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ORIGINAL ARTICLE

Kalanchoe blossfeldiana Extract Induces Cell Cycle Arrest and Necrosis in Human Cervical Cancer Cells

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ABSTRACT

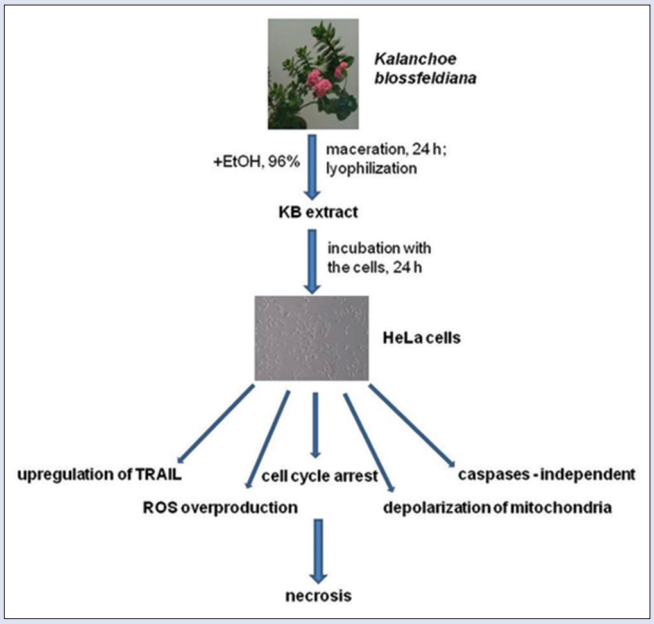
Background: Genus *Kalanchoe* (*Crassulaceae*) comprises plants originating from Africa and Asia. Their pharmaceutical preparations are commercially available in the form of anti-inflammatory and antimicrobial medicaments. Recent studies have shown anticancer activity of *Kalanchoe* plants. However, studies on *Kalanchoe blossfeldiana* (KB) are extremely limited, and its cytotoxic properties have not yet been evaluated. **Objective:** The objective of the study is to estimate the cytotoxic activity of KB on human cervical carcinoma (HeLa) cells and determine the mode of cell death. **Materials and Methods:** The cytotoxic activity of ethanolic extract of leaves of KB was tested on HeLa cells using Real Time xCelligence System (RTCA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell death, the generation of reactive oxygen species (ROS), depolarization of the mitochondria, cell cycle arrest, and the activity of caspases-3/7/9 were assessed by flow cytometry or a luminometer. The activity of poly (ADP-ribose) polymerase (PARP), translocation of apoptosis-inducing factor (AIF) in the cells, and expression of 92 genes associated with apoptosis/necrosis were also estimated. **Results:** According to the results, the IC_{50} values of KB extract were 8.28 ± 0.29 and 9.63 ± 1.07 μg/mL in RTCA and MTT assay, respectively. The results showed a significant increase in the generation of ROS and depolarization of the mitochondria in the dead cell population. Caspases-3/7/9 were not activated during treatment with KB extract. We also found that the pathway of HeLa cell death was induced by tumor necrosis factor-related apoptosis-inducing ligand and was independent of the PARP and AIF. The extract also induced cell cycle arrest in S phase. **Conclusions:** The results of this study indicate that KB extract induces necrosis in HeLa cells and this process is death receptor-mediated leading to the overproduction of ROS, mitochondrial dysfunction, and cell cycle arrest.

Key words: *Bryophyllum*, caspases, mitochondrial membrane potential, nonapoptotic cell death, reactive oxygen species

SUMMARY

- *Kalanchoe blossfeldiana* extract was prepared and investigated on human cervical carcinoma cells. This study demonstrates the strong cytotoxic properties of the extract which induces cell cycle arrest and necrosis by death receptor of tumor necrosis factor-related apoptosis-inducing ligand, reactive oxygen species overproduction, and mitochondrial depolarization. This process is poly (ADP-Ribose) polymerase- and caspases-3/7/9 independent.

Abbreviations used: 7-AAD: 7-aminoactinomycin; 3-AB: 3-aminobenzamide; AIF: Apoptosis-Inducing Factor; BSA: Bovine Serum Albumin; BIRC7: Baculoviral IAP Repeat Containing 7; CARD: Caspase Recruitment Domain; CI: Cell Index; DHE: Dihydroethidium; DMEM: Dulbecco's Modified Eagle's Medium; DMSO: Dimethyl Sulfoxide; FBS: Fetal Bovine Serum; KB: *Kalanchoe blossfeldiana*; MMP: Mitochondrial Membrane Potential; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP: Poly (ADP-Ribose) Polymerase; PBS: Phosphate-Buffered Saline; PI: Propidium Iodide; PS: Phosphatidylserine; RIPK1: Receptor Interacting serine/threonine Kinase 1; ROS: Reactive Oxygen Species; RTCA: Real-Time Cell Analyzer; SD: Standard Deviation; TNF: Tumor Necrosis Factor; TNFSF10: Tumor Necrosis Factor Superfamily Member 10; TRAIL: TNF-Related Apoptosis-Inducing Ligand.



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INTRODUCTION

Kalanchoe (also named *Bryophyllum*) is a genus of plants which mostly come from tropical areas of Africa and Asia. It belongs to the *Crassulaceae* family and includes about 150 species of plants that are used for the local treatment of many diseases, such as arthritis, ulcers, tissue injuries, ear infections, and skin diseases.[1] Pharmaceutical preparations from *Kalanchoe* plants are commercially available. Several compounds from different *Kalanchoe* species have been isolated and identified as

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flavonoid glycosides, anthocyanins, bufadienolides, triterpenoids, fatty acids, and sterols.[1,2] This diversity in the phytochemical composition has been attributed to the various biological activities of *Kalanchoe* species, such as antiviral, antimicrobial, immunomodulatory, anti-inflammatory, antioxidant, and cardiovascular properties.[1,3-7] Some of the recent studies have also demonstrated *in vitro* cytotoxic activity of the plant extracts and the isolated compounds against cancer cells. For example, a strong cytotoxic activity against several cell lines was obtained with cardenolides and bufadienolides isolated from *Kalanchoe tubiflora*.[8] Bufadienolides, isolated from *Kalanchoe gracilis*, showed potential cytotoxic activity against human cancer cell lines, with potency reaching the nanomolar range.[9] *Kalanchoe pinnata* and *Kalanchoe daigremontiana*–well-known species of *Kalanchoe*–were tested on Raji cells. Bufadienolides isolated from the leaves of these two species can be potential cancer chemopreventive agents.[10] The extracts from aerial parts and roots of *K. pinnata* have also examined on human cervical carcinoma (HeLa), human gastric adenocarcinoma (MK-1), and murine melanoma (B16F10) cells *in vitro.* The results showed that extracts from the roots were strongly cytotoxic to MK-1 and HeLa cells.[11,12] Other species, such as *Kalanchoe hybrida*, *Kalanchoe thrysiflora*, and *Kalanchoe marmorata*, were tested on human breast carcinoma (MCF-7) cell line[13,14] among which *K. hybrida* showed significant cytotoxic activity.[13]

One of the more interesting species of the genus *Kalanchoe* is *Kalanchoe blossfeldiana* (KB) which is widely distributed in the tropical regions of India. Depending on the color of the flower, there are varieties of KB, which is why it is popular among commercial growers. Phytochemical analysis of KB has revealed the presence of flavonoids, such as quercetin and its glycosides, diglycosides of kaempferol and pelargonidin, cyanidin, peonidin, petunidin, delphinidin, and malvidin.[15] It also contains sterols, squalene, and gallic acid.[16,17] There are a few studies describing the phytochemical analysis of KB; however, studies describing the biological activity of this species are scarce. A previous study on KB has revealed that the plant possesses high virus-neutralizing activity.[18] Another study has demonstrated the antimicrobial activity of KB extract on clinical isolates (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli,* and *Klebsiella pneumonia*), as well as on standard reference strains.[3] This is a highly encouraging alternative in the treatment of diseases associated with drug-resistant strains. To the best of our knowledge, there is no information regarding the cytotoxic activity of KB extract on tumor cells in the literature.

Therefore, in this study, we explored the effect of an ethanolic extract of KB against HeLa cells *in vitro* with an attempt to evaluate the mechanisms that play a role in the inhibition of cell proliferation and induction of cell death.

MATERIALS AND METHODS

Preparation of *Kalanchoe blossfeldiana* extract

The extract of KB (the pink-flowered cultivar) was prepared from fresh leaves (100.0 g) collected from a commercial garden source (Garden Center Justyna, Gdańsk, Poland). A voucher specimen (No. 21759) has been deposited in the Herbarium of the Medical University of Gdańsk (GDMA herbarium). The leaves were macerated and stirred with 96% ethanol (0.5 L) for 24 h at room temperature. The extract was filtered and concentrated under reduced pressure (40°C). After lyophilization, the extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20.0 mg/mL.

Cell culture

The human cervical adenocarcinoma cell line (HeLa S3) was obtained from the American Type Culture Collection (USA). The cell line was cultured in Dulbecco's Modified Eagle's Medium supplemented with 2 mM L-glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin and 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich, USA). The cells were incubated at 37°C and 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used in the estimation of the cells viability. HeLa cells were seeded in 96 well plates at a density of 5×10^3 cells/well and treated for 24 h with the KB extract at concentrations of 2.0–150.0 μg/mL. The maximal concentration of DMSO (used as the solvent of the extract) was 0.75% (v/v) for this assay. Following treatment, the cells were incubated with MTT (0.5 mg/mL; Sigma-Aldrich). A plate reader (Epoch, BioTek Instruments, USA) was used to measure the absorbance of the formazan solution. The data are expressed as IC_{50} mean values (±standard deviation [SD]) of at least two independent experiments.

RTCA cell proliferation assay

The viability and proliferation of HeLa cells treated with KB extract were determined with the xCELLigence Real-Time Cell Analyzer Dual Plate (ACEA Biosciences, San Diego, CA, USA). The cells were seeded at a density of 2×10^4 cells/well into E-plate 16 (ACEA Biosciences). After 24 h, the extract was added at concentrations of 2.0–150.0 μg/mL. The maximal concentration of the solvent (DMSO) used in the RTCA experiments was 0.75% (v/v). The IC_{50} values were calculated in the RTCA software v. 1.2.1 RTCA software v. 1.2.1 (ACEA Biosciences). All the experiments were performed in three independent repeats.

Dead cell assay

To estimate the cytotoxic effect of KB extract on HeLa cells, we used Annexin V and Dead Cell Assay Kit (Merck Millipore, Burlington, MA, USA). The cells were seeded in 12 well plates (1×10^5 cells/well) and incubated with the extract at concentrations of 2.0–100.0 μg/mL. The concentration of DMSO added to the cells (control) was 0.5% (v/v). Next, the cells were stained with reagents from the assay (annexin V and 7-AAD [7-aminoactinomycin]) and analyzed by Muse Cell Analyzer (Merck Millipore). The experiments were performed in three independent repeats.

Hoechst staining

To observe the effect of KB extract on the HeLa cell nuclei, we seeded HeLa cells in a 12-well plate (1×10^5 cells/well). Next, we added DMSO (0.5% [v/v]) and the extract at concentrations of 20.0, 50.0, and 100.0 μg/mL. Then, the cells were incubated for 24 h and stained with fluorescent Hoechst 33342 dye (0.5 μg/mL; Life Technologies, USA) and observed under a fluorescent microscope (Leica, Switzerland).

Annexin-V-Fluos and propidium iodide staining

HeLa cells were seeded in a 12 well plate with coverslips (1×10^5 cells/well) and incubated with KB extract at concentrations of 20.0, 50.0, and 100.0 μg/mL. DMSO was added to the cells (control) at a concentration of 0.5% (v/v). After 24 h, the cells on the coverslips were stained with Annexin-V-Fluos Staining Kit (Roche, Germany) and observed under the fluorescence microscope.

Immunofluorescence staining

HeLa cells were seeded in a 12 well plate with coverslips (1×10^5 cells/well) and treated with KB extract at concentrations of 20.0 and 100.0 μg/mL. DMSO was added to the cells (control) at a concentration of 0.5% (v/v). After 24 h of incubation, the cells on the coverslips were fixed in 4%

formaldehyde (in phosphate-buffered saline [PBS], v/v) at room temperature for 10 min. Then, the cells were permeabilized with 0.1% Triton-X 100 (Sigma-Aldrich) in PBS for 15 min. Subsequently, the coverslips were incubated in 5% bovine serum albumin (BSA) (in PBS, w/v) for 1 h. Then, the cells were incubated with the anti-apoptosis-inducing factor (AIF) antibody (1:100, rabbit polyclonal, Sigma-Aldrich) in PBS with 2% FBS (v/v) and 1% BSA (w/v) at 4°C for 1 h. Next, the cells were washed thrice with cold PBS and incubated with Alexa Fluor 594-conjugated anti-rabbit secondary antibody (1:1000 in PBS, Thermo Fisher Scientific, USA) for 1 h. After three 5-min washes, the cells on the coverslips were incubated with Hoechst 33342 dye (0.5 μg/mL) at room temperature for 20 min and observed under the fluorescence microscope.

Caspase-9 and caspases-3/7 activity

The cells were seeded in 12 well plates (1×10^5 cells/well) and treated with the extract at concentrations of 2.0–100.0 μg/mL. DMSO was added to the control cells at a concentration of 0.5% (v/v). After 4 h and 24 h of treatment, caspase-Glo 9 Assay Kit (Promega, USA) and Glomax Multi + Detection System (Promega) were used to determine the activity of caspase-9. After 24 h of incubation of the cells with the extract, the activity of caspases-3/7 was assessed with Muse Caspase-3/7 Kit Muse Caspase-3/7 Kit (Merck Millipore) and Muse Cell Analyzer, following the manufacturer's instructions. All the experiments were performed in three independent repeats.

Poly(ADP-ribose) polymerase activity

To estimate the activity of poly (ADP-ribose) polymerase (PARP), HeLa cells were seeded in 24 well plates (5×10^4 cells/well) and treated with KB extract and/or the PARP inhibitor-3-aminobenzamide (3-AB) in the concentration range of 10.0–150.0 μg/mL and/or 2.0–10.0 mM, respectively, for 24 h. After incubation, the cells were harvested and stained with Muse Count and Viability Kit (Merck Millipore) and analyzed with Muse Cell Analyzer. The results were repeated three times.

Mitochondrial membrane potential

Hela cells were seeded in 12 well plates (1×10^5 cells/well) and incubated with the extract at concentrations of 2.0–100.0 μg/mL. The concentration of DMSO was 0.5% (v/v) in a control sample. After 3 h of exposure, the cells were stained with Muse MitoPotential Assay Kit (Merck Millipore). Muse Cell Analyzer was used to determine the percentage of depolarized/live cells. All the experiments were independently repeated three times.

Reactive oxygen species production

HeLa cells (1×10^5 cells/well, 12 well plates) were treated with the extract in the concentration range of 2.0–100.0 μg/mL. The concentration of DMSO in a control sample was 0.5% (v/v). After 24 h of incubation, the cells were stained with Muse Oxidative Stress Kit (Merck Millipore) and analyzed with Muse Cell Analyzer. The experiments were done in three independently repeats.

Cell cycle analysis

HeLa cells were seeded (5×10^5 cells/well, 6 well plates) and incubated with the extract in the concentration range of 2.0–100.0 μg/mL for 24 h. The concentration of DMSO (the control) was 0.5% (v/v). After treating, the cells were prepared with Muse Cell Cycle Assay Kit (Merck Millipore). The percentage of cells in each phase of the cell cycle was determined by Muse Cell Analyzer. The experiments were done in triplicate.

Real-time polymerase chain reaction

HeLa cells were treated with KB extract at a concentration of 20.0 μg/mL and with DMSO (0.1% [v/v]) for 24 h. The cells were harvested, and total RNA from the cells was isolated using the RNeasy Mini Kit (Qiagen, The Netherlands). After measuring the concentration of RNA, cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

Next, cDNA was applied on the TaqMan Array Human Apoptosis Fast 96 well plates (Thermo Fisher Scientific 4418717). Each plate contains 92 assays for genes associated with apoptosis and four assays for control genes. The polymerase chain reaction (PCR) reactions were performed in StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The data were obtained in three independently repeated experiments and analyzed with StepOne software v. 2.3 (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Statistical data were processed using the STATISTICA 12.0 Software Package (StatSoft. Inc., Tulsa, OK, USA). All data were expressed as mean values ± SD. The Student's *t*-test was used to compare the results. The statistical significance was set at $P < 0.05$.

RESULTS

Kalanchoe blossfeldiana extract decreased human cervical carcinoma cells viability

The cytotoxic activity of KB extract on HeLa cells was determined by MTT assay and the xCELLigence RTCA system. MTT assay allows for the determination of IC_{50} values based on one time point, whereas RTCA system detects cellular changes by a parameter called cell index at every time point during the whole experiment.[19,20]

In this study, the results of both MTT assay and RTCA system were similar in case of HeLa cells incubated with the extracts. The IC_{50} values were 9.63 ± 1.07 μg/mL and 8.28 ± 0.29 μg/mL for MTT assay and RTCA, respectively.

Furthermore, the RTCA profiles of the cell proliferation indicate that the extract showed a dose-dependent and time-dependent cytotoxic activity [Figure 1].

Kalanchoe blossfeldiana extract induced necrosis in human cervical carcinoma cells

The effect of KB extract on HeLa cells was estimated after treating the cells for 24 h with the extract. The total percentage of early and late apoptotic cells (total apoptotic) was 5.28% ± 0.21%, 5.35% ± 0.96%, 5.56% ± 0.59%, 7.73% ± 0.25%, 13.03% ± 2.66%, and 28.36% ± 2.66% for KB extract at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 μg/mL, respectively. In contrast, the percentage of dead HeLa cells was 12.91% ± 1.74%, 35.68% ± 4.09%, 49.14% ± 3.98%, 61.22% ± 2.15%, 62.99% ±1.73% and 57.47% ± 3.72% for KB extract at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 μg/mL, respectively [Figure 2]. These results indicate that the cytotoxic effect of the extract on the cells increased in a dose-dependent manner. However, the number of the early apoptotic cells was similar for all the tested concentrations [Figure 2f].

To confirm the results obtained from Muse Cell Analyzer, we stained HeLa cells with annexin and propidium iodide (PI) after incubating the cells with KB extract. We found necrotic and apoptotic cells in the samples treated with the extract in comparison to the control cells [Figure 3].

Furthermore, after 24 h treatment with KB extract, HeLa cells were stained with Hoechst 33342 dye, which mostly showed necrotic

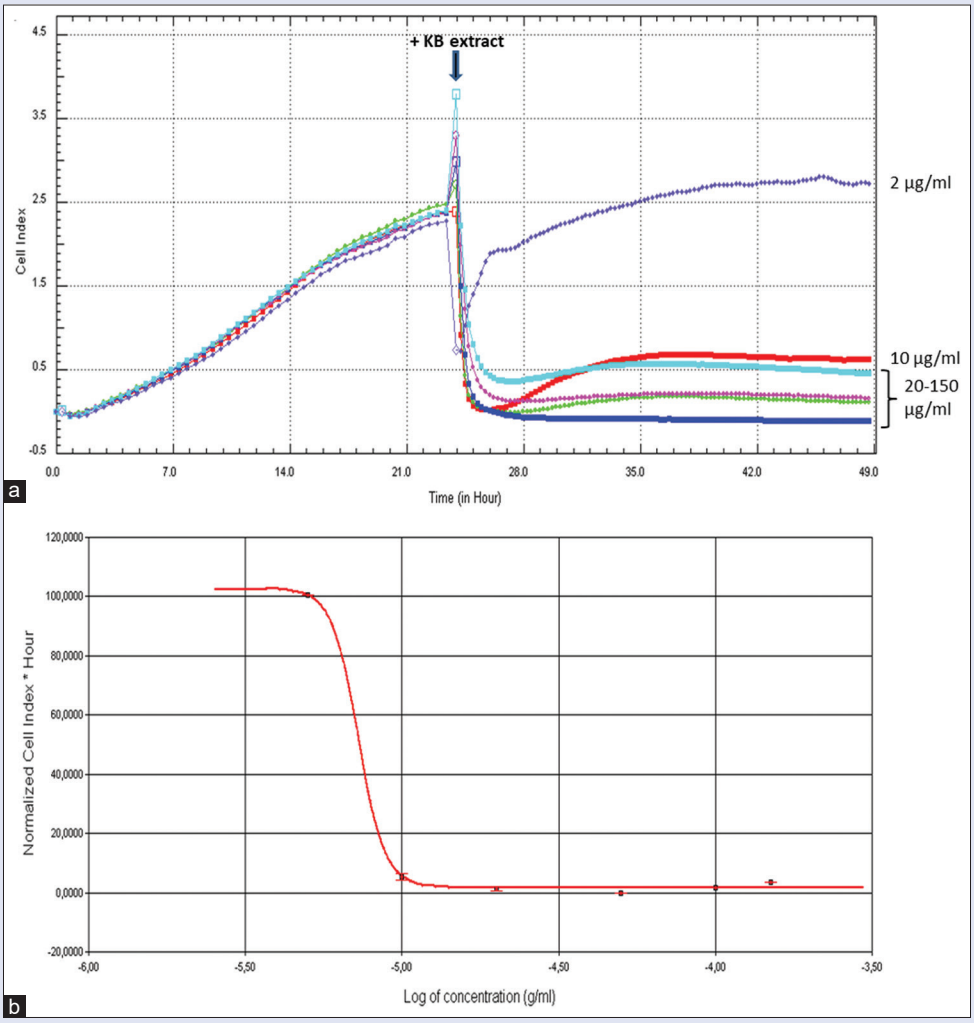

Figure 1: RTCA profiles of human cervical carcinoma cell proliferation and measurement of cell index values during incubation of the cells with *Kalanchoe blossfeldiana* extract. The cells were treated with the extract at different concentrations (2.0–150.0 µg/mL) (a). The IC_{50} value was calculated based on the dose-response curves of the cell indexes by the xCELLigence system (b). Error bars represent standard deviations

nuclei in the cells. The chromatin was disorganized and appeared to have dispersed fragments in the nuclei in comparison to the control cells [Figure 4].

Kalanchoe blossfeldiana extract did not affect poly (ADP-ribose) polymerase activity

After 24 h of treatment with KB extract and/or an inhibitor of PARP–3-AB, HeLa cells were tested for their viability by flow cytometry. We observed that the viability of the cells treated simultaneously with the extract and 3-AB at different concentrations did not significantly differ from the viability of the cells incubated only with the extract (without 3-AB). We also observed that 3-AB did not affect the viability of HeLa cells at 2.0, 5.0, and 10.0 mM concentrations [Figure 5].

Kalanchoe blossfeldiana extract did not trigger the translocation of apoptosis-inducing factor to the human cervical carcinoma nuclei

The translocation of AIF from the mitochondria to the cell nuclei was assessed after 24 h of incubation of HeLa cells with KB extract and subsequent immunostaining. We observed a red fluorescence diffused in the cytoplasm of the cells that were treated with 20.0 and 100.0 µg/mL of the extract. The treated cells, similar to the control cells, did not show the presence of AIF in the cellular nuclei [Figure 6].

Kalanchoe blossfeldiana extract decreased the activity of caspase-9 in human cervical carcinoma cells and did not activate caspases-3/7

The activity of caspase-9 was lower in the cells treated with KB extract than that of control samples at all the tested concentrations and the incubation times (4 and 24 h). Although the treatment up to 4 h did not show any statistically significant activity of caspase-9, after 24 h, its activity was significantly lower for the concentrations above 20.0 µg/mL of the extract in comparison to the control cells [Figure 7a], whereas for the concentrations of 2.0, 5.0 and 10.0 µg/mL the activity was similar to the control.

After incubating the cells with KB extract and determination of caspases-3/7 activity, we found that the executioner caspases were not activated at all the tested concentrations. The amount of early apoptotic cells (the lower right quadrants) in the samples treated with KB extract is not higher than in the control sample [Figure 7b-f]. The percentage of total apoptotic cells (mostly late apoptotic) was 3.95% ±0.30%, 3.87% ± 0.50%,

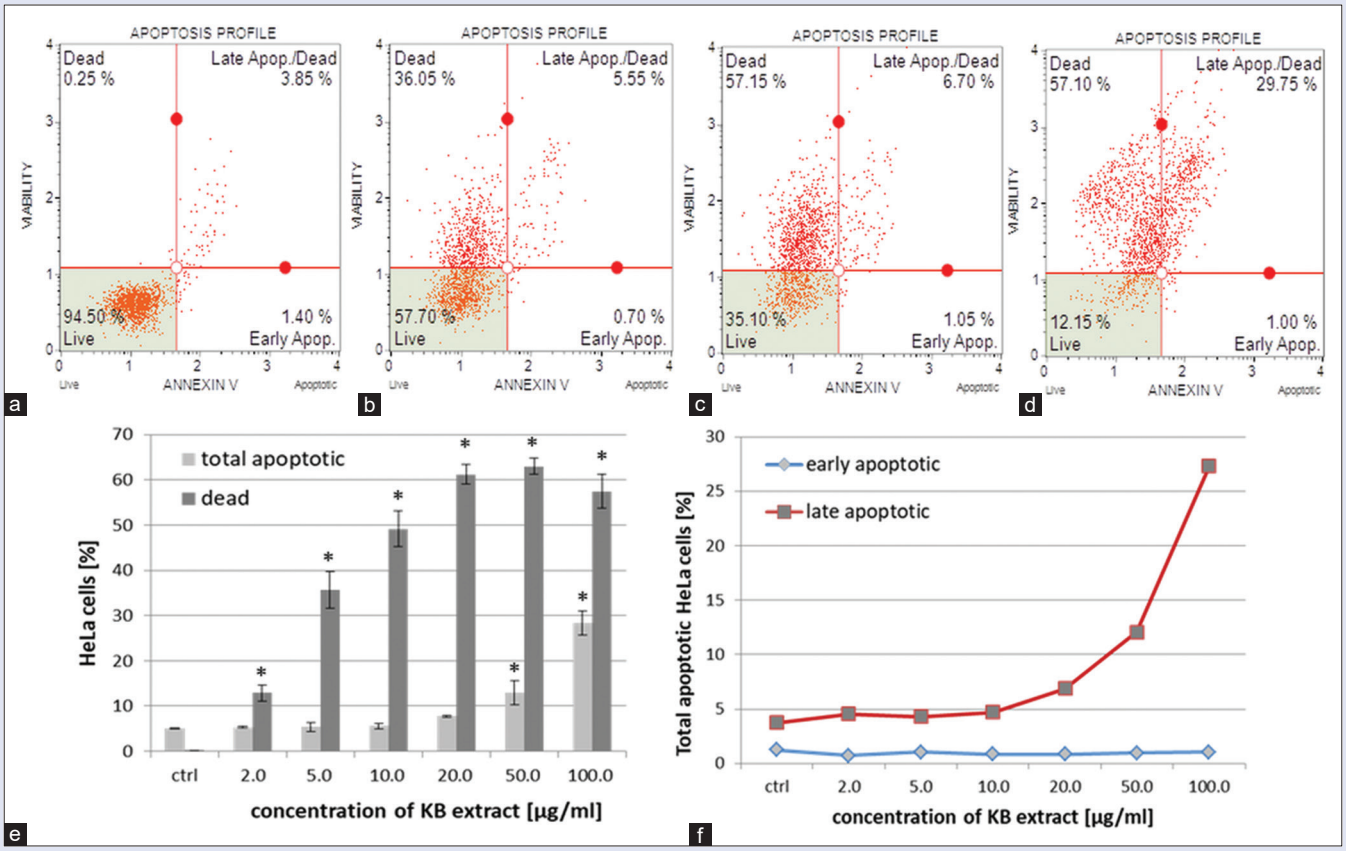

Figure 2: The necrotic and apoptotic effects of *Kalanchoe blossfeldiana* extract on human cervical carcinoma cells. The percentage of the necrotic and apoptotic cells was determined by flow cytometry. The cells were treated with dimethyl sulfoxide (0.5%, control) (a) and *Kalanchoe blossfeldiana* extract at concentrations of 2.0, 5.0 (b), 10.0, 20.0 (c), 50.0 and 100.0 µg/mL (d) for 24 h. The rate of apoptosis and necrosis was estimated in comparison to the control (e). The distribution of early and late apoptotic cells was determined for all the tested concentrations of the extract (f). Each sample was run in triplicate. Error bars represent standard deviations. Significant differences relative to the control are marked with an asterisk "*" ($P < 0.05$)

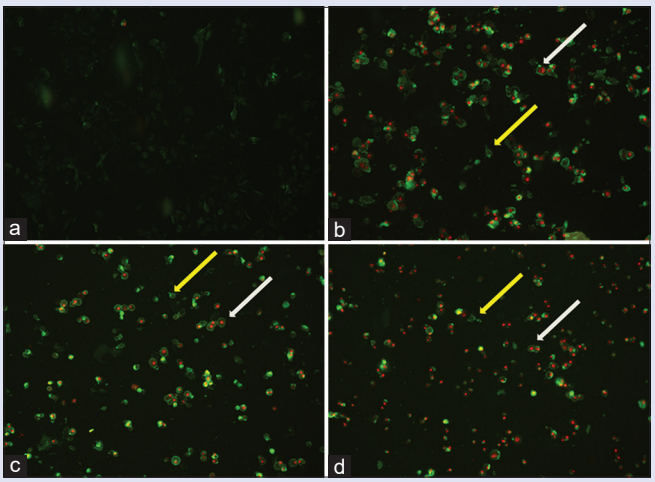

Figure 3: Human cervical carcinoma cells processed with Annexin-V-Fluos Staining Kit after treatment with *Kalanchoe blossfeldiana* extract. The cells were stained with annexin-V-fluorescein (green) and propidium iodide (red) after treating the cells with dimethyl sulfoxide (0.5%, control) (a) and with *Kalanchoe blossfeldiana* extract at concentrations of 20.0 (b), 50.0 (c) and 100.0 µg/mL (d). Arrows indicate the necrotic cells (white arrows; high annexin and high propidium iodide staining) and apoptotic cells (yellow arrows; high annexin staining)

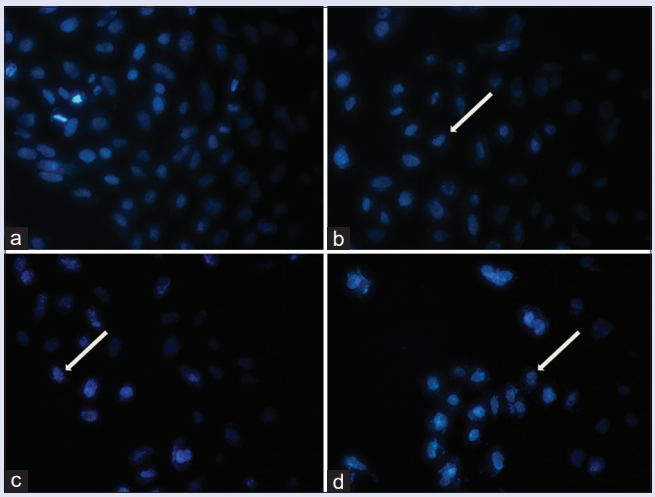

Figure 4: *Kalanchoe blossfeldiana* extract induced necrotic changes in the nuclei of human cervical carcinoma cells. The nuclei were stained with Hoechst 33342 dye after treating the cells with dimethyl sulfoxide (0.5%, control) (a) and *Kalanchoe blossfeldiana* extract at concentrations of 20.0 (b), 50.0 (c) and 100.0 µg/mL (d). The cells incubated with the extract showed dispersed chromatin fragments in comparison to the control sample (dimethyl sulfoxide). Arrows represent nuclear changes in the cells

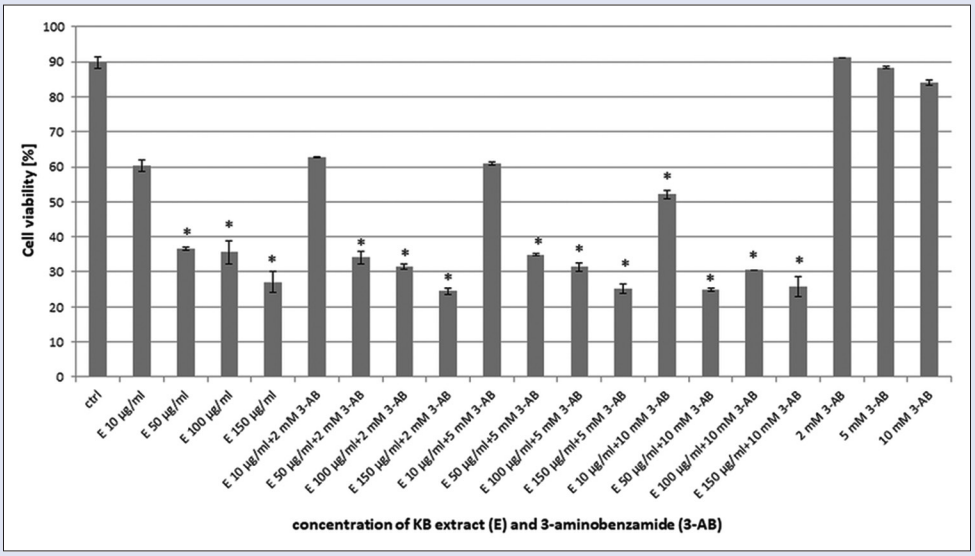

Figure 5: The viability of human cervical carcinoma cells treated with *Kalanchoe blossfeldiana* extract and/or 3-AB. The cell viability was estimated by flow cytometry after 24 h of treating the cells with the extract and/or 3-aminobenzamide in the concentration range of 10.0–150.0 µg/mL and 2.0–10.0 mM, respectively. Each sample was run in triplicate. Error bars represent standard deviations. Significant differences relative to the control are marked with an asterisk "*" ($P < 0.05$)

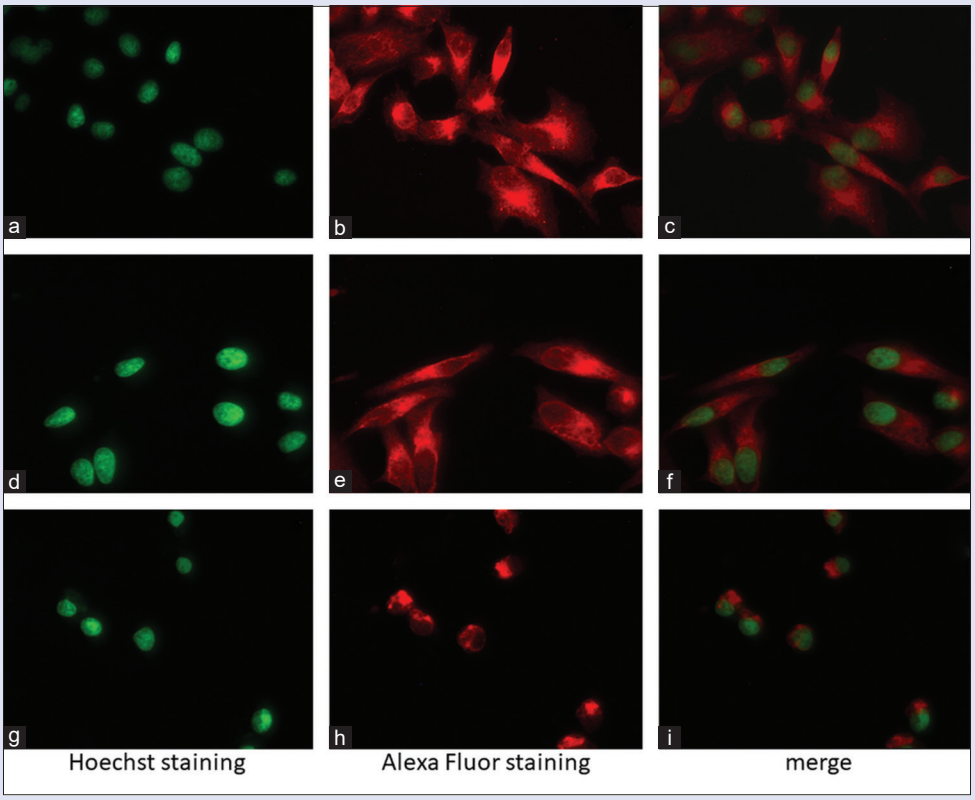

Figure 6: Immunostaining of apoptosis-inducing factor in human cervical carcinoma cells treated with *Kalanchoe blossfeldiana* extract. The cells carcinoma cells were stained with anti-apoptosis-inducing factor and Alexa Fluor 594-conjugated anti-rabbit antibodies and Hoechst 33342 dye after treating the cells with dimethyl sulfoxide (0.5%, control) (a-c) and *Kalanchoe blossfeldiana* extract at concentrations of 20.0 (d-f) and 100.0 µg/mL (g-i). The cells incubated with *Kalanchoe blossfeldiana* extract showed a diffused red fluorescence outside the green-colored nuclei

3.60% ± 0.71%, 5.56% ± 0.48%, 16.98% ± 2.51% and 32.12% ± 2.47% for KB extract concentrations of 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 µg/mL, respectively. The percentage of dead cells was 10.03% ± 0.57%, 23.29% ± 3.09%, 25.15% ± 3.00%, 31.94% ± 2.16%, 45.72% ± 0.41%

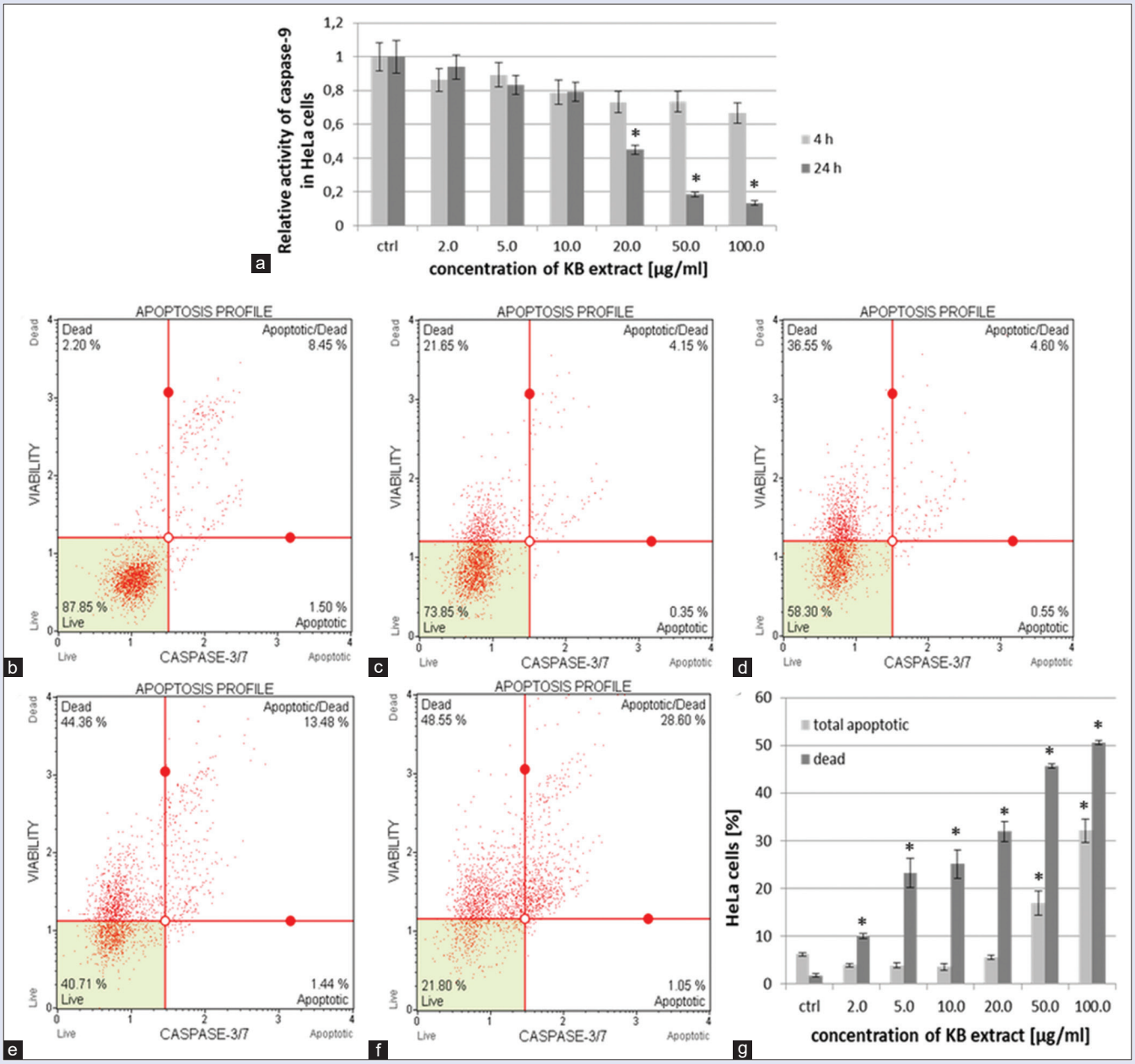

Figure 7: Caspase-9 and caspases-3/7 activity in human cervical carcinoma cells after treatment with *Kalanchoe blossfeldiana* extract. The activity of caspase-9 was measured after 4 h and 24 h of treating the cells with 0.5% dimethyl sulfoxide (control) and *Kalanchoe blossfeldiana* extract in the concentration range of 2.0–100.0 µg/mL (a). The activity of caspases-3/7 was determined after 24 h of treating the cells with 0.5% dimethyl sulfoxide (b) and *Kalanchoe blossfeldiana* extract at concentrations of 2.0, 5.0 (c), 10.0, 20.0 (d), 50.0 (e) and 100.0 µg/mL (f). The activities of the executioner caspases in *Kalanchoe blossfeldiana*-treated cells were compared to the control (g). Each sample was run in triplicate. Error bars represent standard deviations. Significant differences relative to the control are marked with an asterisk "*" ($P < 0.05$)

and 50.59% ±0.49% for concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL, respectively [Figure 7g].

Kalanchoe blossfeldiana extract modulated mitochondrial membrane potential (ΔΨm) in human cervical carcinoma cells

To study the effect of KB extract on the mitochondrial membrane potential in HeLa cells, we incubated the cells with increasing concentrations of the extract. According to the results, there was a significant increase in the loss of mitochondrial potential in KB-treated cells in comparison to the control cells (incubated with DMSO). The percentage of depolarized/live cells was 7.8% ± 1.19%, 9.19% ± 1.12%, 11.08% ±1.16%, 20.98% ± 1.76%, 35.15% ± 0.15% and 62.09% ± 0.97% at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL, respectively [Figure 8].

Kalanchoe blossfeldiana extract increased reactive oxygen species production in human cervical carcinoma cells

To determine the extent of oxidative stress in HeLa cells exposed to KB extract, we monitored the amount of intracellular superoxide radicals

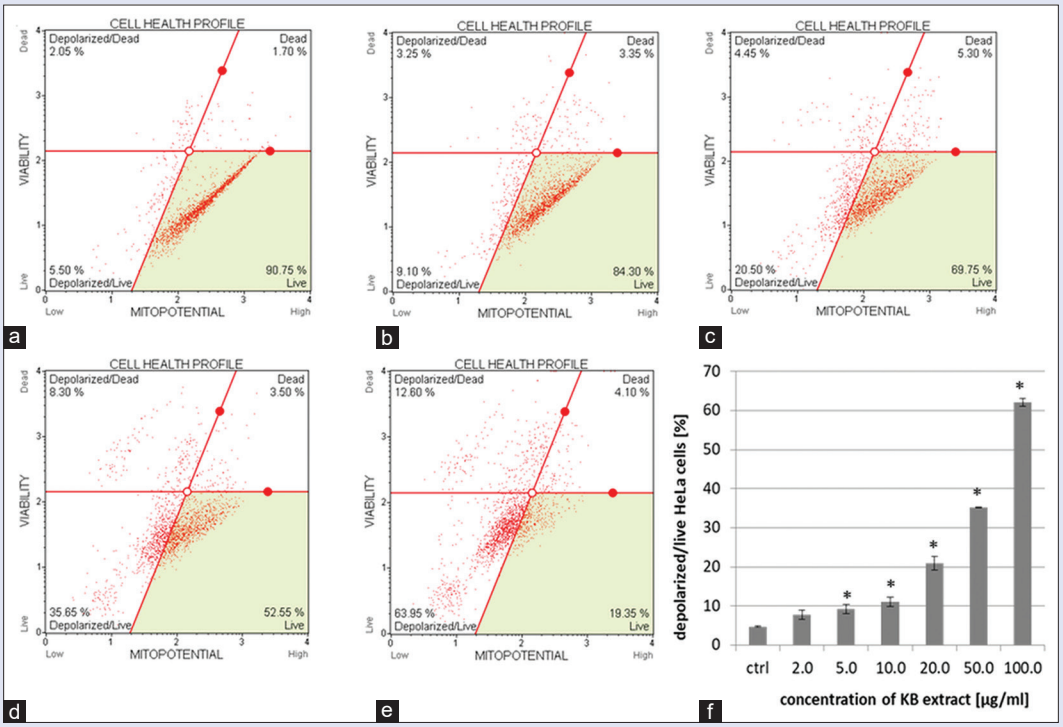

Figure 8: *Kalanchoe blossfeldiana* extract induced changes in the transmembrane mitochondrial potential in human cervical carcinoma cells. The cells were exposed to 0.5% dimethyl sulfoxide (a) as the control and *Kalanchoe blossfeldiana* extract at concentrations of 2.0, 5.0 (b), 10.0, 20.0 (c), 50.0 (d) and 100.0 µg/mL (e) for 3 h. The changes in the mitochondrial depolarization were determined in comparison to the control (f). Each sample was run in triplicate. Error bars represent standard deviations. Significant differences relative to the control are marked with an asterisk "*" ($P < 0.05$)

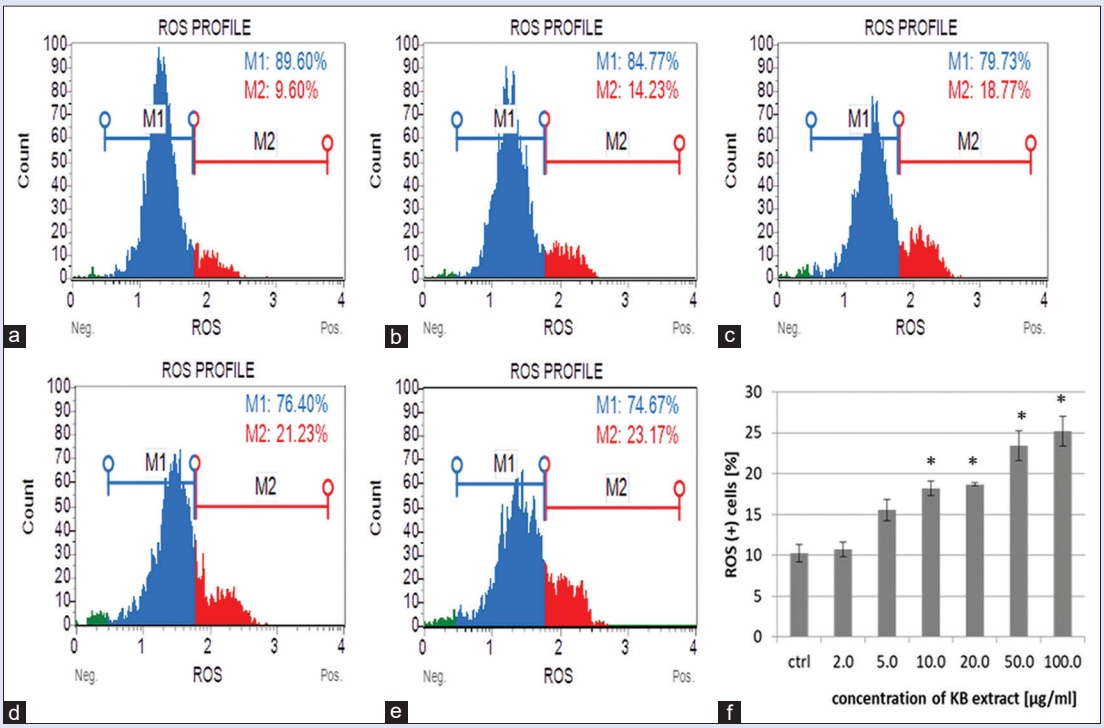

Figure 9: *Kalanchoe blossfeldiana* extract induced the production of ROS in human cervical carcinoma cells. The cells were incubated with 0.5% dimethyl sulfoxide (a) as the control and the extract at concentrations of 2.0, 5.0 (b), 10.0, 20.0 (c), 50.0 (d) and 100.0 µg/mL (e) for 24 h. The level of reactive oxygen species production in the cells was estimated in comparison to the control (dimethyl sulfoxide) (f). Each sample was run in triplicate. Error bars represent standard deviations. Significant differences relative to the control are marked with an asterisk "*" ($P < 0.05$)

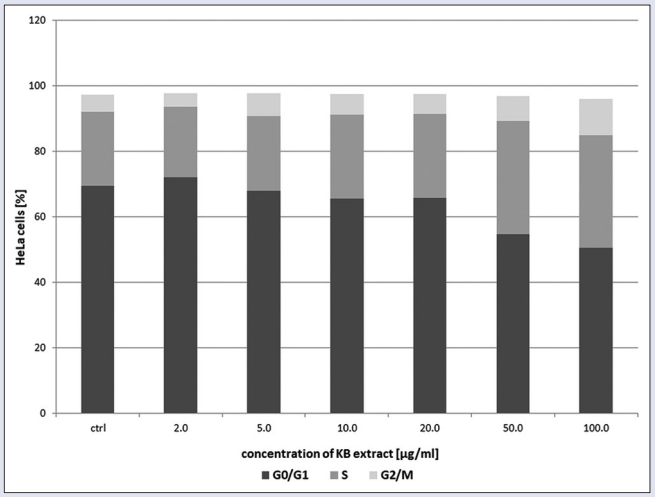

Figure 10: *Kalanchoe blossfeldiana* extract induced cell cycle arrest in S and G2/M phases in human cervical carcinoma cells. The cells were incubated with *Kalanchoe blossfeldiana* extract at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL and 0.5% dimethyl sulfoxide as the control for 24 h. The percentage of cells in each phase was measured by flow cytometry. Each sample was run in triplicate

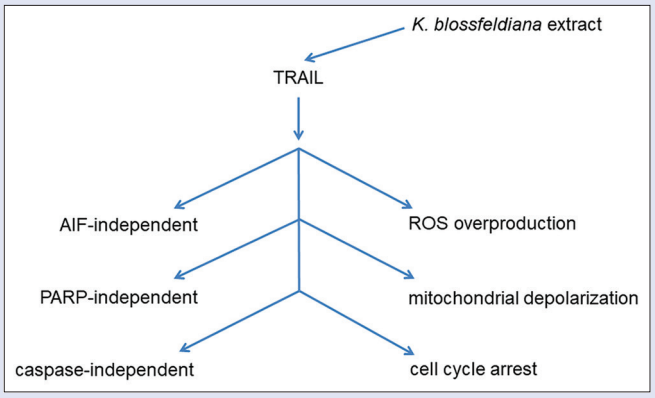

Figure 12: The scheme of death in human cervical carcinoma cells after treatment with *Kalanchoe blossfeldiana* extract

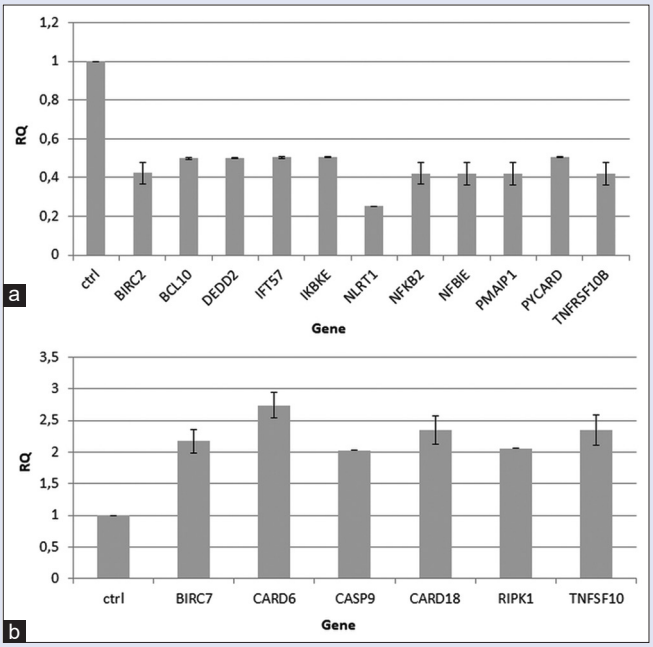

Figure 11: *Kalanchoe blossfeldiana* extract induced changes in the expression of genes in human cervical carcinoma cells. The cells were treated with 0.1% dimethyl sulfoxide (control) and *Kalanchoe blossfeldiana* extract at a concentration of 20.0 µg/mL for 24 h. The expression of genes was normalized to 18 S endogenous control gene, and their levels are presented as a fold-change under (a) or over (b) the value 1.0 (ctrl)

with the use of dihydroethidium (DHE). DHE is cell-permeable and on reaction with superoxide anions, it forms the DNA-binding fluorophore-ethidium bromide. It intercalates with DNA and gives red fluorescence.

After 24 h of incubating the cells with the KB extract, the percentage of cells exhibiting the production of reactive oxygen species (ROS) (ROS [+] cells) was 10.75% ± 0.89%, 15.55% ± 1.27%, 18.18% ± 0.87%, 18.71% ± 0.21%, 23.44% ± 1.80%, and 25.22% ± 1.83% for concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL, respectively [Figure 9].

Kalanchoe blossfeldiana extract induced cell cycle arrest in S and G2/M phases

We determined the effect of KB extract on the cell cycle of HeLa cells. According to the results, KB induced cell cycle arrest in S and G2/M phases in comparison to the control cells. The percentage of cells in S phase was increased from 22.65% ± 1.63% for the control to 21.45% ± 0.64%, 22.75% ± 0.07%, 25.8% ± 0.14%, 25.6% ± 0.85%, 34.6% ± 2.26%, and 34.2% ± 1.41% for the cells treated with KB extract at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL, respectively. Similarly, the percentage of cells in G2/M phase was increased from 5.2% ±0.35% for the control to 4.3% ± 0.42%, 6.0% ± 0.14%, 6.2% ± 0.57%, 6.05% ± 0.34%, 7.75% ± 0.64%, and 11.15% ± 0.92% for the extracts at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL, respectively. Furthermore, there was a decrease in the number of cells in G0/G1 phase (from 69.5% ± 0.42% for the control cells to 54.6% ± 3.54% and 50.6% ± 2.55% for the KB extract at concentrations of 50.0 and 100.0 µg/mL, respectively) [Figure 10].

Kalanchoe blossfeldiana extract increased the expression of genes involved in cell necrosis

To determine the cell death pathway in HeLa cells treated with KB extract, the expression of 92 related cellular death genes was tested with qPCR human apoptosis array. Figure 11 shows the expression of genes only with significant changes in comparison to the control (≥2-fold over or ≤0.5-fold under the control). The highest overexpression we observed for tumor necrosis factor (TNF) superfamily member 10 (TNFSF10) (also named as TNF-related apoptosis-inducing ligand [TRAIL]), baculoviral IAP repeat-containing 7, caspase recruitment domain family member 6 and 18, respectively.

DISCUSSION

In this study, we examined the effect of KB extract on HeLa cells. The extract showed significant cytotoxic activity, which was dose and time dependent. Furthermore, based on the flow cytometric and microscopic analysis, the extract of KB was found to induce necrosis. The extract significantly increased the count of dead cells, which was much greater than the population of the apoptotic cells. The percentage of cells in early apoptosis was unchanged for all the tested concentrations of the extract. Microscopic analysis revealed that most of the cells were stained

with red coming from penetrate PI to the nuclei due to disruption of the cellular membranes and green coming from the binding of annexin to the phosphatidylserine (PS) present in the cellular membranes. It is well-known that externalization of PS on the outer membrane of cells is one of the early hallmarks of apoptosis. However, Zargarian *et al.* showed that cells undergoing necrotic cell death also expose PS and release extracellular vesicles to their surroundings.[21] Furthermore, this externalization of PS may occur in the late stage of cell death.[22] Our results indicate that a dominant form of HeLa cell death in this study is necrosis.

Cells undergoing apoptosis or necrosis differ with respect to their morphological changes. The primary morphological features of apoptosis are severe shrinkage, rounding and blebbing, chromatin condensation, and fragmentation. In contrast, flattened cells, the formation of membrane bubbles and their rupture, disorganization of cytoplasm, homogeneous condensation of chromatin and its final disintegration are the characteristics of necrosis.[23] In this study, KB extract mostly caused flattening of the cells resulting in the strong attachment of these cells to the substrate. Furthermore, Hoechst staining revealed a disorganized chromatin that appeared as dispersed fragments in the nuclei.[23]

Conventionally, necrosis has been defined as an unregulated process which is independent of apoptosis.[24] However, recent studies have shown that necrosis is a genetically controlled process which is triggered by different stimuli; this process is named as regulated necrosis.[25] Among the different types of programmed necrosis, two of the most studied and described processes are the necroptosis, which is initiated by the TNF receptor and the necrosis, which is mediated through the PARP pathway.[26] In the PARP pathway of cell death, the polymerase is activated and induces a massive PARtylation of proteins which leads to the depletion of cellular ATP and an energetic catastrophe. PARP hyperactivation induces the release of AIF from mitochondria to the cytosol and then to the nucleus where DNA is fragmented.[27] In this study, we estimated the selected factors responsible for the activation of cells death as well as examined the role of cellular PARP and AIF after treatment with KB extract. The gene expression analysis revealed that the extract may induce HeLa cell death by the TRAIL (TNFSF10). TRAIL triggers RIPK1-dependent (receptor-interacting serine/threonine kinase 1) necrosis, which is independent of PARP.[22] RIP1 kinase forms multimeric complexes with the membrane receptors and binds to RIP3 forming the necrosome.[28] Our analysis has revealed the significant overexpression of *RIP1* gene in HeLa cells. Furthermore, we found that neither PARP nor AIF participated in the cascade of cell death triggered by the extract. In this study, the PARP inhibitor (3-AB) at different concentrations did not change the viability of the cells incubated with the extract. The immunostaining of the cells after treatment with KB extract showed that AIF was present only in the cytosol and did not get translocated to the nuclei. This indicates that the extract induced receptor-mediated necrotic type of death in HeLa cells. Figure 12 shows the proposed model of this pathway.

Necrosis is also characterized by the mitochondrial dysfunction and production of ROS.[29] ROS are formed as a natural product of normal energy metabolism. The excessive production of superoxide radicals and oxidative stress occurs due to the inefficient cellular antioxidant system. This leads to the damage of lipids, proteins and nucleic acids in the cells and disrupts the mitochondrial membrane integrity.[22,30] Our results showed that HeLa cells treated with KB extract underwent oxidative stress and this effect was dose-dependent. We also observed a significant increase in the population of the depolarized/live HeLa cells just after a few hours of incubating the cells with the extract. This confirms that mitochondrial membrane potential decreased as a result of changes in the mitochondrial membrane permeability.[31,32]

Increase in the level of ROS and the occurrence of mitochondrial dysfunction may result in the inhibition of cancer cell migration and cell cycle arrest.[33] This study showed that KB extract paused cell cycle in HeLa cells in S and G2/M phases. This effect was dose-dependent and was noticeable at higher concentrations of the extract.

Necrosis, in comparison to apoptosis, is a process where no massive activation of caspases is observed. In apoptosis, the initiator caspases cleave the executioner caspases to activate them[34] and other cellular components leading to cell death. In this process, the participation and role of caspases are evident, whereas in necrosis, activation of caspases has been almost never occurred. In our study, to confirm the necrotic pathway of cell death in HeLa cells, we estimated the regulatory level of initiator caspase-9 and executioner caspases-3/7. The results indicated that caspase-9 was not activated during the incubation of HeLa cells with KB extract. The downregulation of caspase-9 was observed both at the initial period of treatment and after 24 h of treatment. The effect was time-and dose-dependent. The extract did not also activate caspases-3/7 at the tested KB concentrations. These results agree with those obtained from the staining of the cells with annexin/7-AAD and flow cytometric analysis.

To the best of our knowledge, cytotoxic properties of KB extract have not yet been studied. There are studies describing other species of genus *Kalanchoe* with an estimation of the role of selected factors in their cytotoxic activity. For example, Hsieh *et al.* analyzed the effects of *K. tubiflora* extract on different tumor cell lines and showed the mechanisms of inhibition of cell proliferation and reduction of cell viability: by inducing multipolarity and by disrupting alignment of chromosomes in metaphase.[35,36] In another study, *K. pinnata* extract and its fraction were tested on human cervical cancer cells, which exhibited growth inhibitory activity. The fraction strongly induced apoptosis by the activation of Bax, suppression of Bcl-2, and cleavage of PARP-1.[12] Kaewpiboon *et al.* studied the effect of *K. laetivirens* extract by estimating its activity on human lung A549 cancer cells resistant to etoposide. They found that the extract reversed this resistance in the cells by the downregulation of NF-κB.[37]

All these actions are partly dependent on the presence of secondary metabolites-bufadienolides and flavonoids in the genus *Kalanchoe*. Thus, bufadienolides isolated from *Kalanchoe* plants have been widely investigated for their cytotoxic activity on cancer cells.[2,8-10,13,38]

CONCLUSION

The findings of this study have indicated that KB extract induces necrosis in HeLa cells and this is a death receptor-mediated process which leads to the overproduction of ROS in the cells, mitochondrial dysfunction and cell cycle arrest. The precise molecular mechanism of this pathway leading to cell death is yet to be further studied, especially that this process could be used in eliminating tumor cells in future anticancer approaches.

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Conflicts of interest

There are no conflicts of interest.

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