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Research Article
Apoptotic Activity and Cytotoxic Effect of *Convolvulus spicatus* on Human Breast Cancer Cell Line (MCF-7)

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Abstract

Background and Objective: Despite advancements in modern therapeutic strategies, breast cancer still the most common cause of the high death rate among women worldwide. Wild plants and their extracts have been used in traditional medicine because of their efficient anti-cancer properties. This study aims to investigate *in vitro* the anti-cancer, anti-proliferative and potential therapeutic effects of *Convolvulus spicatus* (*C. spicatus*) methanolic extract against human breast cancer cell line Michigan Cancer Foundation-7 (MCF-7), besides putting shed on the mechanism of action of this extract. **Materials and Methods:** MTT (dimethylthiazol-diphenyltetrazolium bromide) cytotoxicity assay was done to evaluate *C. spicatus* methanolic extract's cytotoxic effects and its therapeutic potentiality against MCF-7 cells. Flow cytometry was used to clarify the potential impact of the different concentrations of the extract against the cell cycle's evolution. Nuclear densification and apoptotic changes were also analyzed and the Annexin V/propidium iodide staining method was used to ensure the anti-proliferative effect of *C. spicatus* extracts. The expression level of the apoptotic regulatory gene (Bax gene) was evaluated. **Results:** The results proved that cytotoxicity was significantly elevated in a dose-dependent manner under various concentrations. preG1 apoptosis and cell growth arrest at the G_2/M phase was noticed. Bax gene was upregulated at its mRNA level by a 5.6-fold increase, compared to the untreated MCF-7 cells. **Conclusion:** This study gives deep insight into evaluating natural extracts and/or bioactive ingredients derived from the *C. spicatus* plant and eventually exhibited a promising apoptosis-inducing anti-cancer agent.

Key words: Anti-cancer, apoptosis, DNA damage, flow cytometry, *Convolvulus spicatus*, MTT assay, MCF-7 cell line, Bax gene

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a prevailing common health problem with a high global mortality rate[1]. Despite advancements in tumour biology diagnosis and treatment methods, cancer remains the world's second leading cause of death, accounting for approximately 8.7 million deaths per year[2,3]. Cancer spreads and advancement occur via a successive series of epigenetic and genetic changes that result in cell insensitivity, propagation, proliferation and survival, stimulated by an increase in apoptosis resistance and cell cycle progression deregulation[4,5]. The most dangerous and well-known form of cancer in women worldwide is Breast Cancer (BC), with 2 million cases diagnosed in 2018 (23% of all cancers). According to World Health Organization (WHO)[6], the prevalence rates of BC in women vary between developed and developing countries, with the highest rates of 80 per 100,000 women in all developed countries except Japan. The lowest rates of less than 40 per 100,000 women in developing countries. However, in less developed areas, the mortality rate of BC is higher. This high prevalence of BC is due to various factors in the human lifestyle, including demographic problems, vulgar eating habits, reproductive, hereditary and hormonal breast-related causes[7].

The reduction of mortality rates and the improvement of cure chances are conceivable just if the tumour was managed during the primary stages of its occurrence. There are various types of cancer treatments available today and they differ depending on the cause and severity of the disease. These treatment lines include surgery, chemotherapy, radiotherapy and immunotherapy[8]. Unfortunately, these procedures have several side effects that differ depending on the patient and treatment strategies used. Chemotherapies particularly may cause various side effects, which vary depending on the type and dosage of medications given and the treatment duration.

Plants and their extracts appreciate the natural origin of bioactive phytochemical compounds characterized by high biological and clinical impacts, other than the widespread ability in different chronic diseases' treatment and used in folk medicine from ancient years[9,10]. These days, a substantial interest is directed toward the natural sources, highlighting the medicinal plants such as Phenolics and Flavonoids compounds[11] that have significant antioxidant capacities and reduce cancer prevalence and mortality rates[12]. Various types of cultivated and wild plants have been utilized in recent medicine applications as pharmaceutical agents because they posse beneficial antioxidants effects and contain distinct forms of phytochemical constituents[10,13]. These plants have the most efficient antioxidant, anti-viral, anti-fungal, anti-bacterial, anti-inflammatory and anti-cancer effects[14-16].

Convolvulus spicatus is a genus that belongs to the family Convolvulaceae and is considered one of the most clinically, economically and therapeutically family in Angiosperms[17-20]. Many researchers have extensively studied the medicinal properties, bioactivity and structures of phytochemical compounds for *Convolvulus* plants and their physiological mechanisms[18]. Due to their properties, they are broadly utilized as therapeutic agents in traditional medicine, whereby extracts of the Convolvulaceae family have been proven to possess tremendous potential in combating cancer strains, hence explaining its ironic name, "the weed of cancer".

Previous studies have featured the cytotoxic capacity of other members of the genus against several tumour cells along with being a safe, effective and non-toxic chemotherapeutic agent as corroborated by recent research. The aerial parts of *C. arvensis* are utilized in wound healing under its anti-haemorrhagic activities and its anti-spasmodic properties. While it likewise reported being a potent antioxidant against chromium (III) and chromium (VI) stress. Additionally, it has anti-tumour and anti-angiogenesis actions, alongside possessing toxic impacts in high doses. Rodríguez-García *et al.*[12] clarified the importance of cashew and almond extracts as cytotoxic effects in the cancer cell line.

Recently, many investigations studied the antioxidant and biological activities of *Convolvulus*, especially for *C. prostrates*[21] and *C. pilosellifolius*[22]. The pharmacological potential of *Convolvulus* plants comes from the high biological activities of their chemical constituents reported in previous studies, such as the use of *C. arvensis* and *C. pilosellifolius* as tumour growth inhibitor[23,24], *C. pilosellifolius* as an antiulcer genic[25]. Tungmunnithum *et al.*[11] studied the anti-cancer activity of *Convolvulus spicatus* and *Astragalus vogelii*.

Rodriguez *et al.*[26] stated that naturally origin therapeutics plants which are rich in components of Phenolics and Flavonoids contents considered as a robust anti-cancer cell and their manufactured medicines act as a preventive drug through anti-apoptosis regulator as Bcl-2-associated X protein (Bax) or by downregulation of apoptotic molecules as Nuclear Factor κB (NFκB). Elevated Bax gene expression, the apoptotic regulatory gene, was an essential factor in the prognosis of BC[27]. This overexpression is correlated with prolonged patient survival via being more sensitive to the therapeutic agents[28].

The ambition of this work is to investigate and evaluate the efficiency of methanolic extract for *C. spicatus* as an anti-cancer agent. Additionally, to illuminate the main mechanism of action of this extract against MCF-7 cells, via investigating its apoptotic activity, that could be an attractive targeting drug with a high affinity for hindering cancer progress.

MATERIALS AND METHODS

Study area: This study was carried out at Biochemistry Laboratory, College of Medicine, Jouf University, Saudi Arabia from September, 2020-January, 2021.

Plant material: It was obtained from the Al-Jouf region, Saudi Arabia. Then the plant species were identified and authenticated as *Convolvulus spicatu*s belonging to the Convolvulaceae family, according to Boulous[29] by Professor Ahmed Kamal Eldin Osman, Department of Botany, Faculty of Science, South Valley University, Egypt.

Preparation of *C. spicatus* extract: Fresh aerial parts of *C. spicatus* were dried and ground into powder at room temperature to extract its active constituents. The methanolic plant extract was performed using a Soxhlet brand extruder. Briefly, weigh 10 g of *C. spicatus* powder and placed in 250 mL methanol for 6 hrs with Soxhlet system. The methanolic extracts obtained were dissipated to dryness and concentrated at 40-50°C under pressure using a rotary evaporator. The extract was left to cool down to 4°C, then stored in airtight dark bottles.

Cell viability assessment

Cell cultures: The Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt, provided the MCF-7 cells for this research. Cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 from Gibico (USA). This cell culture contains 10% Fetal Bovine Serum (FBS) from Sijixin Inc. in China and 1% penicillin-streptomycin antibiotics (from Invitrogen in the United States). These cells were incubated at 37°C in a humidified CO_2 incubator with a humidity of 95% and a CO_2 concentration of 5%.

***In vitro* cytotoxicity assay:** MCF-7 viable cells were seeded in 96-well tissue culture plates for the MTT assay and 100 L of the medium containing various concentrations of *C. spicatus* methanolic extract (78.12, 156.25, 312.5, 625, 1250, 2500, 5000 and 10000 g mL^{-1}) was added. The cytotoxicity was measured using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) reagent method[30] after 72 hrs of incubation. According to Eskandani *et al.*[31], the half-maximal Inhibitory Concentration (IC50) of *C. spicatus* methanolic extract required to reduce proliferation/viability values by 50% compared to control was calculated. All experiments were done in triplicate and the mean values were used to calculate the results:

$$\text{Cell viability (\%)} = \frac{\text{OD of treated cells}}{\text{OD of control}} \times 100$$

$$\text{Growth inhibition (\%)} = 100\text{-Cell viability (\%)}$$

Cell cycle analysis

Apoptosis assessment: To detect apoptosis, a cell cycle analysis using Propidium Iodide (PI) staining via flow cytometry was performed according to the Annexin V-FITC Apoptosis Detection Kit's manufacturing guidelines. This was done to investigate the effects of methanolic extract of *C. spicatus* on relative cellular DNA CONTENT of human breast line cells.

Apoptosis-regulatory gene RNA isolation and quantitative RT-PCR: All procedures were performed according to Kumar *et al.*[32] protocols with slight modifications as follows. Total RNA was extracted from both treated and nontreated MCF-7 cells with methanolic extract of *C. spicatus.* After 24 hrs of incubation with detected IC50 concentration using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Afterwards, 1 µg of RNA was reverse transcribed to the first-strand cDNA by cDNA Synthesis kit (Biorad, Germany). To check the expression level of the apoptotic regulatory gene (Bax gene), real-time PCR was performed by amplifying the obtained cDNA. The β-actin used as an internal control reference gene. Real-time PCR was performed by Rotor Rotor-Gene 6000 Real-time PCR System (QIAGEN, Germany) using SYBR Green. The primer sequences used in qRT-PCR are (1) Bax F 5′-GTTTCA TCC AGG ATC GAG CAG-3; Bax R 5′-CATCTT CTT CCAGAT GGT GA-3′ and (22) β-actin F 5′ GTGACATCCACACCCAGAGG-3′; β-actin R 5′-ACAGGATGTCAAAACTGCCC-3′. The PCR conditions for cDNA amplification begin with a 15 min initial activation step at 95°C, followed by 40 cycles of denaturation (95°C for 30 sec), annealing (58°C for 20 sec) and extension (72°C for 30 sec) steps. Finally, the data were normalized against β-actin mRNA in each sample using the $2^{-\Delta\Delta CT}$ method to compute relative fold changes of target gene mRNA levels.

RESULTS

Cytotoxic activity of *C. spicatus* methanolic extracts: The present study investigates *in vitro* the cytotoxic effects of *C. spicatus* methanolic extract and therapeutic potentiality against the risk of cell line (MCF-7) breast cancer in humans using MTT assay via reduction of MTT reagent to purple coloured product (formazan) by mitochondrial

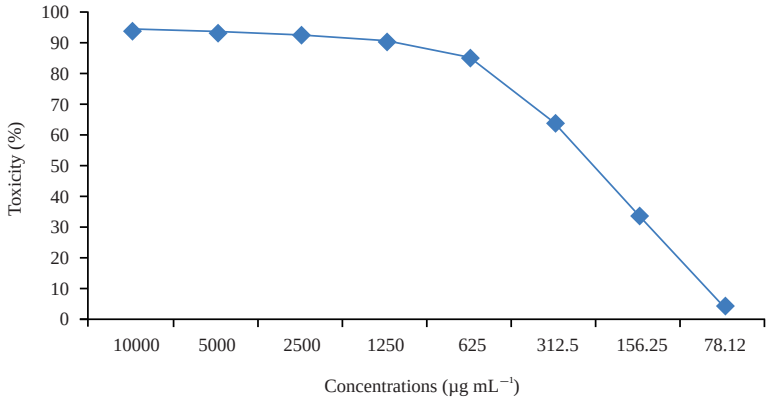

Fig. 1: Effect of *C. spicatus* on human breast cancer cell line (MCF-7) with different concentrations (78.12, 156.25, 312.5, 625, 1250, 2500, 5000, 10000 µg mL^{-1})

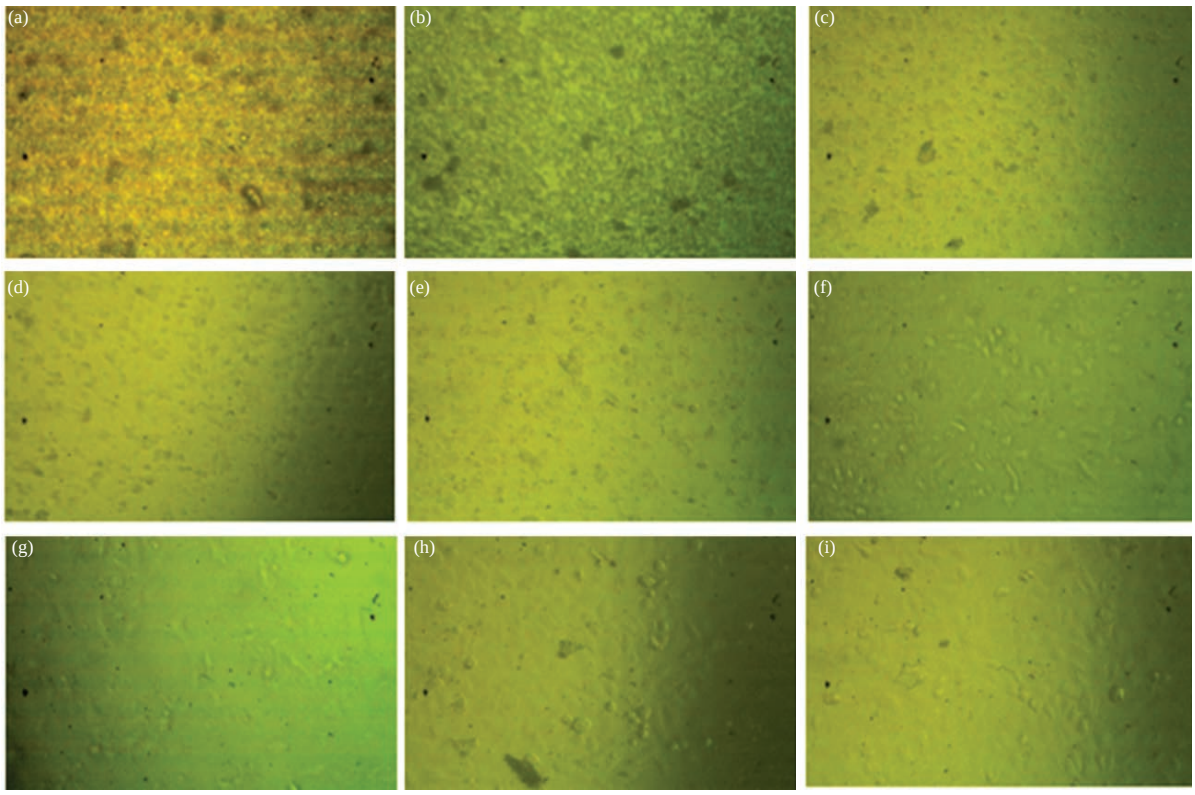

Fig. 2(a-i): Morphology of control and different concentration of *C. spicatus* methanolic extracts treated human breast cancer cell line (MCF-7) (40×Magnification)
(a) 10000, (b) 5000; (c) 2500; (d) 1250; (e) 625; (f) 312.5; (g) 156.25 and (h) 78.12 µg mL^{-1} as compared with (i) untreated MCF-7 cells (control)

dehydrogenase. Different concentrations of *C. spicatus* methanolic extracts (78.12, 156.25, 312.5, 625, 1250, 2500, 5000 and 10000 µg mL^{-1}) were used in the MCF-7 breast cancer cells treatment, the cytotoxicity analysis results presented in (Fig. 1) demonstrated a dose-dependent increase in the cytotoxicity revealing a significant difference between all treated groups and MCF-7 control cell culture. Cytotoxicity of *C. spicatus* extracts impact in breast cancer cell lines was highest using concentrations 625-78.12 µg mL^{-1}. The results reveal the sensitivity of the MCF-7 cells to the potent toxicity effect of *C. spicatus* plant extracts (Fig. 2a-i). The microscopic observations (Fig. 2i) for monitoring the untreated breast

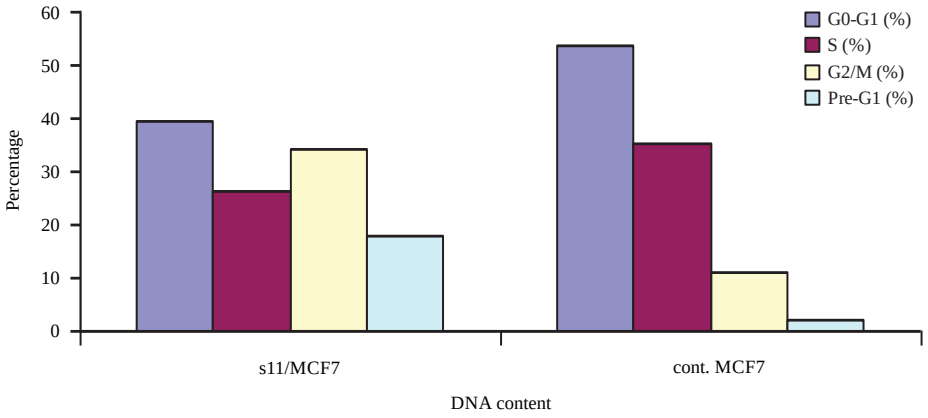

Fig. 3: Effects of different concentrations of *C. spicatus* extracts on cell cycle distribution in human breast cancer cell line (MCF-7) as compared with untreated MCF-7 cells (control)
Different concentration (78.12, 156.25, 312.5, 625, 1250, 2500, 5000 and 10000 µg mL^{-1})

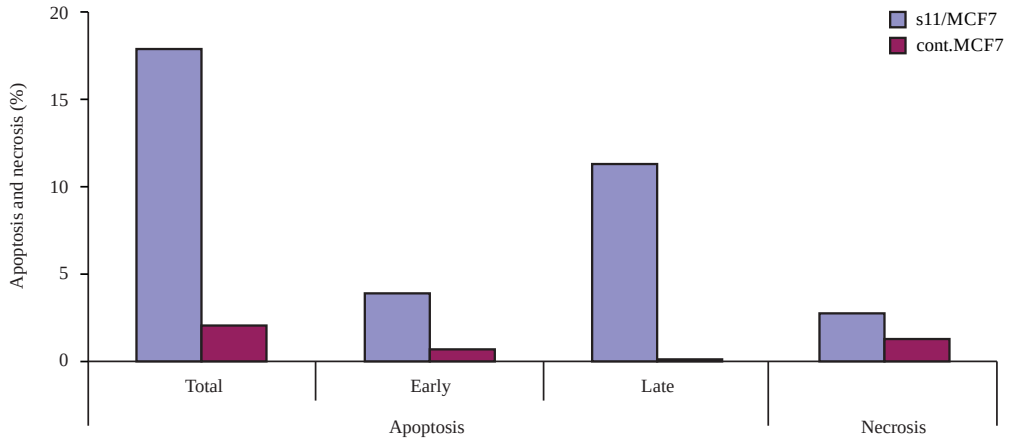

Fig. 4: Induction of apoptosis and necrosis in human breast cancer cell line (MCF-7) by incubation with *C. spicatus* methanolic extract compared with untreated MCF-7 cells (control)

cancer MCF-7 cells compared with MCF-7 cells treated with eight concentrations of *C. spicatus* methanolic extracts (78.12, 156.25, 312.5, 625, 1250, 2500, 5000, 10000 µg mL^{-1}) (Fig. 2h-a). The half-maximal inhibitory concentration (IC_{50}) value was obtained from the log dose cytotoxic effects of the methanolic leaf extract of *C. spicatus* that inhibits the growth of 50% of the MCF-7 cell population was observed at 245.825 µg mL^{-1} concentration of *C. spicatus* plant. The U.S. National Cancer Institute (NCI) protocol of cytotoxicity criteria determined the activity of crude extract varied from active, moderately active and inactive based on the values of the IC_{50} ranged from less than 20 µg mL^{-1} for active mode, 20-100 µg mL^{-1} for moderately active and more than 100 µg mL^{-1} for inactive.

Cell cycle analysis: Flow cytometry was used to clarify the potential impact of the different concentrations from *C. spicatus* methanolic extract against the cell cycle evolution for MCF-7 cells. The obtained results (Fig. 3) recorded that treatment of MCF-7 cancer cells with *C. spicatus* extracts caused preG1 apoptosis and cell growth arrest at the G_2/M phase. MCF-7 cell treatment with *C. spicatus* methanolic extract stimulates significant elevation in the percentage of cells in the G_2/M phase compared to the control group (from 11.13% (control)-34.17% in *C. spicatus* treated cell lines). Furthermore, pre-G_1 percentages were increased from 2.04% (control)- 18.01% in *C. spicatus* treated cells.

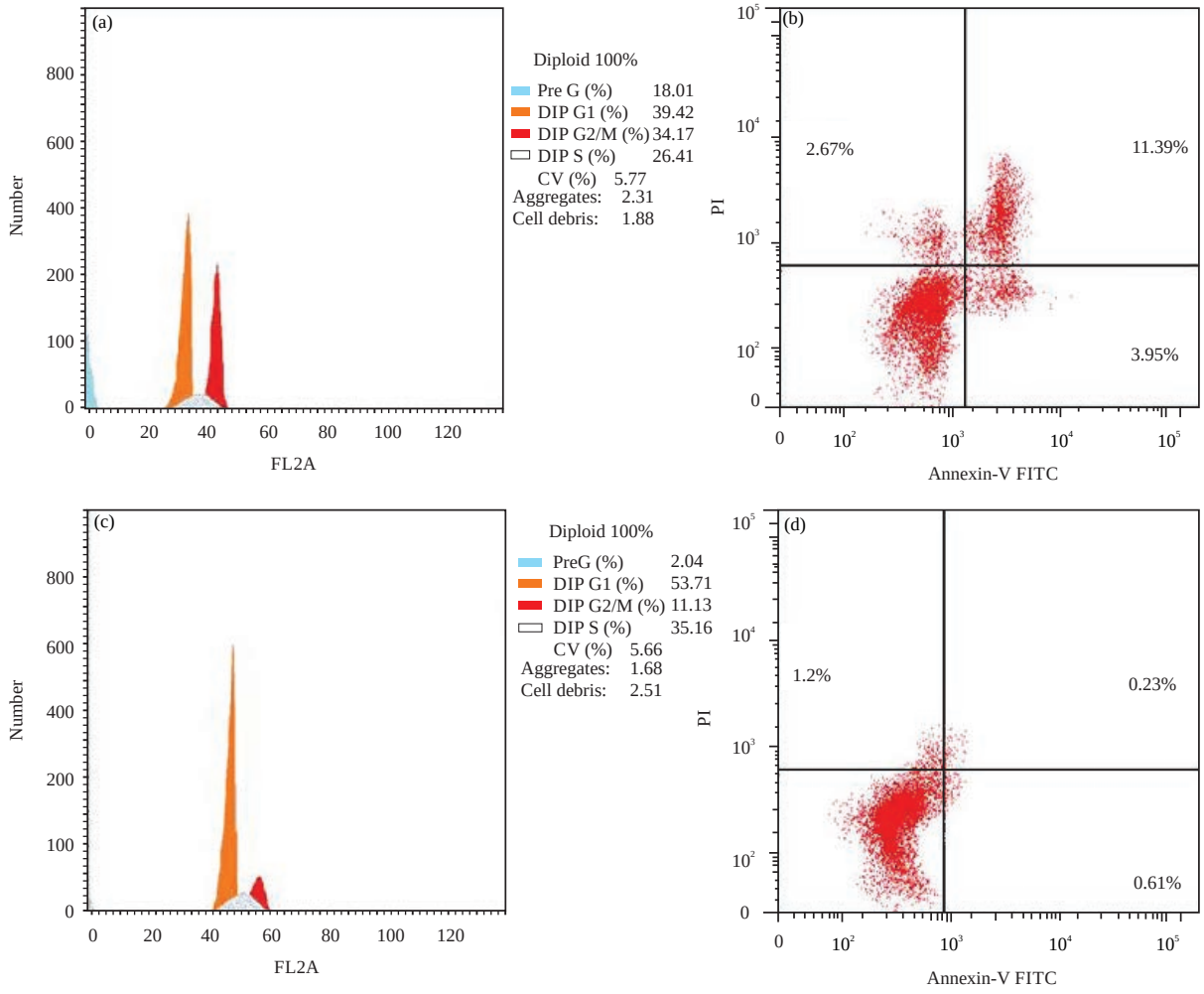

Fig. 5(a-b): DNA damage and the cell cycle distribution in the human breast cancer cell line (MCF-7) under *C. spicatus* methanolic extract treatment (a-b) as compared with untreated MCF-7 cells (c-d)

% is for percentage, Annexin V-FITC (Staining kit), FL2A: Fluorescent Light of the DNA dye-Area, PI: Propidium Iodide

Induction of early apoptosis, late apoptosis and necrosis: The process of staining Annexin V/propidium iodide for nuclear densification and apoptotic variations by flow cytometry analysis was used to ensure the anti-proliferative impact of *C. spicatus* extracts. There were only a few cells in the control group that reacted positively to propidium iodide. In contrast, there is an observed gradual front increase in the percentage of positive cell response against propidium iodide stratified according to the rise in the concentration of *C. spicatus* extracts, while the total apoptotic (18.01%) observed at the 10000 µg mL^{-1} concentration as compared with 2.04% in control MCF-7 cells (Fig. 4). The augmentation in apoptotic cell count in MCF-7 cells treated with *C. spicatus* showed the induction of the apoptotic effect of Methanolic extract from *C. spicatus*.

To investigate the induction of DNA fragmentation, damage and cell cycle distribution in the MCF-7 cancer cells by the induction of oxidation stress under *C. spicatus* methanolic extracts, flow cytometry analysis was performed (Fig. 5a and b). *C. spicatus* significantly reduces the DNA content, shows apoptosis in the G_0/G_1, suggesting that the G_1 phase in the treated cells was lost by programmed cell death. The cell populations in the *C. spicatus* treated MCF-7 cells shift from viable to apoptotic compared with untreated cancer cells (control) (Fig. 5c and d). The apoptosis appears; however, the percentage of MCF-7 apoptotic cells was significantly higher than necrosis in *C. spicatus* treated cancer cells.

Gene expression assay: The mRNA relative expression level of the apoptotic regulatory gene (Bax) was investigated in the

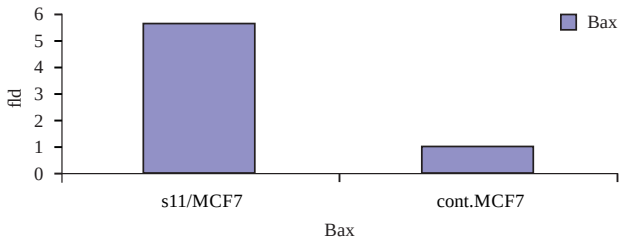

Fig. 6: Effect of *C. spicatus* methanolic extract on the mRNA expression level of the apoptotic regulatory gene (Bax) in the human breast cancer cell line (MCF-7) compared with untreated MCF-7 cells
fld: Fatty liver dystrophy

MCF-7 cell line when treated with methanolic extract of *C. spicatus.* As shown in (Fig. 6), the treatment induced the Bax gene's upregulation by increasing its mRNA level by 5.6-fold compared with the untreated MCF-7 cells.

DISCUSSION

The results of this study revealed the activity of *C. spicatus* methanolic extract as anti-proliferative and anti-cancer potentiality against the MCF-7 cancer cell lines. On the MCF-7 cancer cell lines, the methanolic extract had cytotoxic effects, that were proportional to the treated dose. Natural products play an important role in the production and discovery of new drugs. Initially, more than half of the newly approved drugs were originally extracted from natural product[33]. Because of the chemical compounds present in their extracts, wild plants act as important anti-tumour agents in cancer induction and treatment[34]. All of these products have unique properties that allow them to compete against cancer cells while having minimal side effects. Zhou *et al.*[35] stated that natural products such as green tea and grape skin act as a preventive agent to fight against cancer. Mallhi *et al.*[36] discovered that *phoenix dactylifera* has natural ingredients that serve as an active drug against cancer cell progression. Furthermore, phenolic components can be pro-oxidant and modify the cancer cell redox balance[37,38]. Simultaneously, most wild medicinal plants' cytotoxic potential effect was necessary for cancer therapy and played a significant role in cancer induction and treatment[39]. MCF-7 cells are more sensitive to the cytotoxic activity of *C. spicatus* plant extract than normal cells with lower sensitivity to the extract, according to the IC_{50} results seen in this study. The IC_{50} values showed that MCF-7 cells are more sensitive to the cytotoxic activity of *C. spicatus* plant extract than normal cells with less sensitivity to the extract. IC_{50} values of methanolic extract of *C. spicatus* is about 245.825 µg mL^{-1}. This high value could be due to the short period of treatment[40].

Samarakoon *et al.*[41] studied the cytotoxic activity of about 116 wild plant extracts of chloroform, hexane, methanol and ethyl acetate from leaves and plants' stems. These extracts produce different values of IC50 and had a cytotoxic effect on MCF-7 and hepatocarcinoma cell lines. Similarly, one of the most abundant and common mangrove species, Avicenna Mariana, widely used as traditional medicine, has been found to have cytotoxic potential in its leaf extracts against human cancer cell lines via apoptosis due to the high presence of phenol and flavonoid contents[42].

Apoptosis, the fragmentation of genomic DNA, is an influential model in recent research for cancer diseases. This model induces the rate of cell death without any inflammatory responses, which is considered the origin of different adverse side effects[40]. Moreover, apoptosis effects cause cell morphology changes, such as cell wrinkles, cell adhesion loss and DNA fragmentation[43]. Many studies were carried out to clarify if apoptosis is accused as the potential mechanism of plant extract action. The induction of apoptosis has been reported as a fundamental strategy to combat the proliferation of cancer cells[44]. Besides, Mirzaa *et al.*[45] elaborated that cancer cells can be controlled through cell cycle induction of apoptosis. It would be beneficial to treat cancer disease with fewer side effects. Cell cycle progression has a significant impact on increasing mammalian cell propagation and cellular growth.

Moreover, attacking cancer disease risk is an efficient approach by inhibiting the cell cycle of these cancer cells[46,47]. Meanwhile, the observed cell cycle arresting at the G2/M phase supported the induction of the pre G1 apoptosis causes a significant increase in apoptotic cell populations in our treated cell line model. These results were aggregately supported with Pumiputavon *et al.*[48], who revealed that six plants' methanolic extract could induce cancer cell apoptotic death in a cell type.

As expected, the addition of PI enabled viable, early apoptotic, late apoptotic and necrotic cells to be distinguished[49]. Our results illustrated a significant increase of the necrotic and apoptotic cell populations in the MCF-7 cells after their incubation with various concentrations of methanol extracts of *C. spicatus* when compared with their control cells. These results were aggregately agreeable with Srisawat *et al.*[50], who studied the cytotoxic effect and apoptotic mechanism of MCF-7 and MDA-MB-231 cells via *Vatica diospyroides* fruit extracts. This exhibited apoptotic potentiality of *C. spicatus*

plant extract may be due to the chemical composition profile it owns, as reported by several studies. *C. spicatus* plant is known to be rich in several phytochemicals such as tannins, glycosides, sterols, coumarins, saponin, alkaloids and flavonoids that have been described to have anti-proliferative and anti-cancer actions. In agreement with previous studies that reported the complex chemical profile for many plants belonging to *Convolvulus* genus and having different bioactive gradients and chemical compositions of phytochemicals as resins, coumarins, lignans, phenylpropanoids and anthocyanidins[18]. Besides secondary metabolites like lipids, saponins, alkaloids and tannins[51], besides different essential oils[52] and derivatives of caffeoylquinic acid[53]. Other phytoconstituents in the genus *Convolvulus* were studied as amino acids, carbohydrates, terpenoids, anthraquinones and steroids[22].

Besides the novel role of *C. spicatus* methanolic extract in inducing cancer cell apoptotic death, its impact on the relative expression of the apoptotic regulatory genes (Bax) was evaluated via quantitative RT-PCR in the MCF-7 cells. Bax expression assessment is a recognizable approach used for investigating apoptosis upon treatment with those methanolic extracts. Our obtained results in Fig. 6 illustrated that the *C. spicatus* methanolic extract induced apoptosis elicited through the Bax gene. These results agreed with formerly published studies proving that *Convolvulus arvensis* causes maximum apoptosis and cell cycle arrest[11,54]. Despite these promising findings, further research is needed to determine the optimal therapeutic dosage for the human and further research should be carried out in isolation and characterization of their bioactive chemical constituents that could be beneficial in the treatment of other diseases and cancers. Moreover, studying signalling pathways involved in the development of tumorigenesis in patients at the molecular level using various methods is still required. This future research is expected to provide a deeper understanding of the molecular mechanism as well as the effect of *Convolvulus spicatus* in improving such cases.

CONCLUSION

In conclusion, this study demonstrated the antioxidant and anti-cancer properties of *C. spicatus* methanolic extract against MCF-7 cells. This extract also induced cellular apoptosis, that proportionally related to the treated dose. The effects of this extract may be because of the presences of beneficial and efficient bioactive chemical constituents, which need intensive study in isolation and characterization. Emerging from these data, *C. spicatus* could be exploited as an anti-cancer agent for breast cancer treatment, depending on its ability to induce apoptosis.

SIGNIFICANCE STATEMENT

This study discovered that the methanolic extract of *Convolvulus spicatus* has antioxidant and anti-cancer properties against the Human Breast Cancer Cell Line (MCF-7) and the ability to induce apoptosis in these cells proportionally to the treated dose. Emerging from these findings, the *Convolvulus spicatus* could be used as an apoptosis-inducing anti-cancer agent to treat breast cancer. This study will help a lot in treating the diagnosed cases, which will improve their psychological state. Moreover, this work will enable policymakers to create targeted and more efficient prevention. It will lead to critical preliminary insights that can develop more refined, prospective analyses of breast cancer magnitude and risk to save time, efforts and money offered by the government in treating such diseases.

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