Saudi Pharmaceutical Journal 28 (2020) 985–993

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Anastatica hierochuntica (L.) methanolic and aqueous extracts exert antiproliferative effects through the induction of apoptosis in MCF-7 breast cancer cells

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ARTICLE INFO

Article history:
Received 24 February 2020
Accepted 29 June 2020
Available online 9 July 2020

Keywords:
Anastatica hierochuntica
Apoptosis
P53
Cell cycle
Breast cancer
Natural products

ABSTRACT

Breast cancer therapy using anticancer bioactive compounds derived from natural products as adjuvant treatment has gained recognition due to expensive and toxic conventional chemotherapeutic drugs. The whole plant of *Anastatica hierochuntica* (L.) (*A. hierochuntica*) has been investigated for its pharmacologically important anticancer properties but without categorizing the biological activities of the plant parts. We assessed the anticancer potential of different parts of *A. hierochuntica* (seeds, stems and leaves) and explored their mechanisms of action using the human breast cancer cell line, MCF-7. Currently, we investigated the antiproliferative effects of methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts of *A. hierochuntica* plant parts on the MCF-7 cells using cell viability assays. Flow cytometry, Western Blot, DNA fragmentation, and gene expression assays were performed to evaluate apoptosis and cell cycle regulatory proteins. The results indicate that the methanolic and aqueous extracts decreased MCF-7 cell viability in a dose-dependent manner. The induction of apoptosis was observed in all the methanolic and aqueous-treated MCF-7 cells. The cell death process was confirmed by the visualization of DNA fragmentation and cleavage of the intrinsic apoptotic pathways, caspase-9 and caspase-3, the key enzyme causing apoptosis hallmarks. In addition, the most pro-apoptotic extracts, ASD and ML, up-regulated the expression of pro-apoptotic Bax, tumor suppressor TP53 genes and the cyclin inhibitor CDKN1A gene. In conclusion, of the aqueous and methanolic extracts of *A. hierochuntica* plant parts exerting antiproliferative effects through the induction of apoptosis in breast cancer MCF-7 cells, ASD and ML extracts were the most promising natural-based drugs for the treatment of breast cancer.

1. Introduction

Breast cancer (BC) is a prevalent cancer and the primary cause of cancer-related mortality in women globally (Torre et al., 2017). Despite the number of anticancer treatment strategies for BC, including surgery, radiotherapy, supported by gene therapy, immunotherapy, adjuvant endocrine and chemotherapy, the likelihood of resistance to antineoplastic agents remains high (Tang et al., 2016). Research related to BC treatment has increased globally with the identification of novel anticancer therapeutic agents (phytochemicals) in natural products from the plant kingdom (Shareef et al., 2016). The plant-derived secondary metabolites, which are a part of traditional medicine practice, gained accep-

Abbreviations: AL, aqueous extract of *A. hierochuntica's* leaf; ASD, aqueous extract of *A. hierochuntica's* seed; AST, aqueous extract of *A. hierochuntica's* stem; BC, breast cancer; CDK, cyclin-dependent kinase; MCF-7, Michigan Cancer Foundation-7; ML, methanolic extract of *A. hierochuntica's* leaf; MSD, methanolic extract of *A. hierochuntica's* seed; MST, methanolic extract of *A. hierochuntica's* stem; NP40, Nonidet P-40; STS, Staurosporine; TP53, tumor protein p53.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2020.06.020
1319-0164/

tance due to their safety and efficiency compared to chemically-derived medication (Welz et al., 2018). In addition, phytochemicals exhibit chemotherapeutic-like effects after the induction of cell death by apoptosis in tumor cells, through the mitochondrial intrinsic or death receptor extrinsic pathways. These pathways involve caspases, which activate cell cycle arrest, following the inhibition of the actions of cyclins and cyclin-dependent kinases (CDKs) (Bailon-Moscoso et al., 2017; Shahwar et al., 2019) or by prompting microtubule damage (Paul et al., 2020). The discovery of pro-apoptotic phytochemicals and the evaluation of their molecular mechanisms of action enhanced the anticancer therapeutic approach for the development of natural bioactive compounds, denuded of undesirable side effects caused by toxic conventional chemotherapeutic agents (Oronsky et al., 2016).

Anastatica hierochuntica (L.) (*A. hierochuntica*) is a monotypic species belonging to the *Brassicaceae* family, a desert plant with a tumbleweed habit and popularly known as Kaff Maryam (Mary's hand), True Rose of Jericho or Genggam Fatimah (Saleh and Machado, 2012). This plant is widely spread in the Middle East and North Africa and recognized for its resurrection nature (Friedman and Stein, 1980). *A. hierochuntica* related ethnomedicinal practices are documented as easing childbirth and reducing uterine hemorrhage (Khalifa and Ahmad, 1980) as well as used in the treatment of asthma, respiratory diseases, dysentery, colds, fevers, and headaches (Mossa et al., 1987). It is also used to combat conjunctivitis and sterility (AlGamdi et al., 2011). The chemical constituents of *A. hierochuntica* have been investigated for various pharmacologically important properties including antimicrobial (Daoowd, 2013; Tayel and El-Tras, 2009), anti-inflammatory (Abou-Elella et al., 2016; Rizk et al., 1985), nitric oxide inhibitory effects (Yoshikawa et al., 2003a), hypolipidemic (AlAzzawie and Shaban, 2011; Salah et al., 2011), hypoglycemic (Rahmy and El-Ridi, 2002), hepatoprotective (Yoshikawa et al., 2003b), and gastroprotective (Shah et al., 2014) activities using *in vitro* and *in vivo* studies. In addition, novel bioactive compounds such as Anastatin A and B (Yoshikawa et al., 2003b) and hierochins A, B and C (Yoshikawa et al., 2003a) have been identified in the *A. hierochuntica* plant.

Limited literature is available regarding the antiproliferative activity of *A. hierochuntica* plant extracts on mammalian cell lines (Ali et al., 2014; Abou-Elella et al., 2016; Mohammed et al., 2015). Detailed studies related to the anticancer activity of *A. hierochuntica* is limited to its anti-melanogenesis (Nakashima et al., 2010) and anti-cervical cancer activities (Hajjar et al., 2017), using the methanolic extract of the whole plant. No information about the plant parts with possible antitumor properties against BC, is currently available. The present study was undertaken to assess the antiproliferative effect of *A. hierochuntica* plant part (stem, seed and leaf) crude extracts on a hormone-dependent human BC cell line, MCF-7, and to investigate the underlying cell death molecular mechanisms including apoptosis.

2. Methods

2.1. Plant material collection and extraction

A. hierochuntica (L) was collected from the western region of Mecca City in the Kingdom of Saudi Arabia during February and April 2018. The plant was in the dried condition and authenticated by a native herbalist. The whole plant was segregated in stem, seeds and leaves and ground to fine particles using a mechanical grinder. The samples were extracted from 100 g of each part of the powdered plant material with either 300 mL of methanol, resulting in methanolic extracts from the stem (MST), seed (MSD), and from the leaf (ML) or with water, resulting in aqueous extracts from the stem (AST), seed (ASD), and from the leaf (AL). The extraction was done at an ambient temperature with continuous shaking for 3 days (Buss and Butler, 2010). Each filtrate solvent was filtered through a 0.22 μm filter and concentrated using a RV3V rotary evaporator (IKA, Staufen, Germany) with reduced pressure and low temperature. The yield of each extract was weighed and stored at 4 °C until use. A voucher sample is stored at the laboratory for future reference.

2.2. MCF-7 cell line culture

The hormone-dependent human BC cell line, Michigan Cancer Foundation (MCF)-7, was obtained from the American Type Culture Collections (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% of fetal bovine serum (FBS), 2 mM of glutamine and 1% antibiotics composed of 100 IU/ml of penicillin and 100 μg/ml of streptomycin. All the cell culture reagents were procured from Gibco® (Thermo Fisher Scientific, Waltham, MA, USA). The cell line was maintained at 37 °C in a humidified 5% CO_2-incubator. The MCF-7 cells were passaged when they attained 70–80% confluence or were harvested and used for downstream applications.

2.3. Cell viability assay

The MCF-7 cells were seeded at a density of 5×10^3 cells/well/100 μl in opaque white Costar® 96-well plates (Corning, Thermo Fischer Scientific). After 24 h incubation, cells were treated with various concentrations (10, 100, 250, 500, 1000 μg/ml) of methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts of the plant parts. The wells containing media and cells without treatment served as blank and control, respectively. Cell viability was measured after 72 h using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Luminescence was measured using an EnVision 2105 XCite multimode plate reader (PerkinElmer, Waltham, MA, USA) and the results were given in relative light units (RLU).

2.4. Flow cytometry

The MCF-7 cells were seeded at a density of 5×10^5 cells/ml in a Corning® 24-well plate. After 24 h incubation, cells were treated with either the half-maximal inhibitory concentration on cell viability (IC_{50}) or 400 μg/ml of methanolic (MSD, MST, ML) or aqueous extracts (ASD, AST, AL) of the plant parts for an additional 24 h incubation. The cells exposed to 1 μM of staurosporine (STS) (Santa Cruz biotechnology, Dallas, TX, USA) were the positive control for the induction of apoptosis. The untreated cells, the control and unstained cells, were also used in the apoptotic status analysis. The cells were stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and phycoerythrin (PE)-labeled propidium iodide (PI), according to the manufacturer's instructions for the FITC-Annexin V Apoptosis Detection Kit I (Becton Disckinson Biosciences, San Diego, CA, USA). The apoptotic status of 10,000 cells were analyzed with the Becton Dickinson (BD) FACSCanto™ flow cytometer using BD FACSDiva™ 6.0 software (BD Biosciences), and characterized by Annexin V^{+ve}/PI^{-ve} for early apoptosis and by Annexin V^{+ve}/PI^{+ve} for late apoptosis. Viable and necrotic cells were also detected as characterized by Annexin V^{-ve}/PI^{-ve} and by Annexin V^{-ve}/ PI^{+ve}, respectively.

2.5. DNA fragmentation assay

The MCF-7 cells were seeded at a density of 1×10^6 cells/ml in a Corning® 12-well plate. After attachment, the cells were treated

with either 400 μg/ml of methanolic (MSD, MST, ML) or aqueous (ASD, AST, AL) extracts of the plant parts for 24 h incubation. STS-treated cells were used as the positive control with the untreated cells, the control. The DNA of treated and untreated cells was extracted using the ApoTarget™ Quick Apoptotic DNA Ladder Detection (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA was analyzed in 1.8% agarose gel stained with 0.5 μg/ml of ethidium bromide on HE 33 Mini Submarine Electrophoresis Unit (Thermo Fisher Scientific, Waltham, MA, USA). DNA fragmentation was visualized with the UVP Benchtop ultraviolet illuminator (Thermo Fisher Scientific, Waltham, MA, USA) and photographed.

2.6. Protein extraction and Western blot analysis

The MCF-7 cells were seeded at a density of 5×10^5 cells/ml in a 12-well plate. The next day, the cells were treated with 100 and 400 μg/ml of the methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts of the different parts of the plant and incubated for 24 h. The STS-treated cells were used as the positive control. The cell lysates were prepared by adding 80 μl of NP40 lysis buffer (Invitrogen, Carlsbad, CA, USA) as previously described (Matou-Nasri et al., 2017). The extracted proteins were estimated according to the Qubit® Protein assay kit protocol (Invitrogen, Carlsbad, CA, USA) and the sample protein concentrations were read by the Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA). About 150 μg of the estimated proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN® Tetra cell (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The blocking of the PVDF membranes was done with the Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h, followed by probing the membranes with blocking buffer-diluted mouse or rabbit primary antibodies (1:1000 dilution) targeting pro- and cleaved forms of caspase-3, and caspase-9 (Cell Signaling Technology, Danvers, MA, USA) and targeting GAPDH (Abcam, Cambridge, UK) at 4 °C overnight with continuous shaking. The primary antibodies were detected with infrared fluorescent IRDye® 680RD (red)-conjugated goat anti-rabbit or IRDye® 800RD (green)-conjugated goat anti-mouse secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) which were diluted (1:5000 dilution) in Odyssey® blocking buffers and incubated for 1 h at room temperature with continuous agitation. After washing, the blots were scanned and analyzed using a LI-COR Odyssey CLx Scanner (LI-COR Biosciences, Lincoln, NE, USA).

2.7. RNA extraction and reverse transcriptase-quantitative real-time polymerase chain reaction (RT-qPCR)

The MCF-7 cells (1.5×10^6) were seeded into each well of a Corning® 6-well plate. The next day, the cells were treated with 400 μg/ml of the ML methanolic extract and of the ASD aqueous extract for 24, 48 and 72 h of incubation. Total RNA was extracted by applying the protocol for the purification of total RNA from mammalian cultured cells using the GeneJET™ RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by measuring the absorbance at 260/280 nm on the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A cDNA conversion was performed with the two-step protocol using oligo(dT) primers by GoScript™ Reverse Transcription System (Promega Corporation). A relative quantification procedure was performed in the Applied Biosystems™ 7500 Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA) and selected primer pairs of sequences (Invitrogen, Carlsbad, CA, USA) to monitor the expression of these following genes: *Bcl-2*, 5′-AAGATTGATGGGATCGTTGC-3′ (*forward*), 5′-GCGGAACACTT GATTCTGGT-3′ (*reverse*); *tp53*, 5′-TGT GGAGTATTTGGATGACA-3′ (*forward*), 5′-GAACATGAGTTTTTTATGGC-3′ (*reverse*); *Bax*, 5′-GTGC ACCAAGGTGCCGGAAC-3′ (*forward*), 5′-TCAGCCCATCTTCTTCCAGA-3′ (*reverse*); *Bcl-xL*, 5′-CCCAGAAAGGATACAGCTGG-3′ (*forward*), 5′-GCGATCCGACTCACCAATAC-3′ (*reverse*); *GAPDH*, 5′-AAGGTCGG AGTCAACGGATTTGGT-3′ (*forward*), 5′-ATGGCATGGACTGTGGTCA TAGT-3′ (*reverse*); *CDKN1A*, 5′-G CGATGGAACTTCGACTTTGT-3′ (*forward*), 5′-GGGCTTCCTCTTGGAGAAGAT-3′ (*reverse*); *Cyclin A*, 5′-G TCACCACATACTATGGACATG-3′ (*forward*), 5′-AAGTTTTCCTCT CAGCACTGAC-3′ (*reverse*); *Cyclin D1*, 5′-TCCAGAGTGAT CAAGTGTGA-3′ (*forward*), 5′-GATGTCCACGTCCCGCACGT-3′ (*reverse*); *Cyclin E*, 5′-AATAGAGAGGAAGTCTGG-3′ (*forward*), 5′-AGA TATGACACCTGCATG-3′ (*reverse*). Each monitored gene expression was related to that of the internal control GAPDH. The relative changes in the expression of the specified gene were analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

The data are expressed as mean ± standard deviation (SD) or as mean ± standard error (SE) of experiments performed in triplicate and each experiment was independently repeated at least three times. The cytotoxicity results are expressed as percentage cell survival compared to the untreated control. The IC_{50} values were calculated by a nonlinear dose/response regression model using the GraphPad Prism software, version 6 for Windows (GraphPad Software, La Jolla, CA, USA, http://www.graphpad.com/). The statistical analysis of the gene expression studied was evaluated by Freidman's 2-Way ANOVA by ranks followed by post-hoc pairwise multiple comparison tests. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Methanolic and aqueous extracts of A. hierochuntica inhibit BC MCF-7 proliferation

Methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts of the *A. hierochuntica* plant parts (seed, stem, leaf) were tested for their competency to inhibit the proliferation of BC MCF-7 cells. Cell exposure to increasing concentrations of methanolic (Fig. 1A) and aqueous (Fig. 1B) extracts resulted in a decrease of the cell viability in a dose-dependent manner, compared with the untreated cells, the 100% (Fig. 1). The IC_{50} value corresponding to the concentration causing 50% inhibition of MCF-7 cell viability was determined for each extract. It was revealed that the aqueous ASD extract exhibited the lowest IC_{50} value (282.50 μg/ml, Table 1) followed by the methanolic ML extract (293.27 μg/ml, Table 1). The highest IC_{50} values were obtained from the methanolic MSD extracts (458.90 μg/ml, Table 1) and methanolic MST (539.62 μg/ml, Table 1).

3.2. Methanolic and aqueous plant part extracts induce apoptosis in MCF-7 cells

The BC MCF-7 cells were treated with methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts, examined for the detection of the apoptotic status using flow cytometry based on Annexin V-FITC/PI-PE double staining. The cell treatments with 400 μg/ml of the plant part extracts indicated a significant ($p < 0.05$) decrease in the percentage of viable ($AnnexinV^{-ve}$/ PI^{-ve}) MCF-7 cells compared to the untreated cells, the control (Fig. 2). The concomitant increase in the proportion of early ($AnnexinV^{+ve}$/ PI^{-ve}) and late

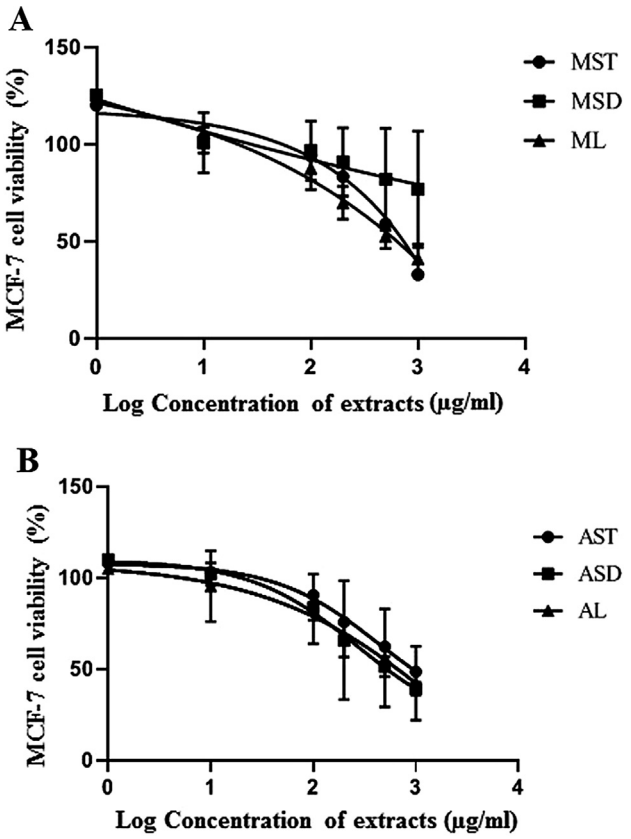

Fig. 1. Effects of methanolic (A) and aqueous (B) extracts on MCF-7 cell viability. The MCF-7 cells were treated with increasing concentrations (10–1000 µg/ml) of methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts for 72 h incubation. Cell viability was determined using CellTiter-glo® kit and expressed as percentage of the control, the untreated cell viability, corresponding to 100%. The results are presented as mean ± SD of three independent experiments.

Table 1
IC_{50} values of methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) of *Anastatica hierochuntica* extract concentrations resulting in 50% decrease of MCF-7 cell viability.

Extracts	IC_{50} values (µg/ml)
MSD	458.899
MST	539.619
ML	293.268
ASD	282.501
AST	448.054
AL	375.226

(AnnexinV^{+ve}/ PI^{+ve}) apoptotic cells were observed in the treated groups compared to the control, with only marginal differences in the percentage of necrotic (AnnexinV^{-ve}/ PI^{+ve}) cells (Fig. 2B). The methanolic and aqueous extracts of the plant parts induced, on average, approximately 40–50% apoptosis (early and late) in MCF-7 cells (Fig. 2). Specifically, both methanolic and aqueous extracts of the plant leaves and STS treatment had similar proportions of late induction of apoptosis in MCF-7 cells, 17.8% ($p < 0.005$), 18.2% ($p < 0.002$) and 21.8% ($p = 0.0001$), respectively. However, there was not a significant difference in terms of apoptosis induction, observed between the STS-treated cells and cells treated with methanolic and aqueous extracts of the plant parts (Fig. 2B). The cell treatments with the plant part extracts tested at their IC_{50} inhibitory concentration (Supplementary Figure) showed no significant differences in the percentage of viable cells, early and late apoptosis compared to the apoptotic status of the cells treated with 400 µg/ml of each plant extract (Fig. 2). The visualization of the DNA fragmentation in apoptotic cells was analyzed by isolating the genomic DNA from the MCF-7 cells, following exposure to (400 µg/ml) crude extracts of the plant parts for 24 h incubation. A "DNA ladder" formation was observed when the degraded DNA extracted from the apoptotic cells was subjected to gel electrophoresis (Fig. 3). Aqueous AL and methanolic extracts of the plant parts (MSD, MST, ML) showed a clear degradation of chromosomal DNA into small internucleosomal fragments (Fig. 3). As expected, intact genomic DNA was observed from the healthy untreated cells (Fig. 3).

3.3. Methanolic and aqueous extracts induced the apoptosis trigger caspase-9 and caspase-3 cleavage in MCF-7 cells

The induction of apoptosis by exposing the MCF-7 cells to 100 µg/ml and 400 µg/ml of methanolic and aqueous extracts of the plant parts were confirmed through the assessment of the expression of proteins involved in apoptotic pathways using a Western blot analysis. Fig. 4 depicts the decreased and increased expression of procaspase-3 and cleaved-caspase-3, respectively in the extracts-treated MCF-7 cell groups. Increased expression levels of cleaved-caspase-9 and a reduced expression of procaspase-9 were also observed in cells treated with the sets of plant extracts (Fig. 4). As expected, no cleavage of caspase-3 and caspase-9 was observed in viable untreated MCF-7 cells, the control (Fig. 4).

3.4. Methanolic and aqueous extracts up-regulate the expression of the pro-apoptotic Bax gene, tumor suppressor TP53 gene and the cyclin-dependent kinase inhibitor CDKN1A gene

The antiproliferative effects of ASD and ML extracts on MCF-7 cells were investigated by monitoring, over time (24, 48, and 72 h of incubation), the expression levels of apoptosis and cell cycle-related genes (Bcl-2, Bcl-xL, Tp53, Bax, CDKN1A, Cyclin A, D1 and E) using RT-qPCR. The basal level of gene expression was determined in each group of untreated MCF-7 cells (i.e. control), harvested at the different time intervals. No significant difference between each basal level of gene expression over the time was observed (data not shown). The MCF-7 cell treatment with 400 µg/ml of ASD, significantly increased the levels of gene expression for Bax (1.9-fold, $p = 0.000005$), TP53 (2.5-fold, $p = 0.000005$) and CDKN1A (3.5-fold, $p = 0.000005$) after 48 h incubation, compared to the basal level of gene expression detected in the control, the untreated cells (Fig. 5A). Bcl2 and Cyclin E gene expression levels were down-regulated by 0.2-fold ($p = 0.000005$) and 0.5-fold ($p = 0.010313$), respectively. However, the Bcl-xL and Cyclin D1 gene expression levels were not statistically significant compared to the control (Fig. 5A). The treatment of the MCF-7 cells with ML, significantly up-regulated the expression of Bax (2.9-fold, $p = 0.000001$ and 2.5-fold, $p = 0.002$), TP53 (3.6-fold, $p = 0.000005$ and 2.9-fold, $p = 0.0056$) and CDKN1A (3.2-fold, $p = 0.000005$ and 2.6-fold, $p = 0.005602$) at 48 h and 72 h of cell treatment, respectively (Fig. 5B). A significant decrease in the expression levels of Bcl-xL (0.1-fold, $p = 0.0000005$), Bcl2 (0.3-fold, $p = 0.002937$), Cyclin D1 (0.1-fold, $p = 0.005602$) and Cyclin E (0.2-fold, $p = 0.005602$) were only detected in the ML-treated MCF-7 cells at 48 h incubation (Fig. 5B). The gene expression level of Cyclin A remained unaffected in both ASD and ML-treated MCF-7 cells, at all the incubation periods (24, 48 and 72 h), compared with the basal level of Cyclin A gene expression detected in the untreated cells, the control (Fig. 5B).

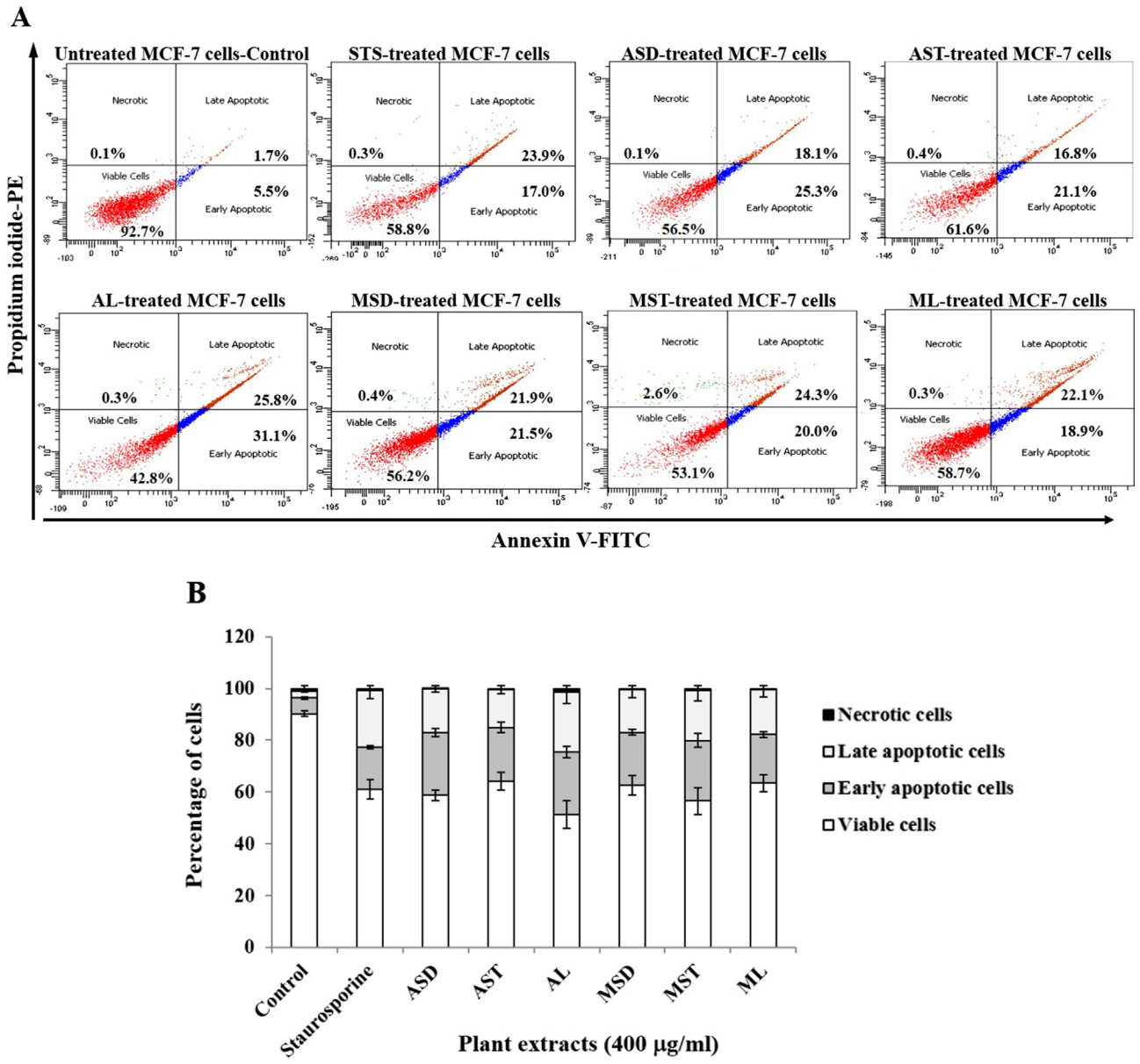

Fig. 2. Effects of both methanolic and aqueous extracts on the induction of apoptosis in MCF-7 cells. The MCF-7 cells were untreated, treated with 400 µg/ml of methanolic and aqueous extracts along with 1 µM STS (positive control) for 24 h followed by FITC-labeled Annexin V/PE-labeled PI double-staining for apoptosis analysis using FACS. (A) Numbers within scatter plots represent the percentage of viable (lower left, Annexin V^-/PI^-), early apoptotic (lower right, Annexin V^+/PI^-), late apoptotic (upper right, Annexin V^+/PI^+), and necrotic cells (upper left, Annexin V^-/PI^+). (B) Bar graphs present the percentage viable, apoptosis, and necrosis of MCF-7 cells in the presence or absence of various concentrations of each extract along with the untreated (control) and STS-treated cells, based on three independent experiments. The results are presented as mean ± SE of six independent experiments.

4. Discussion

The induction of apoptosis and cell growth arrest are the safest approaches for cancer therapy, in contrast to conventional neo-adjuvant anticancer agents, which are toxic with a high risk of triggering inflammation and side effects due to damaged and necrotic cells (Mou et al., 2019; Pfeffer and Singh, 2018; Samadi et al., 2015). From an economical perspective, conventional anticancer agents are expensive. However, natural compounds with potential anticancer activities are mostly inexpensive (Chawla et al., 2018; Seca and Pinto, 2018), underpinning the interest in the discovery of pro-apoptotic natural bioactive compounds for the development of new complementary and alternative anticancer agents. The qualitative and/or quantitative measurement of apoptotic events and the investigation of related downstream target genes are pivotal to establish and validate the mechanisms of action employed by natural product extracts to activate apoptosis (Taraphdar et al., 2001). In the current study, we provided evidence that methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts of *A. hierochuntica's* stem, seeds and leaves inhibited the viability of hormone-dependent human breast cancer, MCF-7, cell line through the induction of apoptosis involving the intrinsic-dependent apoptotic pathway. The up-regulation of the expression of pro-apoptotic target genes including Bax, and p53 with the cyclin-dependent kinase inhibitor CDKN1A (also known as p21), known to cause cell growth arrest, was observed.

It was evident that the plant extracts, after 72 h of treatment, exhibited antiproliferative activities on the MCF-7 cells in a dose-dependent manner. Both ASD and ML were efficient, based on the lowest IC_{50} value (Table 1). This finding supports Ali et al.

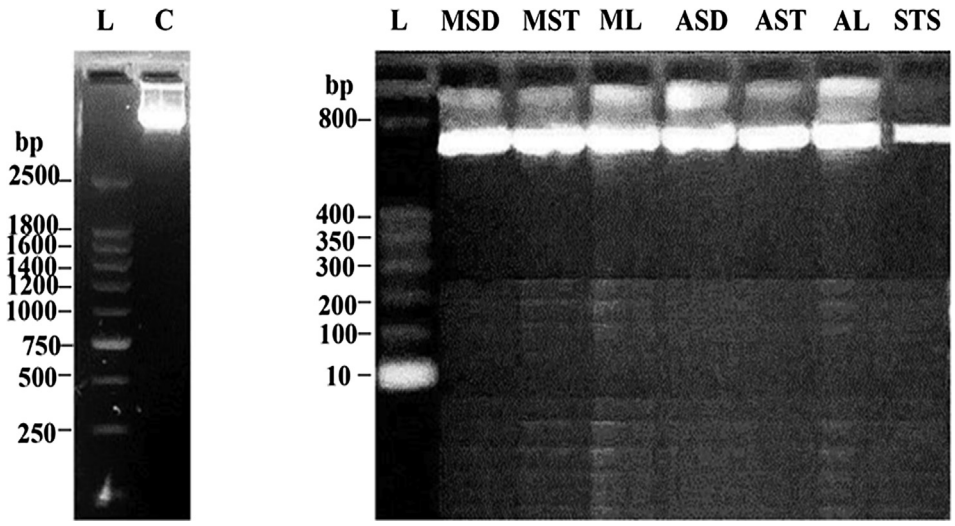

Fig. 3. Effects of both methanolic and aqueous extracts on DNA fragmentation in MCF-7 cells. Representative gels showing DNA fragmentation using ApoTarget™ Quick Apoptotic DNA Ladder detection kit in MCF-7 cells treated with 400 μg/ml of methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts along with STS-treated MCF-7 cells, confirming their apoptotic status after 24 h of cell treatment. Genomic DNA remained intact in untreated MCF-7 cells, the control (C).

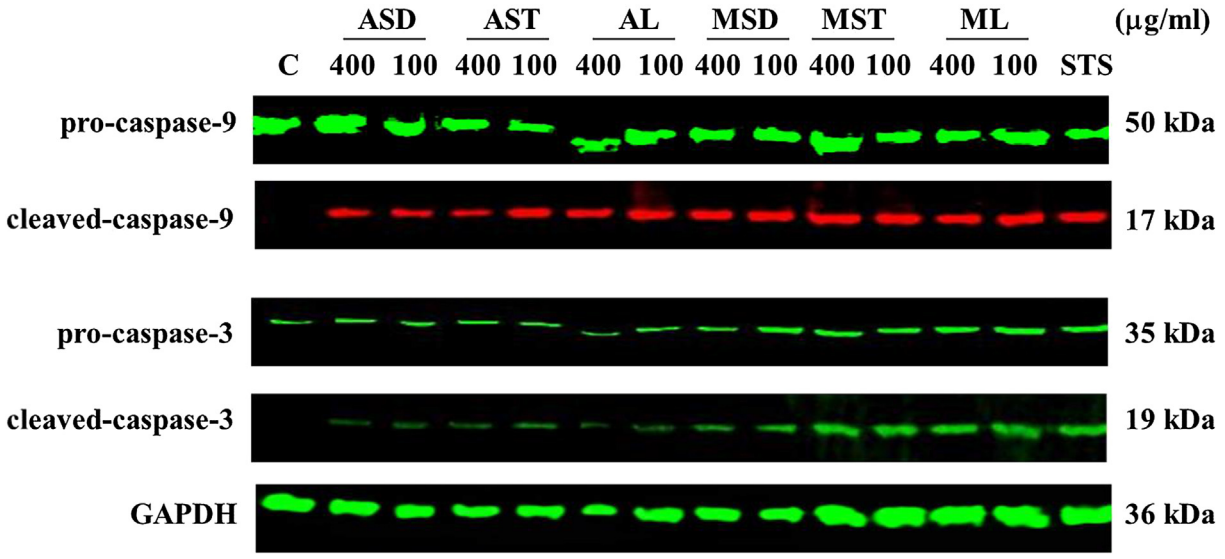

Fig. 4. Extracts-mediated apoptosis trigger caspase-9 and caspase-3 cleavage in MCF-7 cells. Representative Western blot gels showing the cleavage of caspase-9 (cleaved caspase-9) and that of caspase-3 (cleaved caspase-3) in MCF-7 cells after 24 h of treatment with 100 and 400 μg/ml of methanolic (MSD, MST, ML) and aqueous (ASD, AST, AL) extracts along with 1 μM STS, used as a positive control. No cleavage of caspase was observed in untreated MCF-7 cells, the control (C).

(2014) who reported that an ethanolic extract of the whole *A. hierochuntica,* inhibited 30% of the MCF-7 cell proliferation. The potential antiproliferative properties of *A. hierochuntica* have been extensively studied in various human and murine cell lines. Abou-Elella et al. (2016) reported that after 24 h of treatment with an ethyl acetate fraction, it displayed an IC_{50} of 3.6 μg/ml on the proliferation of the HeLa cell line, and Hajjar et al. (2017) demonstrated that a methanolic fraction of the same plant, treated for 24 and 48 h at 50 and 25 μg/ml concentration, inhibited 50% of the HeLa cell proliferation. An aqueous extract of *A. hierochuntica,* tested at 100 mg/ml concentration, inhibited 70% of AMN-3 cells (Mohammed et al., 2015). Previously, Nakashima et al. (2010) isolated 44 pure compounds from an ethyl acetate soluble fraction of *A. hierochuntica,* of which the named "compound 30" showed a high cytotoxic effect with an IC_{50} of 6.9 μM in B16 melanoma 4A5 cells. Similarly, methanolic and ethyl acetate soluble fraction inhibited hepatotoxicity by 68.7% and 80.9% respectively, in a D-galactosamine-induced model (Yoshikawa et al., 2003a). AlSobeai (2016) demonstrated that the *A. hierochuntica* methanolic, ethanolic and aqueous extracts, tested at more than ≥1000 μg/ml, exerted no cytotoxic effects on a Vero cell line, providing evidence that it is only toxic to cancer cells. It was reasonable to study the antiproliferative activities of the aqueous and organic extracts of *A. hierochuntica,* considering the wide range of effective concentrations depending on the type of cancer cells and the duration of treatment.

Natural programmed cell death, occurring due to apoptosis induced by the methanolic and aqueous extracts of the *A. hierochuntica* seed, stem and leaf parts, was evaluated based on Annexin V-FITC and PI-PE double staining using a flow cytometer and DNA fragmentation. During apoptosis, the plasma membrane loses its integrity resulting in phosphatidylserine (PS) residues exposed to the outer membrane, detectable with Annexin V while PI, a DNA-binding dye, is permeable in dead cells. The combination

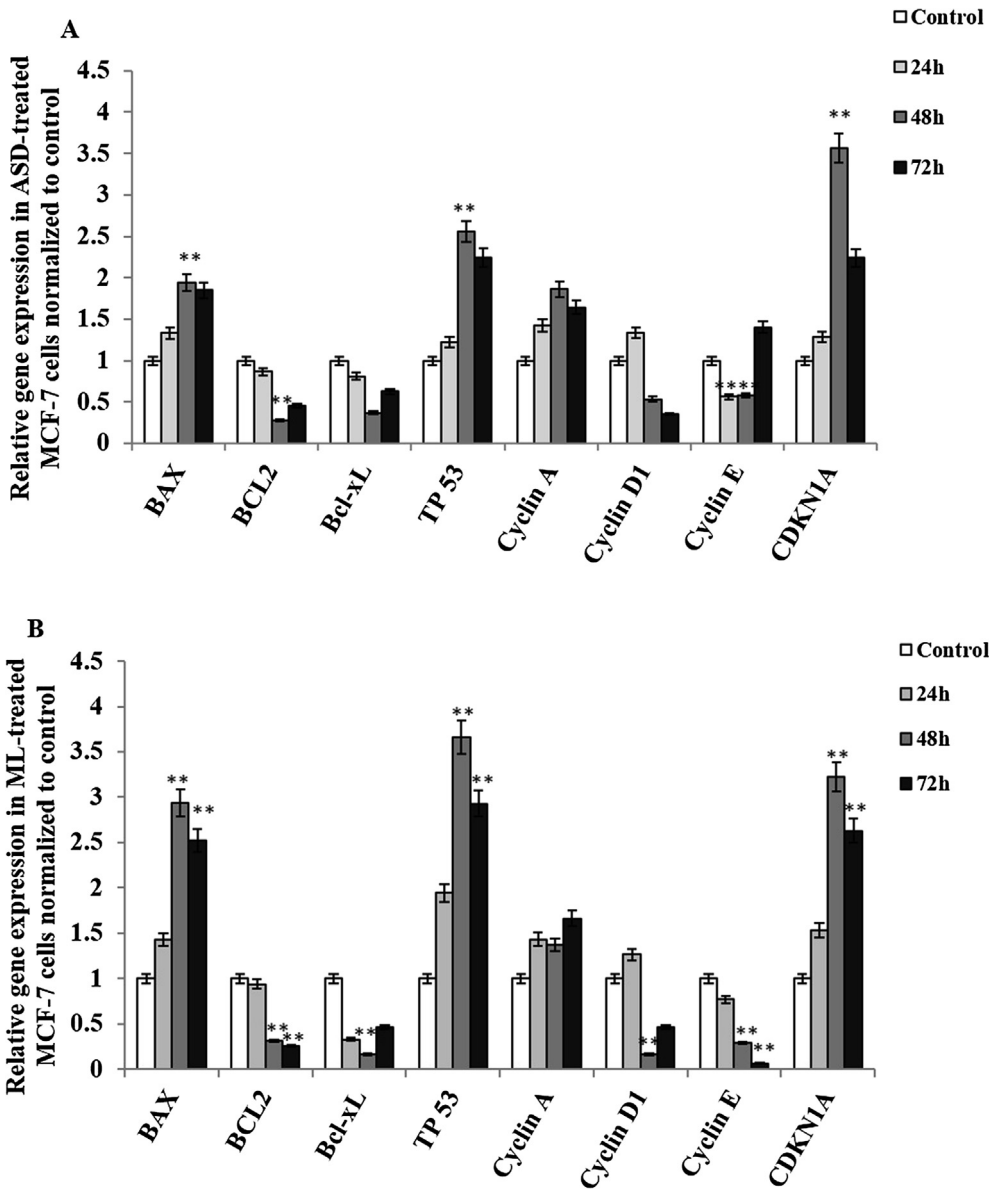

Fig. 5. Effects of both methanolic and aqueous extracts on apoptosis, tumor suppressor p53 and cell cycle-related gene expression detected in MCF-7 cells. The bar graphs show the relative expression of (BAX, BCL2, BC-xL) apoptotic-, tumor suppressor TP53 and (Cyclin A-D1-E, cyclin inhibitor CDKN1A) cell cycle-related transcripts determined by real-time polymerase chain reaction analysis in MCF-7 cells treated with 400 µg/ml of either ASD (A) or ML (B), as compared with the basal level of gene expression monitored in untreated MCF-7 cells, the control.

of both dyes are recommended for the identification of early and late apoptotic cells. Our findings suggest that both AL and MST extracts prompted the translocation of PS from the inner to the outer leaflet of the cell membrane, triggering apoptotic cell death in MCF-7 cells. The formation of a ladder-like pattern of fragmented DNA is an early morphological sign of apoptosis (Zhang and Xu, 2002). The ladder-like appearance of DNA occurs through the activation of an endogenous endonuclease that cleaves the DNA in the linker region between histones, which comprises of about 180–200 base pairs (bps), multiples of the bps are referred to as an "apoptotic" ladder. According to the current findings, the apoptosis inducing potential of MSD, MST, ML and AL extracts prompted DNA fragmentation in the MCF-7 cells. The confirmation of DNA fragmentation and apoptotic status of methanolic and aqueous extracts-treated MCF-7 cells are confirmed by assessing the pro/cleaved-caspase expression and by monitoring the gene expression of apoptotic regulators and tumor suppressor p53, known to be up-regulated following DNA damage (Williams and Schumacher, 2016).

The family of protein caspases plays an important role in several apoptotic pathways. Caspases are produced as inactive precursors (procaspases) and are mainly activated by specific proteolytic cleavage, following cell death receptor activation, the extrinsic apoptotic pathway (Parrish et al., 2013). The intrinsic apoptotic pathway can also be induced through the mitochondrial Bcl-2 family members, through the negative regulation of the anti-apoptotic protein expression of Bcl-xL and Bcl-2 (Burlacu 2003; Miyashita et al., 1994) and the positive regulation of the pro-apoptotic protein Bax, causing the release of cytochrome *c* from the mitochondria, a component of apoptosome, corresponding to the site of activation of caspase-9. Both extrinsic and intrinsic apoptotic pathways cause caspase-8 and caspase-9 activation convergence which activate caspase-3, the main enzyme responsible for plasma membrane reversion, nuclear and cytoplasmic protein degradation and

DNA fragmentation (Chinnaiyan, 1999; Du et al., 2000; Hill et al., 2004). As a general observation in the current study, the increased expression of apoptotic proteins, cleaved caspase-3 and cleaved caspase-9, were observed in MCF-7 cells treated with 100 and 400 μg/ml of each extract. Based on the extraction method, ASD and ML induced the highest caspase-3 cleavage. Reduced and increased expression of the mitochondrial-dependent pathway pro- and cleaved-caspase-9, were respectively detected in the methanolic extracts of the plant parts rather than the aqueous extracts. Hajjar et al. (2017) computed the protein level expression of cleaved-caspase-9 and TP53 in HeLa cells, at 50 μg/ml concentration for the methanolic fraction of the whole plant, by analyzing the fluorescence intensity using an image-based high-content screening (HSC) technique and investigated the capacity of the plant extract to stimulate the release of mitochondrial superoxide through the production of reactive oxygen species (ROS). The involvement of the cleaved forms of caspase-9 and caspase-3 in the current study support the statement that *A. hierochuntica* initiates apoptosis through the mitochondria-dependent intrinsic pathway.

The cell cycle progress after the heterodimer formation of cyclins and CDKs, which phosphorylate or aid in the intracellular localization of the proteins involved in the control of the cell division (mitosis). The CDKs interacting protein/Kinase inhibitory protein (CIP/KIP) inhibits the activities of the cyclins-CDKs complex as well as other proteins and enzymes called cyclin-dependent kinase inhibitors (CDKN). The CIP/KIP family activates the following three proteins, p21 (CDKN1A), p27 (CDKN1B) and p57 (CDKN1C) (Peter, 1997; Roberts et al., 1994; Sauer and Lehner, 1995). The p21 (CDKN1A) is activated by the transcriptional factor p53, which is expressed and activated in response to DNA damage caused by exogenous sources in cancerous cells and causes either cell cycle arrest for repair and survival or induce apoptosis if the attempt at repair failed (Levine and Oren, 2009). The increased expression of p21 acts as an inhibitor to the Cyclin D-CDK4,6 complexes, the cyclin E-CDK2 complex in the G1 phase and the Cyclin A-CDK2 complex in the S phase, by preventing its entry into the next phase of the cell cycle (Malumbres and Barbacid, 2009; Satyanarayana and Kaldis, 2009). The tumor suppressor protein p53 interacts and forms inhibitory complexes with anti-apoptotic Bcl-xL and Bcl-2 in mitochondria causing membrane permeability and cytochrome *c* release (Mihara et al., 2003), and cytosolic p53 activates the pro-apoptotic Bax by protein-protein interactions (Ha et al., 2003). Consequently, in the current study, the expressions of Bcl-2, Bcl-xL, TP53, Bax, CDKN1A, Cyclin A, D1 and E were studied and were effective at 48 h of MCF-7 cell treatment with ASD and ML. The up-regulation of p53 and p21 genes reached the highest level of expression detected in both ASD and ML extracts-treated MCF-7 cells. A previous study demonstrated that the antimitotic effect of the plant's aqueous extract, induced the blockade of DNA synthesis in *Allium cepa* roots (Shehab and Adam, 1983). These results suggest that the *A. hierochuntica* treatment may suppress the cell cycle progression, by down-regulating the expression of Cyclin D1 and E and induce apoptosis through p53 and p21. This could be due to the rich source of phenols and flavonoids, widely reported to be cyclin inhibitors (Mocanu et al., 2015; Zhang et al., 2018), and recently identified in the methanolic leaf extracts of *A. hierochuntica* (Saranya et al, 2019).

Future experiments will involve the identification, separation, isolation as well as purification techniques to reveal the phytochemical(s) in the aqueous and methanolic *A. hierochuntica* extracts responsible for the observed bioactivities. Comprehensive chemical analyses would involve prep and semi-prep liquid chromatography, crystallography, mass spectrometry, and nuclear magnetic resonance (NMR). Novel secondary metabolites found in the *A. hierochuntica* plant, including the neolignans (hierochins A, B and C) (Yoshikawa et al., 2003a) and flavonoids (Anastatin A and B) (Yoshikawa et al., 2003b) would be of particular interest. The future studies could pave the way to identify natural phytochemical-based anticancer agent(s) for breast cancer.

5. Conclusions

We provided evidence that the aqueous and methanolic extracts of different parts (seed, stem and leaf) of *A. hierochuntica* possess significant antiproliferative effects in human breast cancer MCF-7 cells in a dose-dependent manner. The antiproliferative property of the plant extracts was revealed in rapid cell death in the MCF-7 cells via the induction of apoptosis and result in DNA fragmentation. The mechanism of apoptosis in the MCF-7 cells occur through the p53-dependent mitochondrial pathway, evidenced by the activation of the cleaved forms of caspase-9 and caspase-3, as well as the up-regulation of Bax, p53 and the CDKN1A genes and the down-regulation of antiapoptotic and cell cycle genes. Additional experiments will be conducted to identify and isolate potent bioactive phytochemicals and investigate the development of potential chemotherapeutic or chemopreventive agents for breast cancer.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Mr Thadeo Trivilegio, KAIMRC Core Facility, for the FACS analysis and Ms. Rehab Mohammad Aldoshan and Mrs. Beshaier Alanzi from the Department of Forensic Sciences, Naif Arab University for Security Sciences for their assistance in most of the cell-based assays.

Funding

This project was fully funded by King Abdullah International Medical Research Centre (KAIMRC), grant number RC16/175/R.

Author contributions statement

SR and SMN conceived the study. SAM, ZIA, MSA, AV, HA, HA-E performed the assays, collected the data and performed the data analysis. SMN, SR, and SAM wrote the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.06.020.

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