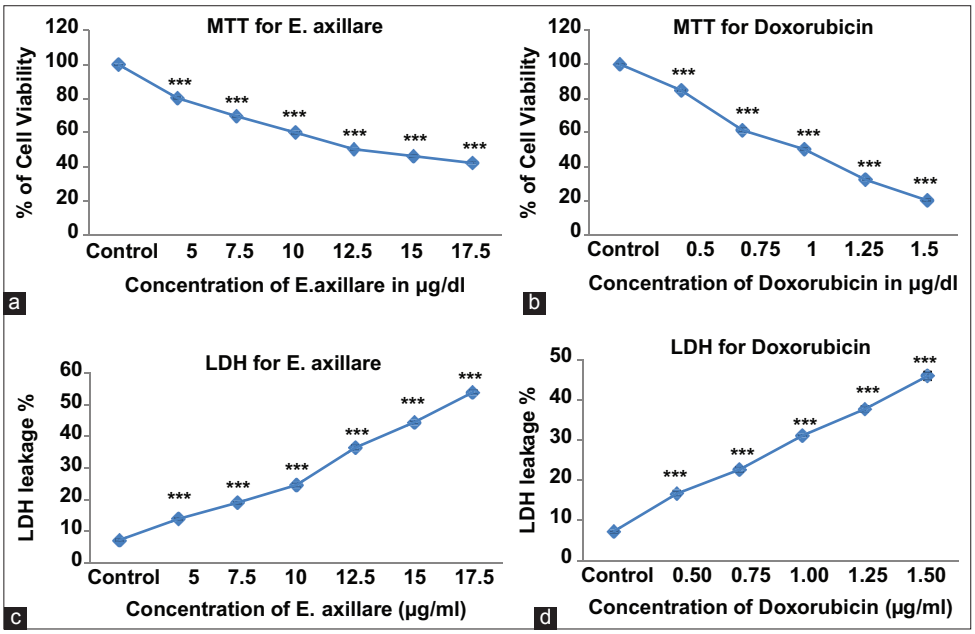

Figure 1: Effect of ethanolic extract of *Enicostemma axillare* (EEEA) on the viability of Michigan Cancer Foundation-7 (MCF-7) cell line. Cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and were incubated with indicated concentration of EEEA and doxorubicin for 24 h. (a) EEEA and (b) doxorubicin inhibits the growth of cells compared to control. The cytotoxic effect of (c) EEEA and (d) doxorubicin on MCF-7 was determined by lactate dehydrogenase assay. Each bar represents the observations. The mean mean±SEM of three independent difference is significant at the 0.05 level ($P < 0.001$ =***, <0.01 =**, <0.05 = *)

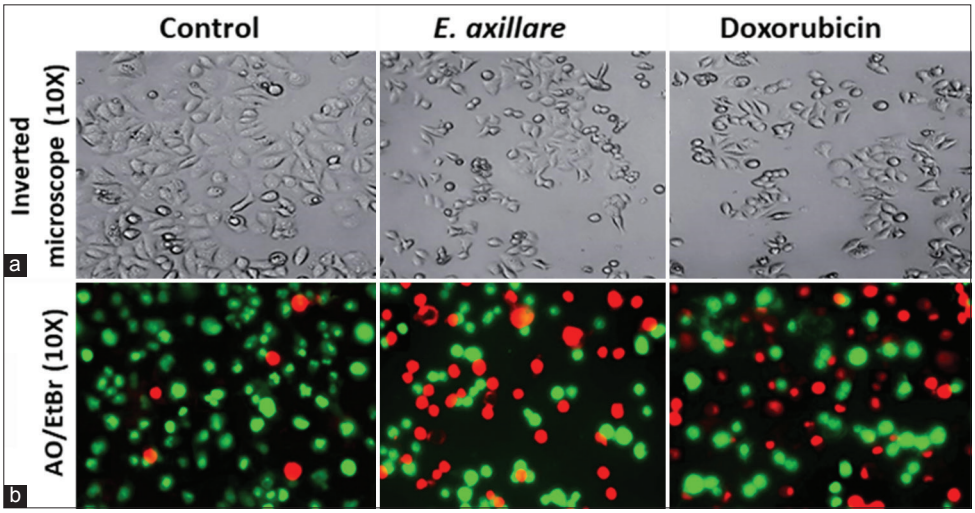

Figure 2: Effect of ethanolic extract of *Enicostemma axillare* (EEEA) on nuclear morphology in Michigan Cancer Foundation-7 (MCF-7) cell line. (a) Cells were treated with 12.5 µg of EEEA and 1 µg doxorubicin as a standard control for 24 h and cells were observed under an inverted microscope (×10). The number of cells decreased after EEEA treatment. (b) AO/EtBr staining of MCF-7 cells, viewed under a fluorescence microscope (×10), the viable cells possess bright green, apoptotic cells have a bright red nucleus

down-regulated the expression of antiapoptotic protein – Bcl-2 in MCF-7 cell line compared to control cells [Figure 3]. These results suggested that Bcl-2 family members play a major role in EEEA-induced apoptosis in breast cancer cells.

Effect of EEEA on the expression of cytochrome c and caspases

This was done to analyze the involvement of mitochondrial release of cytochrome c in breast cancer cells, Treatment of MCF-7 cells with EEEA for 24 h resulted in an increase of cytochrome c levels. These results indicated that EEEA can provoke cytochrome c release from mitochondria into the cytosol [Figure 4], thus supporting that EEEA-induced apoptosis in breast cancer cells involves signaling at the mitochondrial level. To determine whether caspases are involved in EEEA-induced apoptosis, we examined the protein expression of caspases by western blot because the activation of caspases is crucial for mitochondrial-dependent and independent apoptotic pathways. EEEA significantly increased caspase-9 (P = 0.019) and caspase-3 (P = 0.002) expressions in breast cancer cells.

EEEA regulates the expression of extrinsic signaling molecule (Caspase-8)

Extrinsic pathway is mediated by FasL and TRAIL. These ligands activate the death receptor and further activate the downstream signaling molecules like caspase-8, which leads to the induction of apoptosis. We examined the significantly increased level of caspase-8 ($P < 0.001$) protein expression in EEEA treated MCF-7 cells [Figure 4].

DISCUSSION

In this study, the effects of EEEA on the growth of MCF-7 human breast cancer cell lines were studied. Methanolic extract of *E. axillare* showed significant growth inhibitory effect on HeLa cell line, it was found that the percentage of growth inhibition increases with the increasing concentration of the whole plant methanolic extract and the IC_{50} value was 340 µg/ml by MTT assay in the HeLa cell line.[17] This may be due to crude extract or methanol extract on HeLa cell line, which was used for the MTT assay. However, in our study, we

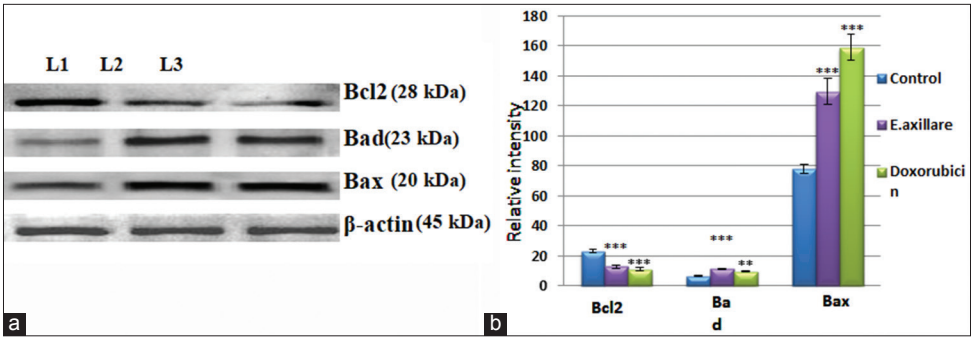

Figure 3: Effect of ethanolic extract of Enicostemma axillare (EEEA) on the protein expression of apoptotic signaling molecules in Michigan Cancer Foundation-7 cells. Cells were treated with 12.5 µg of EEEA and 1 µg doxorubicin as a standard control for 24 h. Protein expression was studied by western blotting, β-actin was used as an internal control. "*" Represents statically significance between control versus drug treatment groups using one-way ANOVA. The mean difference is significant at the 0.05 level (P< 0.001=***, <0.01=**, <0.05=*). L1-Control, L2-EEEA, L3-Doxorubicin

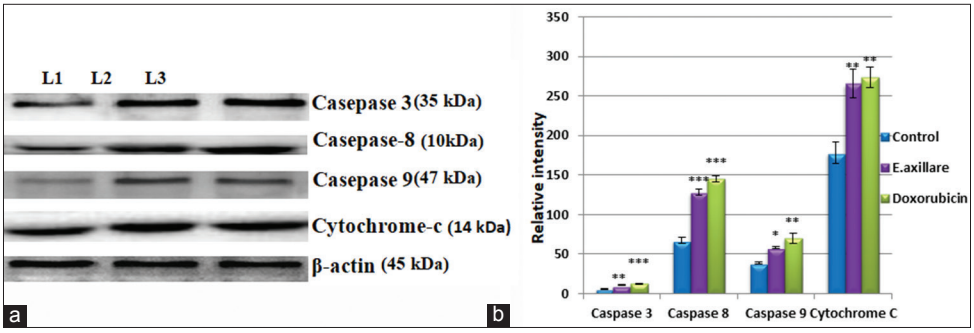

Figure 4: Effect of ethanolic extract of *Enicostemma axillare* (EEEA) on the protein expression of extrinsic and intrinsic apoptotic signaling molecules in Michigan Cancer Foundation-7 cells. Cells were treated with 12.5 µg of EEEA and 1 µg doxorubicin as a standard control for 24 h. Protein expression was studied by western blotting, β-actin was used as an internal control. "*" Represents statically significance between control versus drug treatment groups using one-way ANOVA. The mean difference is significant at the 0.05 level (P < 0.001=***, <0.01=**, <0.05=*). L1-Control, L2-EEEA, L3-Doxorubicin

observed that EEEA significantly inhibited cell proliferation with IC_{50} value of 12.5 µg/ml in MCF-7 cell line.

Further LDH assay was done to investigate the cytotoxic effect of EEEA on MCF-7 cells. LDH is a stable cytosolic enzyme released upon membrane damage in necrotic cells. LDH released into the medium provides an index of cell death and membrane permeability and an increase in LDH activity in the medium occurs as a result of cell membrane fragmentation and enzyme leakage.[19] In the previous report on LDH analysis, the highest doses (150, 200, and 400 µg/mL) of GYZ resulted increases in the LDH levels compared with the controls.[20] In this study, we observed that the LDH level was significantly increased with the increase concentration of EEEA when compared with control. Thus, it is clear that EEEA has good cytotoxic effect on MCF-7 cell line.

We investigated the EEEA-induced apoptosis using AO/EtBr dual staining in MCF-7 cells. After 24 h treatment, the nuclear structure was examined under a fluorescent microscope. Acridine orange penetrates both live and dead cells, while EtBr penetrates only dead cells and stains them red by connecting to their DNAs. In this method, apoptotic cells are stained bright red, due to the infiltration of both AO and EtBr into the cells.[21] Similarly, in this study, we observed that the apoptotic cells were stained bright red, whereas the viable cells were stained green color. These results confirmed that EEEA significantly induced apoptosis in breast cancer cells.

Further, we investigated the protein expression of Bcl2 family members by western blot. Bad, Bax, and Bcl2 activate Cytochrome C which leads to up-regulation of apoptosis. Apoptosis is a complex process regulated by a variety of factors.[22,23] The Bcl-2 family consists of proapoptotic and antiapoptotic members. As an antiapoptotic protein, Bcl-2 confers a negative control in the pathway of cellular suicide machinery, whereas Bax and Bad (proapoptotic) protein promote cell death by competing with Bcl-2. With the Bax:Bcl2 ratio of 1.6:0.5 for EEEA which is close to the standard drug doxorubicin and DMSO control. Thus, it is clear that EEEA induces apoptosis through the intrinsic pathway. Apoptosis-related proteins were found to contribute to the inhibitory effect of acetylshikonin on SGC-7901 cells were studied, the relative Bcl-2 and Bax expression levels induced by acetylshikonin.[24]

The caspase family consists of cysteine-rich proteins, which are divided into initiator caspases (caspases 8 and 9) and executor caspases (caspases 3, 6, and 7). Caspases are activated by two major pathways. One involves the release of cytochrome C from the mitochondria to the cytoplasm, which leads to the activation of caspase-9, it is known as the intrinsic pathway of apoptosis. The other pathway involves the death receptors, caspase-8 gets activated in this pathway, it is known as the extrinsic pathway of apoptosis.[25] In apoptosis, apoptotic Bcl-2 proteins such as Bax and Bak created pores in the mitochondrial outer membrane, through which cytochrome c is released to the cytosol. Binding of cytochrome c to Apaf-1 creates an apoptosome complex that activates caspase-9, which in turn activates caspase-3.[26,27] Studies have been done to assess the levels of cleaved Caspase 3 and 9 in response to epoxyazadiradione by western blot. The data revealed that epoxyazadiradione-induced cleaved Caspase 9 and 3 in a dose-dependent manner.[28] Thus, it was of interest to precise the interaction between caspase-3 and caspase-9. Indeed, the ability of caspase-3 to interact with caspase-9 might, at least in part, contribute to explain its antiapoptotic activity. We observed that caspase-3 expressed in MCF-7 cells interacted with active caspase-9 when these cells were exposed to apoptotic stimuli. Caspases play a pivotal role in apoptosis; it possesses inactive zymogens, in response to apoptotic signals, the inactive zymogens are cleaved to yield active forms of the proteins that are associated with the induction of apoptosis.[29] In the present study, EEEA treatment significantly increased the protein expression of cytochrome c, caspase-8, -9, and -3. It is proved that EEEA induces activation of caspase-3 by the initiation of both the intrinsic and extrinsic apoptotic signaling pathways; thus, EEEA induces intrinsic and extrinsic pathway mediated apoptosis.

This study demonstrates that the EEEA shows good activity in apoptosis with IC_{50} value of 12.5 µg/ml and also it has a great cytotoxic effect in MCF-7 cells. EEEA up-regulates the proapoptotic proteins and down-regulates antiapoptotic, EEEA treatment significantly increased the protein expression of caspase-8, 9, and 3; thus, EEEA induces extrinsic and intrinsic pathway mediated apoptosis. EEEA suppressed the proliferation of MCF-7 breast cancer cells. Our findings suggest that EEEA may be a potential therapeutic agent for the management of breast cancer in the future. Further study on the molecular mechanism is needed. Thus, the present study proves that *E. axillare* induces apoptosis in breast cancer cells.

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AUTHOR'S CONTRIBUTIONS

1. First author – Kamalam Ravi – Principle investigator of the study, this is part of my PhD work, PhD was registered in Saveetha Medical College and presently working in Sri Ramachandra Medical College and RI
2. Second and Corresponding author – Krishnamoorthy Gunasekaran – Guide to the first author and I am working in Rajas Dental College and Hospital

3. Third author – Vijayaraghavan Rajagopalan – He is the co-guide to the first author and he is working in Saveetha Medical College
4. Fourth author – Santhi Silambanan – She is working in Sri Ramachandra Medical College and RI, she helped to the first author in this study.

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