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Apoptosis Induction, Cell Cycle Arrest and in Vitro Anticancer Potentiality of *Convolvulus Spicatus* and *Astragalus Vogelii*

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ABSTRACT

The target of this research is to investigate the anticancer potentiality of the Convolvulus spicatus and Astragalus vogelii. This study concentrated on the molecular pathways including apoptosis and cell cycle arrest of the anticancer cytotoxic effect. Methanol extract of each plant was prepared and utilized. Human Colon (Caco-2) and lung (A549) cancer cell lines were used. Antioxidant power and some phytochemical contents of every extract were measured. The cytotoxic activity of each extract was assessed as well as apoptosis induction. The mRNA level of Bax and Bcl2 apoptosis regulatory genes was detected. The obtained data showed that, the C. Spicatus methanolic extract had the highest total phenolic content, total flavonoid content and antioxidant power in comparison with A. vogelii. Both plants revealed cytotoxic effect on Caco-2 and A549 with different IC50 values and antioxidant power. C. spicatus and A. vogelii extracts exerted clear cytotoxic activity against human Caco-2 and A549 cells, foremost by means of cell cycle arresting at the G2/M phase associated with the pre G1 apoptosis induction. An apparent increase in the mRNA level of Bax and a concomitant decrease in Bcl-2 mRNA level were observed in the Caco-2 and A549 cells treated with C. spicatus and A. vogelii respectively. This study concluded that, Convolvulus spicatus and Astragalus vogelii induced apoptotic cell death and suggests that Convolvulus spicatus and Astragalus vogelii possibly can be utilized as new sources of an apoptosis-inducing anticancer agent for colon and lung cancer treatment with further detailed studies.

Keywords: *Anticancer, Apoptosis, Astragalus Vogelii, Convolvulus Spicatus, Gene Expression.*

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1. INTRODUCTION

In 2018, Cancer is the second major reason of death globally, and is the cause of an estimated 9.6 million deaths. worldwide, about 17% of deaths is caused by cancer. Cancers of the lung, female breast, and colorectal represented the highest incidence and ranked within the upper five in term of mortality. Worldwide, they represent one third of the cancer incidence and mortality burden (Bray *et al.,* 2018).

Different kinds of chemotherapies failure have been reported, it might be caused by adverse reactions as well as drug resistance and drug specificity. There is now need to develop medications that overcome the problems stated above by using natural compounds, it could influence multiple sites with lower side effects and which are effective versus different cancer kinds (Alfarouk *et al.,* 2015).

Orlikova & Diederich (2012) reported that plant extracts and their active ingredients are from the valuable sources of cancer chemotherapeutics. Moreover, many structural analogues have been introduced via molecular changes of the natural Phytochemical and have reinforced the anticancer arsenal (Gordaliza, 2007). More than 70% of anticancer drugs are natural compounds or natural product-derived agents (Karikas 2010).

The Convolvulaceae family includes a huge number of plant which have the treatment potentiality of many diseases (Al- Al-Asady *et al.,* 2014). *Convolvulus* is the enormous genus of family Convolvulaceae. Worldwide *Convolvulus* species are widely distributed and some of them have medicinal activity such as cytotoxic effect, antioxidant, anti-inflammatory, antiasthma, antijaundice, anticancer, and antiulcer activities (Al-Rifai *et al.,* 2017).

Astragalus L., is one of the largest genera of flowering plants in the Leguminosae family. As Annual herbs, sub-shrubs, or shrubs, the plants of *Astragalus* L. are a widely distributed in particular in the temperate and arid areas (Li *et al.,* 2014). Furthermore, the dried roots of some species of *Astragalus* in Asia are utilized in folk Chinese medicine to treat a great array of diseases as nephritis, diabetes mellitus, hypertension, cirrhosis, leukemia, and uterine cancer (Avunduk *et al.,* 2008; Choudhary *et al.,* 2008).

So far, no reports are found about the anticancer activity of *Astragalus vogelii* and *Convolvulus spicatus*. So, the objective of this research is to investigate the anticancer effects of the methanolic extract of these two plants against human lung and colon cancer.

2. MATERIALS AND METHODS

2.1. Plant material

The plant materials were collected and dried from Arar, Northern Border, Saudi Arabia region. The dried plant samples were ground into powder. The plant species were identified and authenticated as *Convolvulus spicatus* and *Astragalus vogelii* belonging to Convolvulaceae and Leguminosae family by Prof. Dr. Ahmed Kamal Eldin Osman, Professor of Botany, Botany Department, Faculty of Science, South Valley University, Egypt.

2.2. Preparation of methanolic extract

The powdered plant materials were used to prepare the methanolic plant extract via Soxhlet extraction procedure. The methanolic extracts were evaporated to dryness and concentrated under pressure at temperature 40 to 50º C in a rotary evaporator. The extracts were subsequently collected and stored in airtight and dark bottles until use.

2.3. Phytochemical analysis and antioxidant effects

The total phenolic content (TPC) and total flavonoid content (TFC) were estimated in *C. spicatus* and *A. vogelii* plant extracts using a colorimetric assay according to (Koldas *et al.,* 2015) based on procedures described by (Singleton and Rossi, 1965) and (Chang *et al.,* 2002), respectively. Total phenolic content was illustrated as mg/g Gallic acid equivalent (GAE). The total flavonoid content (mg/g) was measured by the calibration curve of quercetin and illustrated as mg quercetin equivalents.

Antioxidant activity of the examined extracts was studied according to the methods described by (Oyaizu, 1986), with slightest modification as applied by (Puranik *et al.,* 2018). Briefly, methanolic extract of *C. spicatus* and *A. vogelii* in different concentrations ranging from 100 µL to 500 µL were mixed separately with 2.5 mL of 0.2 mM phosphate buffer (pH 7.4) and 2.5 mL of potassium ferricyanide, (1% W/V).

The obtained mixture was incubated at 500 C for 20 min. Then, 2.5 mL of trichloroacetic acid (10% W/V) was added and centrifuged at 3500 rpm for 8 min, followed by 2.5 mL of distilled water and later 0.5 mL of ferrous chloride (0.1% W/V).

Finally, the absorbance at 700 nm was measured. Ascorbic acid was utilized as positive reference standard.

2.4. Cell lines and Cell cultures

Human Caco-2 (Colon cancer) and A549 (Lung cancer) cancer cell lines were used. Cell lines were obtained from the Holding Company for Biological Products & Vaccines, Egypt VACSERA). Cells were cultured in RPMI 1640 medium (Gibico, USA) which was enhanced with 10% fetal bovine serum (Sijixin Inc., China) and 1% penicillin–streptomycin mixture (Invitrogen, USA) and incubated at 37 ºC in CO_2 incubator with 5% CO_2.

2.5. *In vitro* cytotoxicity assay

For the cytotoxicity assay, 1 × 10^5 cells / ml (100 µg / well) were seeded in 96-well tissue culture plates and incubated at 37°C for 24 h to produce a complete monolayer sheet. Then cells with and without each plant extract were incubated at 37°C, using the methanol extracts at various concentrations ranged from 7.812-1000 µg/mL. After 72 h incubation, the cytotoxicity was estimated using the MTT assay as reported by (Van Meerloo *et al.,* 2011). To obtain the half inhibitory concentration (IC50), the percentages of cell viability and growth inhibition were calculated by the following equations according to Eskandani *et al.* (2014).

Cell viability (%) = [(OD of treated cells / (OD of control] × 100.

Growth inhibition (%) = 100 – Cell viability (%).

2.6. Cell cycle analysis

To investigate the effect of methanolic extract of *C. spicatus* and *A. vogelii* on relative cellular DNA content, cell cycle analysis was performed using propidium iodide (PI) staining via Flow cytometry according to the manufacture instruction of Annexin V-FITC Apoptosis Detection Kit. Briefly, the Caco-2 and A549 cells were seeded in 96-well plates at a concentration of 1 × 10^5 cells per well, and then treated with IC50 concentration and incubated for 48 h.

Cell**s** were stained with PI (10 µg/ml PI, 200 µg/ml RNase) for 15 min at room temperature in the dark. Untreated cells, as control, were simultaneously measured. Cellular DNA content was analyzed using flow cytometry and the percentage of cells in the G0/G1, S, G2/M and pre-G1 phases of the cell cycle were determined.

2.7. Induction of apoptosis

2.7.1. RNA isolation and quantitative RT-PCR of apoptosis-regulatory genes

All procedures were based on Kumar *et al.* (2017) with some modifications as follow. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. Total RNA was extracted from both untreated and treated Caco-2 and A549 cells with *C. spicatus* and *A. vogelii* extracts after 24 h incubation with detected IC50 concentration. One µg of RNA was reverse transcribed to first-strand cDNA. The obtained cDNA was amplified to check the expressions of Bcl-2 and Bax genes. An internal control β-actin was utilized as a standard for the real-time PCR reaction. The sequences of the primers used were as follows; (1) Bcl-2 F 5'-CCTGTG GAT GAC TGA GTA CC-3'; Bcl-2 R 5'-GAGACA GCC AGG AGA AAT CA-3'; (2) Bax F 5'-GTTTCA TCC AGG ATC GAG CAG-3; Bax R 5'-CATCTT CTT CCA GAT GGT GA-3' and (3) β-actin F 5'-GTGACATCCACACCCAGAGG-3'; β-actin R 5'-ACAGGATGTCAAAACTGCCC-3'. The PCR conditions for the amplification of cDNA were 95°C for 35 seconds followed by 40 cycles of denaturation (95°C for 5 seconds), annealing at 58°C for 10 seconds (β-actin) and 55°C for 10 seconds (Bax and Bcl-2), and extension at 72°C for 30 seconds. Reaction with water instead of cDNA template was considered a non-template control.

After amplification, all the amplified PCR products were electrophoresed in 2.5% agarose gel with 100 bp DNA ladder (Fermentas, USA). All gels were visualized by ethidium bromide under UV irradiation. Bcl-2, Bax, and β-actin mRNA levels were quantified by quantitative real-time (qRT-PCR).

The quantitative RT-PCR was performed by a Real Time PCR kit (BIORAD iScriptTMOne-Step RT-PCR Kit) using SYBR Green. A negative control of Diethyl pyro carbonate (DEPC) water was used instead of the cDNA template. The results of Bcl-2 and Bax mRNA expression were presented relation to the expression of β-actin. To specificity of the amplification, the PCR products were determined by melting curve analysis for each primer pairs. Data were analyzed by the 2^ ΔΔCT method. The results of target mRNA levels were normalized against β-actin mRNA in each sample. All target genes results were shown as relative fold change (RFC) to negative control.

3. RESULTS

3.1. Phytochemical analysis and antioxidant activity

The TPC, TFC and antioxidant power were determined for *C. spicatus* and *A. vogelii*. The obtained results are summarized in (Table 1). The results showed that, TPC, TFC and antioxidant power of *C. Spicatus* methanolic extract were higher than *A. vogelii*.

Table 1: Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant power of *C. spicatus* and *A. vogelii* methanolic extract.

Plant	Total phenolic content(mg/g extract)	Total flavonoid content (mg/g extract)	Antioxidant mg/g extract (as ascorbic acid)
C. spicatus	39.4	2.9	53.64
A. vogelii	15	0.9	11.7

3.2. Cytotoxic activity

Cytotoxic activity of *A. vogelii* and *C. spicatus* methanol extract on human Caco-2 (Colon cancer) and A549 (Lung cancer) cell lines was assessed using MTT assay. The test identifies the reduction of MTT by mitochondrial dehydrogenase to purple colored product, namely formazan. Cancer Cells were treated with *C. spicatus* and *A. vogelii* methanol extract at concentrations ranging from 7.81-1000µg/mL. The percentage of cell toxicity was analyzed. Figures (1-4) demonstrates the cell toxicity values for different concentrations. The results indicated that, Caco-2 and A549 cell lines were responded to the cytotoxic effects of the *C. spicatus* and *A. vogelii* methanol extract in a dose- dependent fashion.

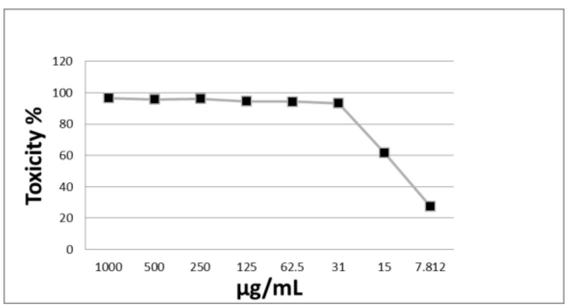

Figure 1: Effect of *Convolvulus spicatus* on Caco-2 cells with different concentrations.

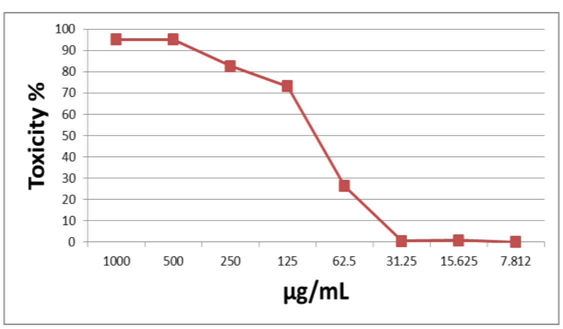

Figure 2: Effect of *Convolvulus spicatus* on A549 cells with different concentrations.

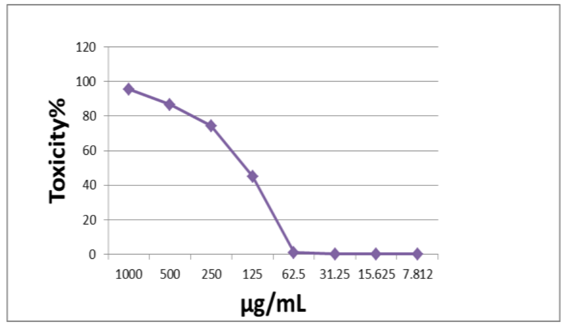

Figure 3: Effect of *Astragalus vogelii* on Caco-2 cells with different concentrations.

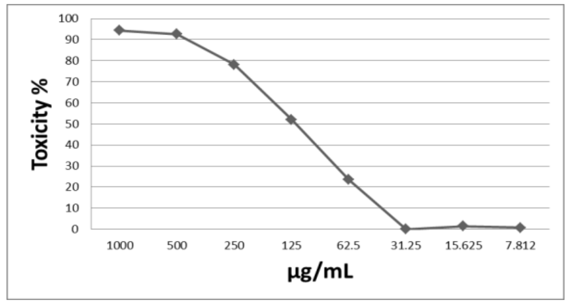

Figure 4: Effect of *Astragalus vogelii* on A549 cells with different concentrations.

Table 2: IC50 values of *C. spicatus* and *A. vogelii* methanol extract on Caco-2 and A549 human cancer cell lines

Cancer cell line	IC50 (µg/mL)	
	C. spicatus	*A. vogelii*
Caco-2	14.2	171
A549	94.1	152

IC50 values indicate the concentration of the extract that prevents the growth of 50% of the cells. The standard of cytotoxicity determined by the U.S. National Cancer Institute (NCI) considers a crude extract as active, moderately active or inactive, when the IC50 values are lower than 20 µg/mL, from 20 to 100 µg/mL, or greater than 100 µg/mL, respectively (Ramos-Silva *et al.*, 2017). The methanol extract of *C. spicatus* induced strong cytotoxicity in Caco-2 cells (IC50=14.2 µg/mL), and showed moderate activity (IC50=94.1 µg/mL) against A549 cell line. In contrast, the methanol extract of *A. vogelii* was inactive against both Caco-2 and A549 cells (IC50=171 and 152), respectively (Table 2).

3.3. Cell cycle analysis

The effect of the *C. spicatus* and *A. vogelii* methanol extract on cell cycle progression on Caco-2 and A549 cancer cells was determined by flow cytometry. The obtained results showed that, the treatment of Caco-2 and A549 cancer cells with *C. spicatus* and *A. vogelii* extracts caused preG1 apoptosis and cell growth arrest at G2/M phase (Figure 5).

Treatment of Caco-2 and A549 cells with *C. spicatus* methanol extract induce statistically significant greater in the percentage of cells in G2/M phase in comparison with control group from 4.25 to 23.13 with Caco-2 cells and from 14.68 to 50.39 with A549 cells. Moreover, the percentages of pre-G1 were increased from 2.21 to 11.47 for Caco-2 cells and from 2.49 to 25.39 with A549 cells.

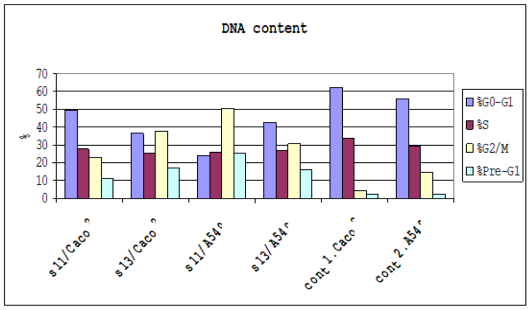

Figure 5: Effects of *C. spicatus* (S11) and *A. vogelii* (S13) methanol extract on cell cycle distribution

In the same direction, treatment of Caco-2 and A549 cells with *A. vogelii* extract also increased the percentages of cells at G2/M phase and cells in pre G1in comparison with control group. The percentages of cells at G2/M phase were increased from 4.25 to 37.87 for Caco-2 cells and from 14.68 to 30.48 for A549 cells. The percentages of cells at pre G1 were increased from 2.21 to 17.34 in Caco-2 cells and from 2.49 to 15.99 with A549 cells

3.4. Induction of early apoptosis, late apoptosis and necrosis

In flow cytometry analysis, Annexin V/propidium iodide (AnnV/PI) staining is based on the ability of the protein Annexin V to bind to phosphatidylserine (PS), which is externalized in the outer cell membrane leaflet upon induction of apoptosis. In viable cells, PS is located in the internal membrane leaflet, but upon induction of apoptosis it is translocated to the external membrane leaflet and becomes available for Annexin V binding. The addition of PI enabled viable (AnnV-/PI-), early apoptotic (AnnV+/PI), late apoptotic (AnnV+/PI+), and necrotic (AnnV-/PI+) cells to be distinguished (Baskic *et al.,* 2006).

The flow cytometry analysis of Caco-2 and A549 cells showed that, through the treatment with *C. spicatus* and *A. vogelii* the cancer cell populations tend to shift from viable to apoptotic in comparison with control group. Moreover, the early apoptosis events were increased than late apoptotic events in all treatments with all cancer cell lines as shown in (Figure 6).

The apoptotic and necrotic cell populations increased significantly in both Caco-2 and A549 cells due to treatment with methanol extracts of *C. spicatus* and *A. vogelii* when compared with the control cells. But the apoptosis percent was increased significantly than necrosis in all treatment of utilized cancer cells as shown in (Figure 6).

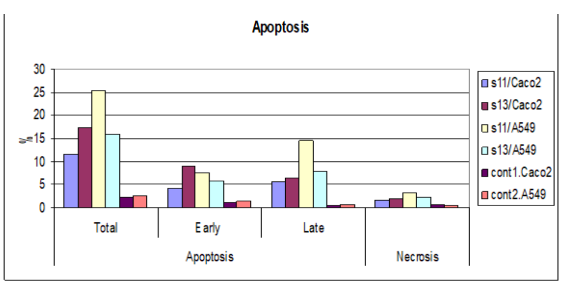

Figure 6: Induction of apoptosis and necrosis among Colon (Caco2) and lung (A549) cancer cells due to incubation with *C. spicatus* (S11) and *A. vogelii* (S13) methanol extract.

3.5. Determination of the expression levels of apoptosis-regulatory genes

The mRNA level of apoptosis-related genes Bax and Bcl-2 in Caco-2 and A549 cell lines was determined (Figure 7).

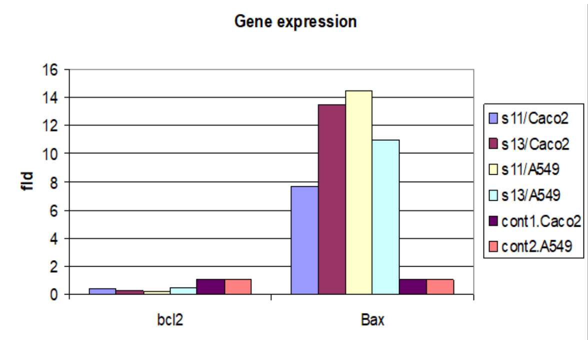

Figure 7: Effects of *C. spicatus* (S11) and *A. vogelii* (S13) methanol extract on Bax and Bcl-2 mRNA expression levels among Colon (Caco2) and lung (A549) cancer cells.

The mRNA levels of Bax and Bcl-2 were estimated by real- time PCR. Treatment with *C. spicatus* as well as *A. vogelii* extracts induced up regulation of Bax gene in both utilized cancer cell lines and the down regulation of bcl-2 gene was observed in the both cell lines.

Treatment with *C. spicatus* and *A. vogelii* caused significant increase of mRNA level ($P \leq 0.001$) in Bax gene (7.6 and 13.5 folds) in Caco-2 and (14.5and 10.9 folds) in A549 cancer cells respectively. In contrast, the Bcl-2 mRNA level was decreased due to treatment with *C. spicatus* and *A. vogelii* (0.4 and 0.27 folds) in CAco-2 and (0.16 and 0.5 folds) in A549 cancer cells, respectively.

4. DISCUSSION

Natural products play a significant function in the discovery of new medications. More than 50% of the newly approved drugs are of natural product origin or designed based on the structure of natural product, whereas the synthetic and synthetic with natural product mimic compounds share forty percent of the newly developed drugs (Newman & Cragg, 2007). The target of the present work is to examine the anticancer potentiality of *C. spicatus* and *A. vogelii*; in addition, to investigate the molecular pathways including cell cycle arrest and apoptotic induction of *C. Spicatus* and *A. vogelii*.

Methanol extract of each plant was prepared and utilized. Two cancer cell lines of Colon (Caco-2) and lung (A549) were used. The TPC, TFC and antioxidant power of each plant extract were measured. The cytotoxic activity of each extract against the utilized two cancer cell lines was assessed. Induction of apoptosis was examined. The mRNA level of Bax and Bcl-2 apoptosis regulatory genes was detected.

The TPC, TFC and antioxidant power for *C. Spicatus* methanolic extract was higher than *A. vogelii*.

The observed highest antioxidant power of *C. spicatus* than *A. vogelii* might be due to the highest TPC and TFC fractions of *C. spicatus* than *A. vogelii* (Malik *et al.,* 2016). Reactive oxygen

species (ROS) play an important role in the oxidative damage of biological systems (Malik *et al.,* 2016). ROS readily combine and oxidize bio-molecules and thus making them indolent with subsequent damage to cells, tissues, and organs leading to cancer progression (Ghagane *et al.,* 2017). Herbal extracts rich in phenolic, flavonoids and tannins, have a natural antioxidant activity may be caused by their redox characteristics and chemical structures (Sanaye and Pagare, 2016). Strong correlation between antioxidant activity, TPC and TFC of Moroccan Pomegranate was reported (Eddebbagh *et al.,* 2016).
In the scope of IC50, cytotoxic effect of *C. spicatus* on the cancer cell lines Colon (Caco-2) and lung (A549) was higher than *A. vogelii*. This result might be due to the increase of TPC and TFC within *C. Spicatus* methanolic extract than *A. vogelii*. Mahmoudi *et al.* (2016) reported that, the biological activities were correlated with phytochemical contents of the plant extracts. High correlation coefficient (R^2) between cytotoxic activity and TPC and TFC among different cancer cell liens was reported (Eddebbagh *et al.,* 2016).
Moreover, the obtained results indicated that, the C. *spicatus* and *A. vogelii* extracts exerted clear cytotoxic activity against human Caco-2 and A549cells, foremost via cell cycle arrest at the G2/M phase associated with the preG1 apoptosis induction, which was verified by the significant increase in apoptotic cell populations. It is recognized, that cellular growth and proliferation of mammalian cells are mediated by cell cycle progression. Moreover, inhibition of the cell cycle has been an effective strategy for eliminating cancer cells (Cho *et al.,* 2011). Guo *et al.* (2012) reported that *Astragalus* saponins can prevent HT-29 human colon cancer cell distribution during the collection in S phase and G2/M arrest, and elevate apoptosis in HT-29 cells meanwhile Caspase 3 activation and poly (ADP-ribose) polymerase cleavage and Auyeung *et al.* (2010) confirmed these results. Ye *et al.* (2011) likewise, explained that the *Astragalus mongholicus* treatment may inhibit proliferation induce apoptosis in human breast cancer cell lines. Whoever, in comparison with control cancer cells, the treated cells with methanolic extract of *C. spicatus* than *A. vogelii* tends to shift from viable to apoptotic cells. Apoptotic activity of Convolvulaceae family members was reported by (Al-Asady *et al.,* 2014; Dewanjee *et al.,* 2015). It has been widely reported that the induction of apoptosis is one of the active strategies for arresting the proliferation of cancer cells (Pistritto *et al.,* 2016). Apoptosis is the greatest concentrate and target for cancer study since the cells killed through this mode of cell death do not induce an inflammatory reaction which may lead to different adverse side effects (Rahman*et al.,* 2017).
The results revealed that, the apoptotic and necrotic cell populations increased significantly in both Caco-2 and A549 cells due to incubation with methanol extracts of *C. spicatus* and *A. vogelii* when compared with the control cells (Figure 6). But the apoptosis percent was increased significantly than necrosis in all treatment with all utilized cancer cell lines. Early apoptotic events were higher than late apoptotic events among two cancer cell lines due to the treatment with plant extracts.
The exhibited apoptotic potentiality of *C. spicatus* and *A. vogelii* may be due to the TPC and TFC as reported by several studies (Sukardiman *et al.,* 2000; Yamashita and Kawanishi 2000; Al-Asady *et al.,* 2014; Esmaeili *et al.,* 2015, Fitriansyah *et al.,* 2018). It has been reported that, the TPC and TFC are promoting apoptosis and induction of cell cycle arrest through the inhibition of DNA replication (Sukardiman *et al.,* 2000; Yamashita and Kawanishi 2000; Panche *et al.,* 2016). Obtained results pointed to, that all the two plant extracts exerted cytotoxicity on Caco-2 and A549 cells via apoptosis.
The effect of *C. spicatus* and *A. vogelii* on the expression levels of apoptosis regulatory genes (Bax and Bcl-2) was investigated among Caco-2 and A549.Previously published two studies have shown that *Astragalus* L .and Convolvulaceae family members (*Astragalus polysaccharide* and *Convolvulus arvensis*) respectively stimulate induction of cancer cell apoptosis and cell cycle arrest (Al-Asady *et al.,* 2014; Wu *et al.,* 2017).
The obtained results showed that, the *C. spicatus* and *A. vogelii* induce apoptosis which is elicited through Bax and Bcl-2 genes. The Bcl-2 family of genes had contained the pro-apoptotic and anti-apoptotic members as bax and bcl-2 respectively. (Miyoshi *et al.,* 2003). Bcl-2 as a key regulator of apoptosis promotes cell survival either by inhibiting agents for the activation of caspases (Ling *et al.,* 2002) or by regulation of apoptosis though active functional antagonism through the formation of heterodimers with other Bcl-2 family members. Bax, a pro-apoptotic member, furthermore, binds to the anti-apoptotic Bcl-2 protein and therefore acts by antagonizing the function of Bcl-2 to abrogate apoptosis. Moreover, the induction of Bax is also observed to promote cytochrome c release from the mitochondria, which finally leads to apoptosis (Thomas *et al.,* 2000). In this research, an apparent increase in the expression of Bax and an associated lowering in Bcl-2 mRNA expression levels, as detected by quantitative RT-PCR, were reported in the Caco-2 and A549 treated with *C. spicatus* and *A. vogelii* methanol extracts. Its suggested that both the Bcl-2 family of proteins (Bax and Bcl-2) play a pivotal role in *Convolvulus spicatus* and *Astragalus vogelii* induced apoptotic cell death of Caco-2 (Colon cancer) and A549 (Lung cancer) cells. Thus, the results suggest that, the observed up- regulation of Bax and the corresponding down-regulation of Bcl-2 genes may be one of the critical mechanisms through which *C. spicatus* and *A. vogelii* induces apoptosis in Caco-2 and A549 cells.

5. CONCLUSION

This study concluded that, that *Convolvulus spicatus* and *Astragalus vogelii* induced apoptotic cell death and suggests that *Convolvulus spicatus* and *Astragalus vogelii* could be used as an apoptosis-inducing anticancer agent for colon and lung cancer treatment with further larger detailed studies.

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