Full Length Research Article

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Research Journal of Agricultural Sciences
Volume 15; Issue 02 (Mar–Apr 2024); pp 420–424

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

In-vitro Anticancer Activity of Ethanolic Extract of *Allium fistulosum* against Human Breast Adenocarcinoma Cell Line (MCF-7)

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Received: 04 Jan 2024; Revised accepted: 13 Mar 2024; Published online: 27 Mar 2024

Abstract

Plant-derived compounds, known for their safety and non-toxic attributes, are increasingly sought after in the pursuit of effective anticancer agents. This study aimed to elucidate the *in-vitro* anticancer potential of ethanol extract from *Allium fistulosum* against the human breast adenocarcinoma cell line (MCF-7) by inducing apoptosis. The cytotoxicity against MCF-7 was evaluated using the MTT assay. The apoptotic effect of the ethanol extract was characterized through mitochondrial membrane potential (MMP) analysis, reactive oxygen species (ROS) assay, and Acridine Orange-Ethidium bromide staining method. The MTT assay revealed concentration-dependent cytotoxicity, with IC_{50} values measured at 45.85 µg/ml, while demonstrating minimal toxicity against normal endothelial cells. Furthermore, treatment with the ethanol extract induced a reduction in MMP and an increase in ROS levels within breast cancer cells. These findings suggest that the ethanol extract may possess potent anticancer and apoptogenic activities by inducing mitochondrial dysfunction and oxidative stress damage in breast cancer cells.

***Key words*:** *Allium fistulosum*, MCF-7, Mitochondrial membrane potential, Reactive oxygen species, MTT assay, Acridine Orange-Ethidium bromide staining method

Breast cancer is a growing global health issue, constituting 1 out of 8 cancer diagnoses and contributing to a cumulative total of 2.3 million newly reported cases across both genders [1]. Conventional cancer therapies encompass surgical procedures, radiotherapy, and chemotherapy, providing temporary alleviation, prolongation of life, and intermittent complete recovery [2-3]. Despite ongoing initiatives to enhance awareness, facilitate early diagnosis, and introduce innovative interventions, persistent challenges arise from drug resistance, rising expenses, and increased toxicity linked to anticancer medications [4-5]. Natural products have been fundamental to healthcare for a long time. Those derived from plants have gained significant attention compared to synthetic medicines because of their safety and non-toxic nature [6]. Ongoing extensive research on natural compounds, with a focus on traditional medicinal plants, has unveiled their potent therapeutic properties. They show promise in preventing chronic diseases, such as cardiovascular conditions, neurodegenerative disorders, and certain types of cancers. The exploration of these plant-derived compounds has revealed potential benefits in mitigating risk factors associated with chronic illnesses, offering new avenues for preventive healthcare strategies [7]. In the ongoing efforts to advance treatment strategies for breast cancer, plants stand out as promising candidates for effective anticancer agents. However, a pressing requirement exists for the development of novel anticancer agents that demonstrate both high efficacy and specificity while simultaneously minimizing adverse outcomes [8]. While plant-derived compounds hold great promise as anticancer agents for breast cancer treatment, addressing challenges such as standardization, bioavailability, and clinical validation is essential for their successful development and integration into clinical practice.

Welsh onion (*Allium fistulosum* L.), a member of the Alliaceae family, holds significance as a culinary ingredient and traditional medicine, with widespread cultivation in China, Japan, and Korea. It is a key component in Asian dishes, valued not only for its flavor but also as a nutrient-rich traditional remedy. The active compounds within *A. fistulosum* contribute to its varied biological activities [9], with some exploration into antioxidant [10], antibacterial [11], lipid-lowering, anti-hypertensive, and cardiovascular disease prevention effects [12]. The anticancer effects of these compounds have been well- established in previous studies [13-16]. In this study, we aimed to evaluate the anticancer effect of ethanol extract against human breast adenocarcinoma cell line (MCF-7) using the cell viability assay. We also performed mitochondrial membrane potential analysis (MMP) and a reactive oxygen species (ROS) assay to gain mechanistic insights into the damage induced by *A. fistulosum* extract in breast cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

DMEM medium, Fetal Bovine Serum (FBS), Total reactive oxygen species (ROS) kit, Invitrogen (USA), 1X PBS

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Citation: Vetrikodi N, Jeyanthi KA. 2024. In vitro anticancer activity of ethanolic extract of *Allium fistulosum* against human breast adenocarcinoma cell line (MCF-7). *Res. Jr. Agril. Sci*. 15(2): 420-424.

was from Himedia, (India). DMSO (Dimethyl sulfoxide), MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml) were from Sigma (USA), Mitochondrial membrane potential assay kit was from Sigma, (USA), 96 well tissue culture plate and wash beaker were from Tarson (India), Fluorescent Imaging System, (ZOE, Bio-Rad, USA). Penicillin/Streptomycin antibiotic solution, Trypsin-EDTA was purchased from Gibco (USA), EtBr, and Acridine orange was purchased from Sigma Aldrich (USA).

Sample extraction

The stems of Welsh onion were cut, frozen at 80°C, lyophilized, and then reduced to a powdered form. The samples were extracted as follows: 1 g of lyophilized powder was stirred in nine mL of aqueous ethanol (70%) and sonicated for 1 h. The mixtures were filtered, and the supernatants were concentrated under reduced pressure. The extracts were then lyophilized and stored at 20°C for further analyses.

Cell lines

MCF-7 (Human breast cancer cells) cell line was purchased from NCCS, Pune, and was cultured in a liquid medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin, and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37°C.

Cell viability assay

The *in-vitro* cytotoxicity of the test sample was assessed using MCF-7 cells through the MTT assay [17]. Cultured MCF-7 cells were trypsinized, pooled, and plated at a density of 1×10^5 cells/ml in a 96-well tissue culture plate with DMEM medium containing 10% FBS and 1% antibiotic solution. After 24-48 hours of incubation at 37°C, the wells were washed with sterile PBS and exposed to varying concentrations of the test sample in serum-free DMEM medium. Each sample was replicated three times, and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 hours. Following incubation, MTT solution (10 µL of 5 mg/ml) was added to each well, and the cells were incubated for an additional 2-4 hours until purple precipitates were visible under an inverted microscope. Subsequently, the medium containing MTT (220 µL) was aspirated, and the wells were washed with 1X PBS (200 µL). To dissolve formazan crystals, DMSO (100 µL) was added, and the plate was shaken for 5 minutes. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA). The percentage cell viability and IC50 value were calculated using Graph Pad Prism 6.0 software (USA) with the formula:

$$\text{Cell viability (\%)} = (\text{Test OD}/\text{Control OD}) \times 100.$$

Mitochondrial membrane potential analysis

The MCF-7 cells (20000–50000 cells/well) were seeded into a 24-well plate and incubated for 24 hours in DMEM growth medium. Subsequently, the plate was rinsed with PBS and exposed to 45.85 µg/ml of *A. fistulosum* ethanol extract in serum-free DMEM medium. Following treatment, the plate was placed in a humidified 5% CO_2 incubator at 37°C for another 24 hours. MMP for both treated and control cells was conducted following the manufacturer's guidelines. Briefly, cells were treated with 100 µl/well of JC-10 dye loading solution, and the plate was shielded from light during incubation for 30–60 minutes at 37°C in a 5% CO_2 environment. Afterward, 100 µl/well of assay buffer B was added, and the plate was centrifuged at 800 rpm for 2 minutes. Fluorescence was then assessed using a Fluorescent Imaging System (ZOE, Bio-Rad, USA) [18].

Reactive oxygen species (ROS) assay

In brief, cultured MCF-7 cells were harvested through trypsinization and consolidated in a 15 ml tube. Subsequently, the cells were seeded at a density of 1×10^6 cells/ml into a 24-well tissue culture plate with DMEM medium containing 10% FBS and 1% antibiotic solution and were incubated for 24 hours at 37°C. The wells were then washed and treated with 45.85 µg/ml of ethanol extract in serum-free DMEM medium, followed by an additional incubation at 37°C for 24 hours. After this incubation period, 1 ml of ROS assay buffer was introduced, and 100 µl of 1X ROS assay staining solution was added to the wells, gently mixed. The plate was incubated for 60 minutes in a 37°C incubator with 5% CO_2. Following the incubation, the immediate evaluation of ROS production was performed using a fluorescence imaging system (ZOE, BIO-RAD) [19].

EtBr/AO staining

Briefly, 5×10^5 cells/ml of MCF-7 cells were seeded into a 96-well tissue culture plate and incubated for 24 hours in DMEM growth medium. Following incubation, the cells were exposed to 45.85 µg/ml of the *Allium fistulosum* sample in serum-free DMEM medium. The plate was then placed in a 37°C, 5% CO_2 incubator for an additional 24 hours. After this incubation period, 10 µl of 1 mg/ml acridine orange and ethidium bromide were added to the wells and gently mixed. Subsequently, the plate was centrifuged at 800 rpm for 2 minutes and promptly evaluated within an hour. The assessment involved the examination of at least 100 cells using a Fluorescent Imaging System (ZOE, Bio-Rad, USA) [20].

Statistical analysis

Results were analyzed by version 7 of GraphPad Prism, using one-way analysis of variance (ANOVA), and differences were considered statistically significant at the level of p-values 0.05.

RESULTS AND DISCUSSION

Assessments of cytotoxicity of Allium fistulosum extracts by MTT assay

The response of cytotoxic effects of ethanol extracts of *Allium fistulosum* against MCF- 7 cells are given in (Fig 1). Following the concentrations increase from 10 to 300 µg/mL of various ethanol extracts of *Allium fistulosum*, the cell viability of MCF-7 cells displayed a gradient response, increasing from 53.33% to 89.63%. Notably, at concentrations of 10, 100, and 500 µg/mL, the cell viability rates were recorded as 89.63%, 63.74%, and 53.33%, respectively shown in (Fig 2). Upon treatment with *Allium fistulosum* extract, MCF-7 cells demonstrated a substantial reduction in viability, yielding an IC_{50} value of 45.85 µg/mL after 24 hours of exposure. Ethanol extract of Allium fistulosum exhibits cytotoxic effects against MCF-7 breast cancer cells in a concentration-dependent manner.

Morphological changes induced by exposure of ethanol extract of Allium fistulosum

In this study, we examined the morphological alterations elicited in MCF-7 cells following exposure to different concentrations of ethanol extract derived from *Allium fistulosum*. Notably, the treated cells exhibited classical apoptotic features, including cellular rounding, shrinkage, membrane blebbing, and detachment from the substratum. Additionally, distinct changes in nuclear morphology were evident, characterized by nuclei that appeared condensed and

vacuolated, further indicating the activation of apoptotic pathways. Moreover, observations revealed instances of loss of contact with neighboring cells, detachment from the substratum, and cellular clumping, collectively underscoring the profound impact of the *Allium fistulosum* extract on MCF-7 cell morphology.

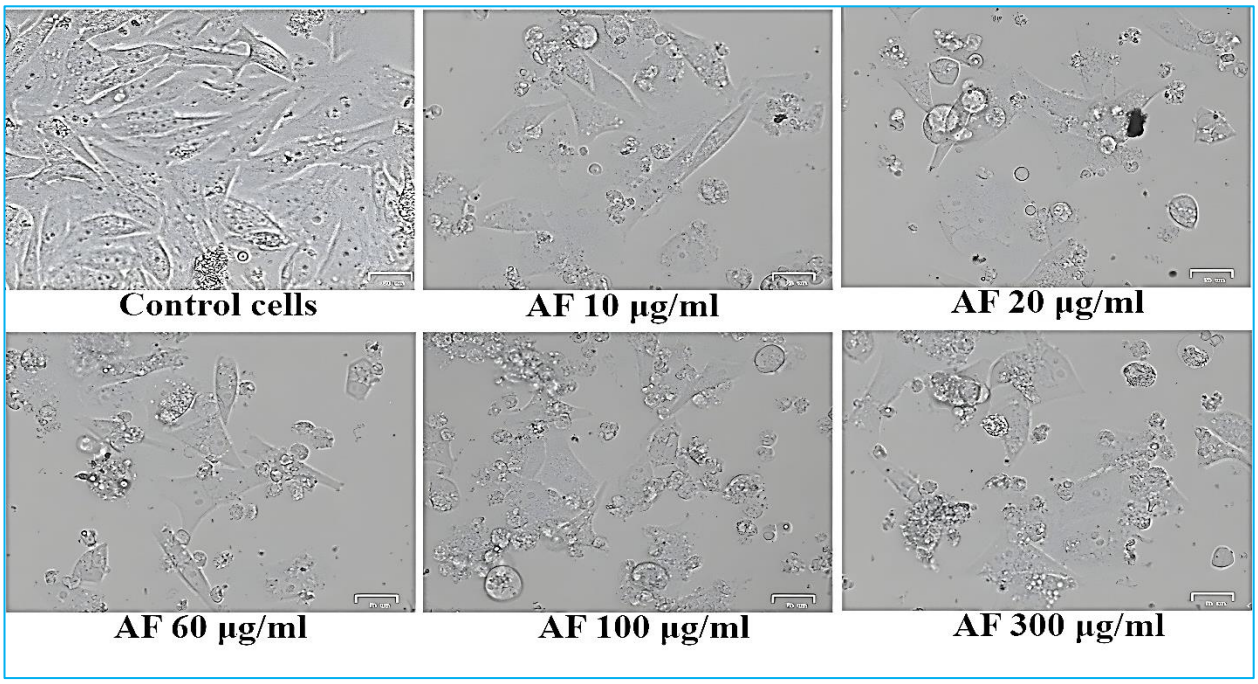

Fig 1 Cytotoxic potential of *A. fistulosum* extract on MCF-7 cells measured by MTT assay. Cells were exposed to different concentrations (10–300 µg/mL) of ethanol extract for 24 h. Results are expressed as the mean ± S.D. of three independent experiments

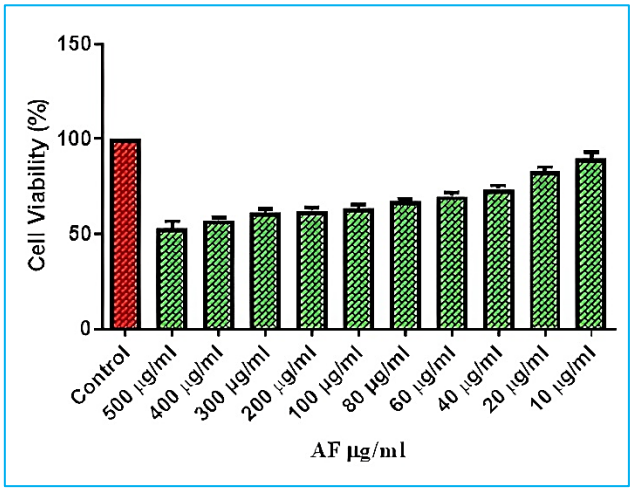

Fig 2 MTT assay on MCF-7 cells. The bar chart represents cell viability after treatment with various ethanol concentrations of *A. fistulosum* (AF) extract. Mean values of three independent experiments are plotted with SEM values

Assessment of oxidation stress using ROS assay

In order to elucidate the impact of the ethanol extract on the induction of oxidative stress in MCF-7 cells, the ROS Assay was conducted using DCFDA fluorogenic dye. This dye, upon oxidation, emits green fluorescence, thereby serving as a marker for oxidative stress. Under fluorescence microscopy, distinct observations were made regarding the response of MCF-7 cells to the ethanol extract treatment. The intensity of green fluorescence, indicative of ROS levels, exhibited a pronounced increase in MCF-7 cells exposed to the ethanol extract at a concentration of 45.85 µg/mL, as illustrated in (Fig 3). Notably, this increase in fluorescence intensity was notably higher in comparison to the untreated control group.

The fluorescence microscopy analysis provides evidence of the ethanol extract ability to induce oxidative stress within MCF-7 cells. This observation suggests a potential mechanism through which the ethanol extract may exert its cytotoxic effects, highlighting the significance of oxidative stress modulation in the context of anticancer activity.

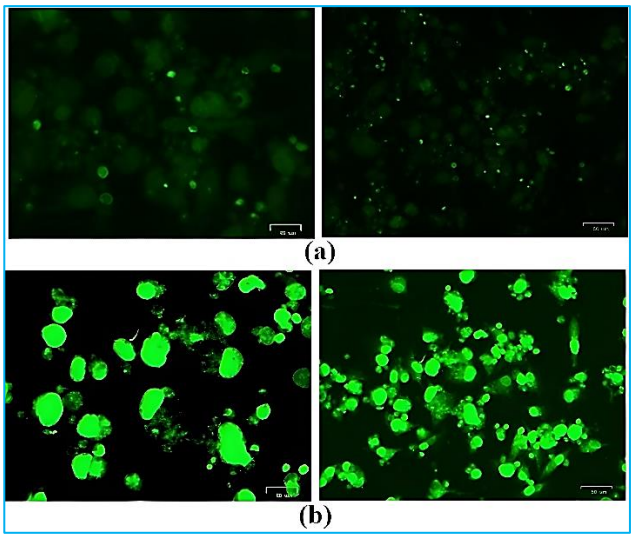

Fig 3 Fluorescence microscopy images of DCFDA stained MCF-7 cells (a) untreated (Control) and (b) Treated at 45.85 µg/mL. Intercellular ROS production in MCF-7 cells after the exposure of ethanol extract of *A. fistulosum*. Scale bar represents 25 µm and 50 µm

Detection of apoptosis by Mitochondrial Membrane potential assay

The assessment of apoptosis via the MMP assay revealed intricate dynamics within the MCF-7 cell line upon treatment with the ethanol extract of *A. fistulosum*. To delineate alterations in MMP levels, we employed JC-1 fluorescent dye, which selectively accumulates within mitochondria. In cells maintaining an intact mitochondrial membrane and high MMP,

JC-1 undergoes polymerization into J-aggregates, emitting a red fluorescence signal. Conversely, in instances of mitochondrial depolarization, JC-1 remains in its monomeric form, yielding a green fluorescence signal. The ratio of red to green fluorescence intensity served as a quantitative indicator of MMP levels (Fig 4).

Control MCF-7 cells exhibited vibrant orange-red fluorescence indicative of robust mitochondrial potential. In contrast, cells subjected to treatment with the *A. fistulosum* extract displayed a progressive decline in orange J-aggregate fluorescence, accompanied by the dispersion of green monomer fluorescence throughout the cytoplasm, signifying mitochondrial transmembrane depolarization. Notably, cells treated with the *A. fistulosum* extract at concentrations of 45.85 µg/mL, and in comparison, with 5-fluorouracil, exhibited heightened green fluorescence intensity relative to the control group. MMP loss is recognized as a pivotal mechanism in the pathway of cell demise. Our investigations revealed a significant reduction in MMP levels within MCF-7 cells subsequent to exposure to the ethanol extract (Fig 4). These findings strongly suggest that the ethanol extract induces cell death in MCF-7 cells through the attenuation of MMP levels, highlighting a critical aspect of the apoptotic cascade instigated by *A. fistulosum* extract.

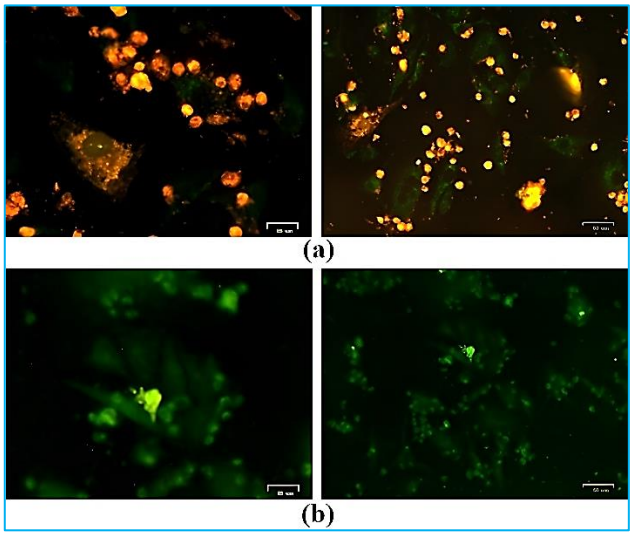

Fig 4 Fluorescence microscopy images of JC-1 stained MCF-7 cells. Evaluating the mitochondrial membrane potential of *A. fistulosum* ethanol extract induced apoptotic cells using JC-1 dye revealed the following observations: (a) In healthy MCF-7 cancerous cells, mitochondria exhibited high polarization, resulting in the accumulation of JC-1 dye within the mitochondrial matrix, forming conspicuous bright red fluorescent J-aggregates. Notably, the absence of green fluorescence indicated the lack of apoptosis. (b) Conversely, in more apoptotic cells, mitochondria showed high depolarization, causing the dispersion of JC-1 dye throughout the entire cell except within mitochondria. This led to a transition from negligible red fluorescence to intense green fluorescence, indicating a higher degree of apoptosis. Scale bar represents 25 µm and 50 µm

Detection of apoptosis by Acridine Orange-Ethidium bromide staining

The assessment of apoptosis through Acridine Orange-Ethidium bromide staining revealed distinct cellular responses following the incubation of MCF-7 cells with the ethanol extract at an IC_{50} concentration of 45.85 µg/ml for a duration of 24 hours. Quantitative analysis demonstrated that the treatment resulted in a diverse population of cells, with 3.05±0.35% identified as dead cells, 8.12±0.9% as necrotic cells, 34.01±3.79% as pro-apoptotic cells, and 54.82±4.88% as apoptotic cells (Fig 5). This distribution underscores the extract's ability to induce apoptosis within the MCF-7 cell population, highlighting its potential as an effective agent in cancer therapy.

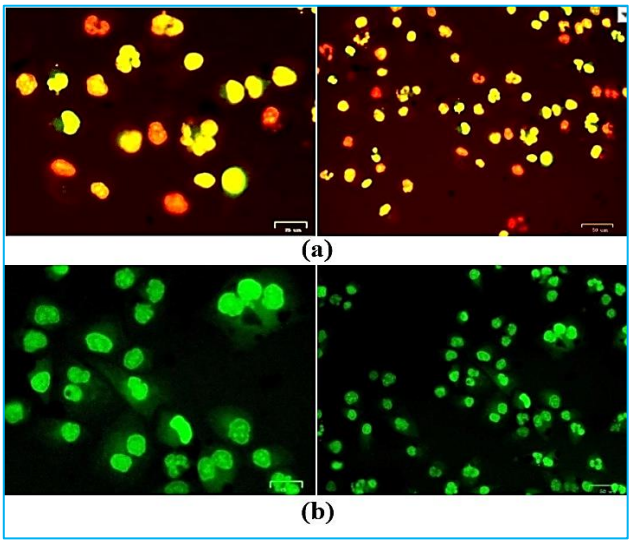

Fig 5 Fluorescence microscopy images of AO/EtBr stained MCF-7 cells (a) untreated (control) and treated at 45.85 µg/mL. Note that AO/EtBr images are recorded using excitation at 460 nm. Scale bar represents 25 µm and 50 µm

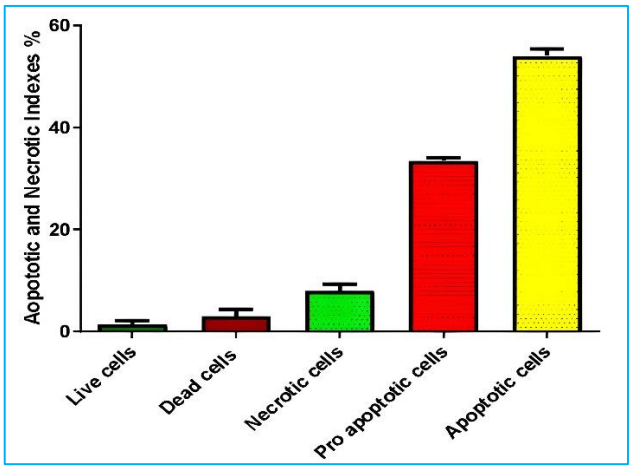

Fig 6 The bar graph represents the percentage of apoptotic and necrotic cells determined by Acridine Orange-Ethidium bromide staining. Value represents the mean ± SE

Cancer cells, renowned for their rapid proliferation and inherent resistance to programmed cell death, pose significant challenges in cancer treatment. Apoptosis, a tightly regulated process of cell self-destruction vital for normal tissue development and homeostasis, becomes dysregulated in cancer, fueling uncontrolled cell growth, tumor progression, and resistance to conventional therapies such as chemotherapy.

Assessing the potential of natural compounds in inducing apoptosis represents a promising avenue in cancer research. In this context, the ethanolic extract of *A. fistulosum* emerges as a subject of investigation for its anticancer properties against the human breast adenocarcinoma cell line (MCF-7). Employing acridine orange/ethidium bromide staining and MMP assay methods, the study delves into the extract's ability to trigger apoptosis within the MCF-7 cell population.

The findings reveal a noteworthy increase in intracellular levels of reactive oxygen species (ROS) upon treatment with

the *A. fistulosum* extract, suggesting its apoptotic potential. ROS accumulation, recognized for its role in apoptosis induction, manifests in the disruption of mitochondrial membrane integrity, further accentuating the apoptotic cascade. Remarkably, the ethanol extract of *A. fistulosum* demonstrates cytotoxic effects against MCF-7 cells, as evidenced by a significant reduction in cell viability with an IC_{50} value of 45.85 µg/mL after 24 hours of treatment. This study underscores the promising anticancer activity of *Allium fistulosum* extract, elucidating its mechanisms of action through ROS-mediated apoptosis induction and MMP modulation. The observed cytotoxicity against MCF- 7 cells underscores the extract's potential as a candidate for further exploration in cancer therapy.

CONCLUSION

In conclusion, the ethanolic extract of *Allium fistulosum* demonstrates significant potential as a novel therapeutic agent in the treatment of human breast adenocarcinoma (MCF-7). Through its ability to induce apoptosis via the generation of intracellular reactive oxygen species and the disruption of MMP, the extract exerts profound cytotoxic effects against MCF-7 cells. The findings from this study underscore the importance of exploring natural compounds for their anticancer properties and highlight *A. fistulosum* as a promising candidate for further preclinical and clinical investigations. Harnessing the apoptotic pathways targeted by *A. fistulosum* extract may pave the way for the development of more effective and selective anticancer therapies, ultimately im- proving outcomes for patients battling breast adenocarcinoma and other malignancies. Further elucidation of the molecular mechanisms underlying the extract's anticancer activity is warranted to optimize its therapeutic potential and facilitate its translation into clinical practice.

Conflict of interest

The authors have no conflicts of interest regarding this investigation.

Acknowledgments

The authors would like to thank Department of Biotechnology, Thanthai Hans Roever College (Autonomous) (Affiliated to Bharathidasan University), Perambalur for creating an environment that encourages research.

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