

In vitro Investigation of Conessine-Mediated Apoptosis on Human Breast Cancer Cell Line (MCF-7) via Mitochondrial Pathway and APAF-1 Gene

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ABSTRACT

Background: Breast cancer was the second most deadly cancer, especially in women. Recent data show that the incidence rate has been slowly rising over time and now it is becoming a global problem. This increasing problem shows how important it is to move away from traditional therapies and toward treatments that use bioactive compounds, which may be safer and more effective. The current study examines the antiproliferative effect of the steroidal alkaloid Conessine on the Human Breast Adenocarcinoma Cell Line (MCF-7). **Materials and Methods:** The Tetrazolium Dye-Based Method (MTT) was used to test Conessine's cytotoxicity. Fluorescence microscopy was used to confirm apoptosis by using Acridine orange and Ethidium Bromide to stain the cells. The Muse™ Mito Potential kit by Muse™ cell analyzer was used to measure the potential of the mitochondrial membrane. Then, quantitative PCR was used to find out the gene expression status of apoptotic-related genes (Apaf-1, Caspase 3, and Caspase 9) and an Anti-Apoptotic Gene (BCLxL). **Results:** The IC₅₀ value of Conessine against the MCF-7 cell line was 123.5981 µg/mL. Morphological analysis also demonstrated dose-dependent effects following treatment with Conessine. Dual staining with Acridine orange and Ethidium Bromide identified apoptotic cells in the treated groups, a finding corroborated by the MMP assay, which indicated an increased proportion of depolarized cells. At the molecular level observation denotes apoptotic genes (Apaf-1, Caspase 3, and Caspase 9) were upregulated and Anti-Apoptotic Genes (BCLxL) were down regulated. **Conclusion:** The results of this study indicated that Conessine possesses anticancer efficacy against the MCF-7 cell line. The upregulation of apoptosis-related genes signifies the activation of the mitochondrial-mediated apoptotic pathway, primarily initiated by APAF-1 (apoptotic protease-activating factor), followed by sequential activation of caspase cascade components. In conclusion, Conessine exhibits significant potential as an anticancer agent, necessitating further exploration across additional cancer cell lines to validate its therapeutic efficacy.

Keywords: APAF-1, Apoptosis, Breast cancer, Conessine, Intrinsic pathway, Mitochondrial Membrane Potential.

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INTRODUCTION

Breast Cancer (BC) is one of the most life-threatening cancers worldwide leading in rank second among women.¹ It develops due to the unrestrained proliferation of breast cells, leading to

the formation of a typically painless lump. The condition often remains undetected in its initial stages. Once the cancer begins to metastasize, it progresses to advanced stages (III or IV), where treatment becomes highly challenging or incurable.² Mammography is often considered a painful diagnostic technique, and the subsequent treatment approach varies depending on the type of breast cancer. Patients with Estrogen Receptor (ER)-positive and Progesterone Receptor (PR)-positive typically undergo hormonal therapy, whereas those with hormone receptor-negative cancers are treated with chemotherapy. In early-stage cases, radiotherapy is commonly employed. Despite the availability of various therapeutic options, most treatments



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are associated with considerable discomfort and significant adverse effects.³ Recent statistical data indicate that approximately one in eight women is affected by breast cancer. In 2022, around two million new cases were reported globally, and this number is projected to rise to nearly 3-4 million by 2040-2050.⁴⁻⁶

Statistical projections indicate a substantial increase in the global incidence of breast cancer in the coming years. BC progressions are highly associated with genetic susceptibility and dysregulation of various molecular signalling pathway. Apoptosis related molecular targets, oncogenes, tumour suppressor genes involved in the uncontrolled cell division/proliferation, metastasis and drug resistance in BC cells. In recent days, bioinformatics research highly focused on the molecular targets and gene involved in BC pathogenesis responses. These kinds of molecular approaches explore novel drug targets and compounds in the field of drug designing.⁷ Conventional treatment approaches to control the BC condition in some extent which may not be sufficient for effective prevention and management. Exploring alternative phyto-based strategies could play a significant role in cancer prevention, potentially minimizing both the pain and adverse effects associated with traditional therapies.⁸ Conessine, a steroidal alkaloid found in the roots, leaves, and seeds of *Holarrhena floribunda*, *Holarrhena antidysenterica*, *Holarrhena curtisii*, *Holarrhena pubescens*, and *Holarrhena mitis*.⁹⁻¹¹ The Apocynaceae family is rich in many steroidal alkaloids. Conessine is one of them with potential pharmacological properties such as anti-plasmodial, anti-bacterial, anti-cercarial, fungicidal, anti-parasite, anti-diabetic, anti-feedant, anti-oxidant, antidiarrheal, antitrypanosomal, and CNS stimulant.¹¹⁻¹⁴

In traditional medicine practice, *Holarrhena antidysenterica* has been widely used by tribal communities for the treatment of various ailments, including epilepsy, abdominal pain, anemia, cholera, protozoal infections, and skin disorders. Among its bioactive constituents, the steroidal alkaloid conessine has been the most extensively studied for its diverse pharmacological activities.⁹ Conessine is known as a histamine H3 receptor antagonist capable of crossing the Blood-Brain Barrier (BBB) through the Central Nervous System (CNS). Owing to this property, it has potential therapeutic applications in the treatment of Alzheimer's disease.¹⁵ Despite Conessine having high-profile pharmacological benefits, its anti-cancer properties have not been explored yet. There is little research evidence on Conessine's anticancer properties. To date, limited evidence is available regarding its direct cytotoxic effects. Herbal formulation (HC9) consists of *Holarrhena antidysenterica*, which shows the potent anti-cancer property against human breast cancer cell lines such as MCF-7 and MDA-MB.¹⁶ Another research reported that Conessine showed inhibitory activity in proliferation, migration, and invasion in liver cancer cell lines (Huh 7 and Mahlavu cells).¹⁷

In this context, our study aimed to explore the anticancer properties of Conessine for the first time in the human breast

cancer cell line MCF-7 through cytotoxicity assessment and apoptosis evaluation, followed by molecular analysis of apoptosis-related gene expression by quantitative PCR.

MATERIALS AND METHODS

Conessine was purchased from Sigma Aldrich. All other reagents and kits, such as Dulbecco's Modified Eagle's Medium (DMEM - Sigma Aldrich, USA), sodium bicarbonate (Merck, Germany), Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA), Muse™ Mito potential kit, and SYBR green kit, were procured from commercial suppliers.

Methods

Acquisition of cell line

MCF-7 Human Breast Cancer cell line and MCF-10A (human mammary epithelial cell line) were purchased from ATCC, USA. Cell line maintenance was processed by utilizing the following Dulbecco's modified Eagles' medium, Fetal bovine serum and Penicillin-streptomycin-Amphotericin B of standard concentrations.

Cytotoxicity assessment

MTT assay was performed to understand the ability of Conessine to inhibit the growth of the MCF-7 cell line. Cells were seeded in a 96-well cell culture plate and incubated under appropriate conditions to achieve 70% confluency. Sample (1 mg of Conessine was dissolved in 0.1 % DMSO and filtered through a 0.2 µm syringe filter) was introduced to each well as 6.25, 12.5, 25, 50, and 100 µg/mL. The treated and control cells were incubated for 24 hr and visualized under a phase-contrast microscope to assess the inhibitory effect, and images were recorded (Olympus CKX41 with Optika Pro5 CCD camera) to analyze morphological changes. Then the media with the sample was replaced by the media with MTT (5 mg/mL) for each well and incubated in the dark for 2-4 hr. The MTT reagent attached to the surface of live cells was extracted using absolute DMSO. The extracted bluish-purple solution was read at 570 nm by an ELISA reader.¹⁸ The percentage viability was calculated as

$$\% \text{ viability} = (\text{OD Mean(sample)}/\text{OD Mean(control)}) \times 100$$

Apoptosis investigation by Dual staining

Acridine orange/Ethidium bromide (AO/EtBr) dual staining was used to assess the apoptotic status of MCF-7 cells following Conessine treatment. 1×10^5 cells were seeded in 6 well cell culture plate and incubated for 3/4th confluency. IC₅₀ (123.6 µg/mL) concentration of Connesin was added to the cells and incubated for 24 hr. Untreated cells were also maintained as a control group. 1 mL of 100 µg/mL of AO and EtBr each was added to the wells and incubated for 10 min at RT. Cells were washed twice with 1xPBS and observed under a fluorescence microscope (Olympus CKX41 with an Optika Pro5 camera).

Mitochondrial membrane stability measurement: Flow cytometry

Mitochondria are a crucial player in most cellular mechanistic pathways. It involves almost all energy metabolisms, homeostatic equilibrium, the pivotal apoptotic pathway initiator, etc. Loss of Mitochondrial Membrane Potential (MMP) disrupts all cellular processes. So, considering the investigation of MMP is predominant. Muse™ Mito potential kit was used to detect MMP through flow cytometry. For this experiment, a standard cell count was seeded in 6-well plates and incubated to reach maximum confluency. Treated (IC_{50} concentration of Conessine - 123.59 $\mu\text{g/mL}$) and untreated cells were washed and trypsinized. Harvested cells were centrifuged and resuspended with 95 μL of 1x assay buffer (Muse™ Mito Potential Dye (MPD) diluted as 1:1000). Then, they were incubated for 20 min at 37°C. At the end of the incubation, 5 μL of 7-AAD reagent was added and incubated for 5 min at RT. Subsequently, the altered MMP value was determined by flow cytometry (Muse™ Cell analyser). High membrane potential causes the Mito Potential dye to be observed more and exhibits increased fluorescence, while a depolarized membrane shows decreased fluorescence intensity. 7-AAD (7-amino-actinomycin D) is a dead cell marker that binds to the DNA and reflects the status of the DNA. Muse cell analyser results different cell population as (i) MPD (-) and 7-AAD (-): Live cells with depolarized Mitochondrial Membrane (MM), (ii) MPD (+) and 7-AAD (-): Live cells with intact MM, (iii) MPD (+) and 7-AAD (+): Dead cells with depolarized MM, (iv) MPD (-) and 7-AAD (+) Dead cells with intact MM.

Quantitative PCR: Gene expression

Cells treated with the IC_{50} concentration of Conessine (123.59 $\mu\text{g/mL}$) and untreated cells were subjected to RNA isolation using the TRIzol method. 1 mL of TRIzol was added to each well and incubated for 5 min. The cells were collected and added with 200 μL of Chloroform. The mixture was centrifuged at 14000 rpm for 10 - 15 min. To the aqueous layer, 0.5 mL of absolute isopropanol was added and left for 10 min at RT. Centrifuged at the same conditions as the previous step. The pellet obtained was washed twice with 75% ethanol by centrifugation. The final pellet was air-dried and resuspended in Tris-EDTA buffer as required. Quantitative and qualitative analysis of isolated RNA was performed, then cDNA was synthesized using the iScript

cDNA synthesis kit (Cat. No: 1708891, Bio-Rad), following the manufacturer's instructions. Synthesized cDNA was used in the qPCR analysis. SYBR Green Master Mix (Cat. No: 786-5062, G-BIOSCIENCE) was used. Gene expression was analysed using LightCycler 96 (Roche). cDNA template, master mix, and forward and reverse primers (APAF1, Cas3, Cas9, BCLxL, GAPDH) were added as instructed by the manufacturer, and PCR conditions were set to 2 min of initial activation followed by denaturation at 95°C and annealing for 1 min at the appropriate primers' optimized temperature. Primer sequences - forward and reverse were tabulated in Table 1. An extension step was performed for 1 min at 72°C for 34 cycles. All the experiments were triplicated. The $\Delta\Delta C_t$ method was used to calculate the gene expression. Qualitative check has also been performed for the confirmation.

Statistical assessment

Graphpad PRISM was used to analyze the statistical significance difference between the control and Conessine-treated groups. All the experiments were triplicated, and the results were provided as Mean $\pm$ SD. One-Way Analysis of Variance (ANOVA) was used to determine the significance, and Tukey's multiple comparison test was used to compare the control and treated groups. $p < 0.05$ is considered significant.

RESULTS

Cytotoxicity assessment

MTT results revealed that Conessine inhibits the growth of the MCF-7 cell line in a dose-dependent manner. Figure 1A represents the data obtained from the cytotoxicity investigation of Conessine against MCF-7 cells. Control showed 100% viability. Treated groups such as 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$ of Conessine exhibited 98.72 $\pm$ 0.761, 89.37 $\pm$ 0.890, 82.85 $\pm$ 0.915, 74.49 $\pm$ 0.197, and 60.68 $\pm$ 0.79 viability percentage, respectively. Based on these data, the exact IC_{50} value was calculated by ED₅₀ Plus V1.0 software as 123.5981 $\mu\text{g/mL}$. Meantime, the normal human mammary epithelial cell line employed in the research showed an IC_{50} value of 174.27 $\mu\text{g/mL}$. Statistical analysis of cytotoxicity showed a significant difference ($p < 0.01$) between the control and treated groups, except at 6.25 $\mu\text{g/mL}$, which showed no significant difference from the control group. Morphological changes observed are shown in Figure 1B. The cell damage was corroborated by the results obtained from the MTT analysis.

Table 1: Primer Sequences.

Oligo nucleotides	Forward (5'- 3')	Tm	Reverse (5'- 3')	Tm
APAF1	GCCAAGCAGGAGGTCGATAATG	62.1	GACCATCCTCAGAAAAGCAGGC	62.1
Cas 3	TTCAGAGGGGATCGTTGTAGAAGTC	63.0	CAAGCTTGTCGGCATACTGTTTCAG	63.0
Cas 9	ATGGACGAAGCGGATCGGCGGCTCC	71.2	GCACCACTGGGGGTAAGGTTTTCTAG	66.4
BCLxL	CTGGTGTTGACTTTCTCTC	58.9	GAGTTCATTCACCTGTTC	55.2
GAPDH	ACTCAGAAGACTGTGGATGG	57.3	GTCATCATACTTGGCAGGTT	55.3

Control showed intact cell nuclei and cell walls. In contrast, cell blebbing, disintegration of cellular organelles, deterioration of the cell wall, loss of cell adhesion, and cell shrinkage were observed in conessine-treated groups in a dose-dependent manner.

Dual staining: AO/EtBr staining

The Conessine-treated group and the untreated group were subjected to the AO/EtBr staining procedure. The resultant images are depicted in Figure 2. Control/untreated groups showed green

fluorescence in almost all cells, indicating the cells' viability (Figure 2A). In contrast, in the treated group, mixed cell fluorescence was observed (Figure 2B), with green fluorescence indicating normal cells with distinct nuclei and orange fluorescence indicating apoptotic cells. Early apoptotic and late apoptotic cells were also observed as green- and orange-fluorescent condensed nuclei, respectively. The anti-proliferative property of conessine also leads to the apoptotic cycle, as confirmed by the AO/EtBr dual staining method.

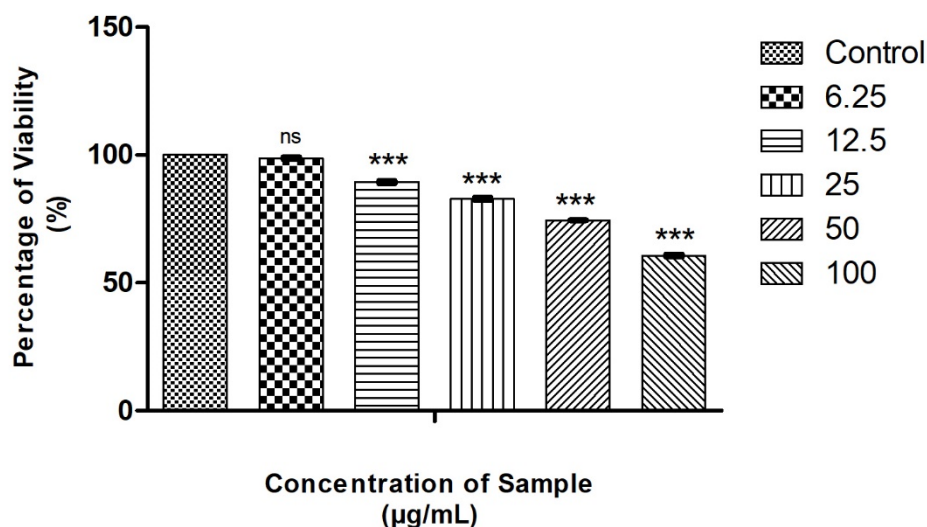


Figure 1A: MTT assay revealed the anti-proliferation activity of Conessine against the MCF-7 cell line in a dose-dependent manner. The X axis shows various concentrations of Conessine, while the Y axis shows cell viability as a percentage. ANOVA results indicate significant differences between the control and treated groups ($p < 0.01$), except for the 6.25 µg/mL group, which showed no significance.

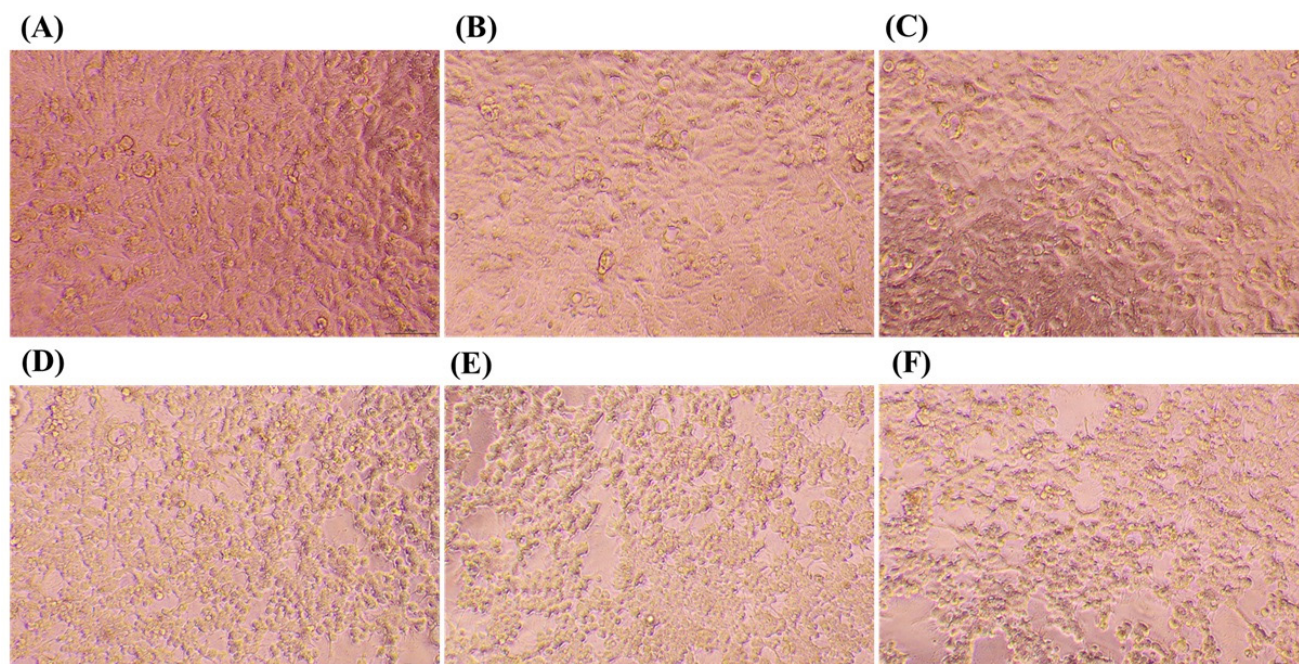


Figure 1B: Morphological changes of MCF 7 cell lines upon treatment with Conessine are depicted in the figure. Control showed distinct morphology, while the treated groups exhibited the deterioration of cells in a dose-related manner.

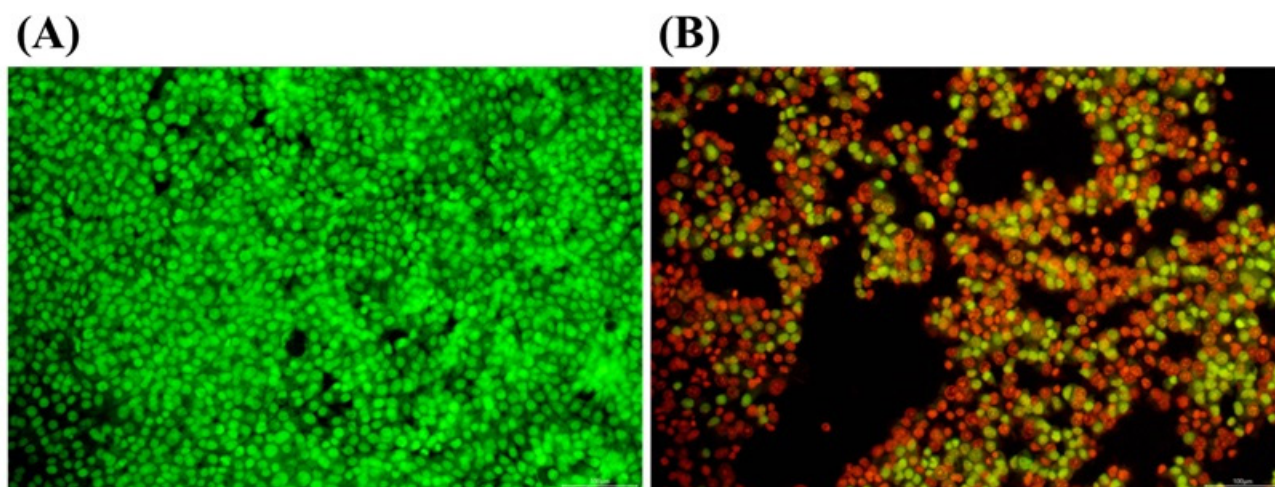


Figure 2: Acridine orange/Ethidium bromide staining. Images showed (A) the control group and (B) the treated group. The control group showed a live cell population, whereas the treated group exhibited a mixed population, including early- and late-apoptotic cells and dead cells.

Mitochondrial membrane potential

Mitochondria act as a central regulator of the caspase cascade-mediated apoptotic pathway. Investigating Mitochondrial Membrane Potential (MMP) is essential in apoptosis studies, as the loss of membrane stability leads to the release of apoptotic triggers from the mitochondria. MMP was assessed by using the Mito potential kit. Figures 3A and 3B showed the results of the MMP analysis for the control and Conessine-treated groups, respectively. The results revealed a decrease in the live cell percentage between the control and treated groups, from 80.41 to 60.06. In contrast, the rate of depolarized live cells, depolarized dead cells, and dead cells was elevated in the treated group compared to the control group. 2 % of depolarized live cells in the control group showed a prominent increase to 19.89 % upon treatment with Conessine (IC_{50} dosage 123.59 $\mu\text{g/mL}$). Similarly, depolarized dead cells were absent in the control group, which was increased to 0.19 % in the treated group. 17.60 % of dead cells were observed in the control group, which increased to 19.85 % in the treated group. From the results, it was clear that conessine treatment has a substantial impact on mitochondrial membrane stability, consistent with the mitochondrial-mediated apoptotic signalling pathway.

Gene expression studies by qPCR

Conessine has been confirmed for its anti-proliferative activity that induces apoptosis. To further elucidate its molecular effects on MCF-7 cells, apoptosis-related gene expression is the primary focus of analysis. Apoptotic regulators such as APAF-1, Caspase 3, and Caspase 9, and the anti-apoptotic gene BCLxL were investigated in control/untreated and conessine-treated groups. The qPCR results were shown in Figures 4A - APAF-1, 4B - Caspase 3, 4C - Caspase 9, and 4D - BCLxL. From the graph, it was understood that the gene expression results also corroborated the previous results of apoptotic staining and MMP analysis. The

APAF-1, Caspase 3, and Caspase 9 genes in the conessine-treated group showed elevated expression compared to the untreated groups. The APAF-1 gene showed a 1.937-fold change compared to the control group. Similarly, Cas3 and Cas9 also showed upregulated gene expression as 1.616 and 1.840, respectively, compared with the control group. The anti-apoptotic gene BCLxL showed downregulated expression, with a fold change of -1.519 compared to the control group. The gel picture (Figure 5) also showed increased band width for APAF-1, Cas 3, and Cas 9 in the treated groups, whereas the lower band width for BCLxL was observed in the untreated group.

DISCUSSION

Recent statistics reported that approximately 2 million new breast cancer cases were diagnosed worldwide, with around 0.6 million related deaths in 2022. Further, the report insisted that both the incidence and mortality rates are projected to triple by 2050, a huge number that underscores the urgent need for attention.⁴ The major contributing factors to poor outcomes among breast cancer patients include limited awareness of early detection, metastatic progression, and tumor recurrence following surgery.⁵ Surgical removal of the primary tumor alone does not ensure a permanent cure; therefore, the effective eradication or induction of apoptosis in breast cancer cells is essential to control excessive proliferation and invasiveness. Chemo and radiotherapy always end up with a plethora of adverse effects. The plant-based bioactive compound of interest, which targets its primary apoptotic signalling pathway, represents a promising and safer alternative for cancer treatment.

Steroidal Alkaloids (SAs) have demonstrated potent anticancer activity against several malignancies, including breast, liver, colon, and pancreatic cancers, etc. SAs are predominantly derived from higher plant families such as Apocynaceae, Solanaceae, and Buxaceae^{19,20} mainly isolated from the seeds of *Holarrhena antidysenterica*, stems of *Holarrhena floribunda*, respectively.

It is known for its wide range of pharmacological properties, including antibacterial, antifungal, and antiparasitic.^{20,21} Although the antioxidant potential of Conessine has been reported, its anticancer efficacy remains unexplored mainly, and no data has been reported yet.²² The present study, therefore, aims to investigate the antiproliferative and apoptosis-inducing potential of Conessine in the human breast adenocarcinoma cell line (MCF-7).

Cytotoxicity was assessed at various concentrations of Conessine, and the results showed dose-dependent inhibition in MCF-7 cells, confirming the anti-proliferative and anticancer efficacy of Conessine, which was well compared with normal mammary cells. The morphometric observation also exhibited a similar trend with respect to the MTT analysis, such as the treated group showed dose-related deterioration of the cell wall and internal organelles, while the control group showed intact cell morphology with nuclei. The preliminary study by Dua *et al.* (2013) demonstrated that Conessine exhibited cytotoxicity in the rat skeletal muscle (L-6) cell line, with an IC_{50} value of 14 μ g/mL.²³ Another study indicated that C2C12 and MCF-7 cells showed no significant cytotoxic response up to 10 μ M of Conessine; however, at 20 μ M, the compound induced partial cell cycle arrest in the S and G2/M phases.²² Holamine and Funtumine are two distinct steroidal compounds, similar to Conessine, isolated from *Holarrhena floribunda*, which exhibited anti-proliferative properties in MCF-7 with IC_{50} of 42.82 μ M and 52.69 μ M, respectively.²⁴

AO/EtBr dual staining resulted in a higher proportion of apoptotic cells in the Conessine-treated group compared to the control, with distinct visualization of early apoptotic, late apoptotic, necrotic, and viable cells under fluorescence microscopy. Consistent results were obtained from Mitochondrial Membrane Potential (MMP) analysis, indicating that Conessine treatment induced oxidative stress in cells. The percentage of cells remaining in the viable or necrotic phase is lower than the percentage of depolarized live and dead cells in the treated group, indicating a pronounced disturbance in mitochondrial homeostasis and a consequent loss of cellular equilibrium. Molecular level investigation was carried out by gene expression method through qPCR, which demonstrated the upregulated gene expression of APAF-1, cas 3, cas 9 (apoptotic-related gene), and downregulated for BCLxL (anti-apoptotic gene) in the treated groups compared to the control group.

Reactive oxygen species, or free radicals, play a crucial role in initiating the sequential apoptosis mechanism. When cells enter a stressful environment, homeostatic equilibrium is disturbed, releasing large numbers of free electrons that disrupt other cellular functions.²⁵ The uncontrolled environment leads to downregulation of the Bcl2 and BCLxL genes, which inhibits the release of cytochrome C from mitochondria. Enormous pores will be generated in the mitochondrial membrane, termed as mitochondrial permeability transition pores (mPTP). The excessive ROS disturbs all the ion channels, which tends to release augmented Ca^{2+} ions and vice versa. These overloaded Ca^{2+} ions are the primary cause of MMP loss.²⁶ This MMP loss

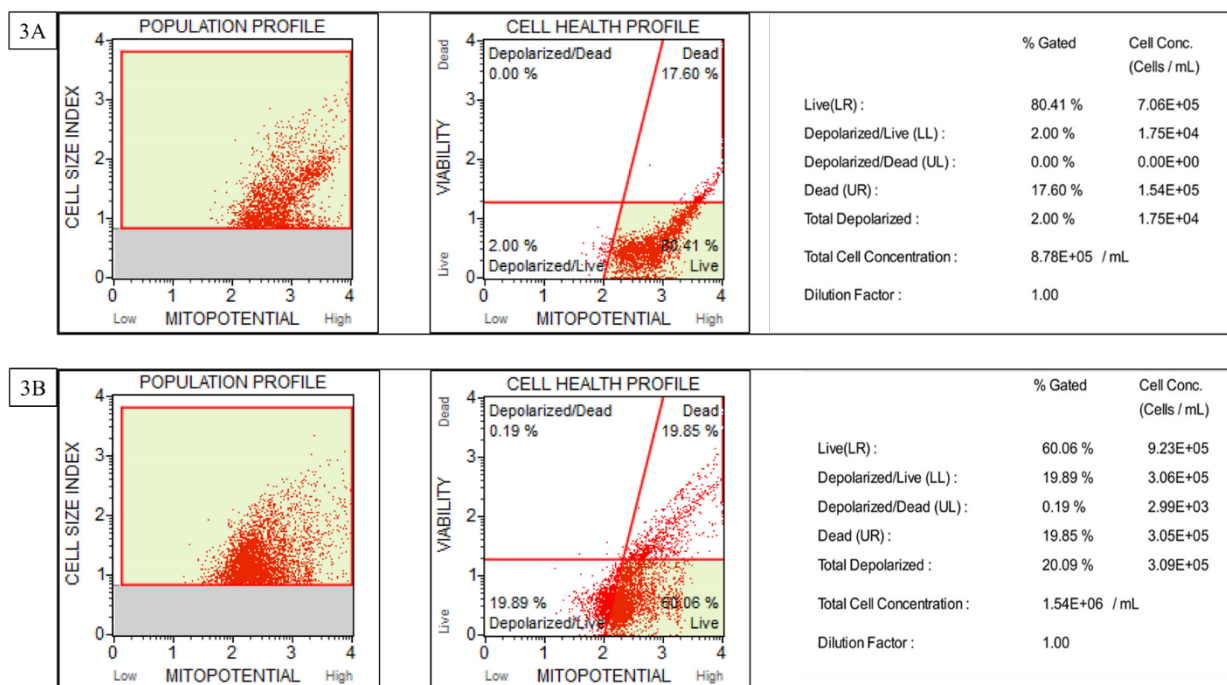


Figure 3: MMP assay. 3A - Control group and 3B - Connesine-treated group. MMP results showed that depolarized cells increased significantly in the treated group compared with the control group, confirming MMP loss. Images showed the cell size index, viability profile, and percentages of live, dead, depolarized live, and depolarized dead cells.

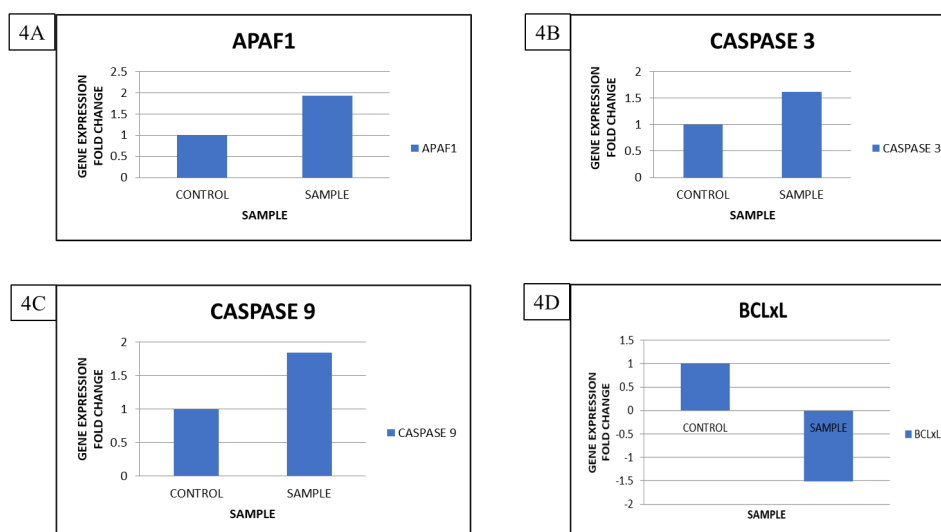


Figure 4: Gene expression analysis. Apoptotic and anti-apoptotic gene expressions were analyzed for Connessine-treated and untreated groups, which were depicted in Figure 4A - Apaf-1, 4B - Cas3, 4C - Cas9, and 4D - BCLxL genes, respectively. Upregulated gene expression was observed for apoptotic genes and downregulated for anti-apoptotic genes.

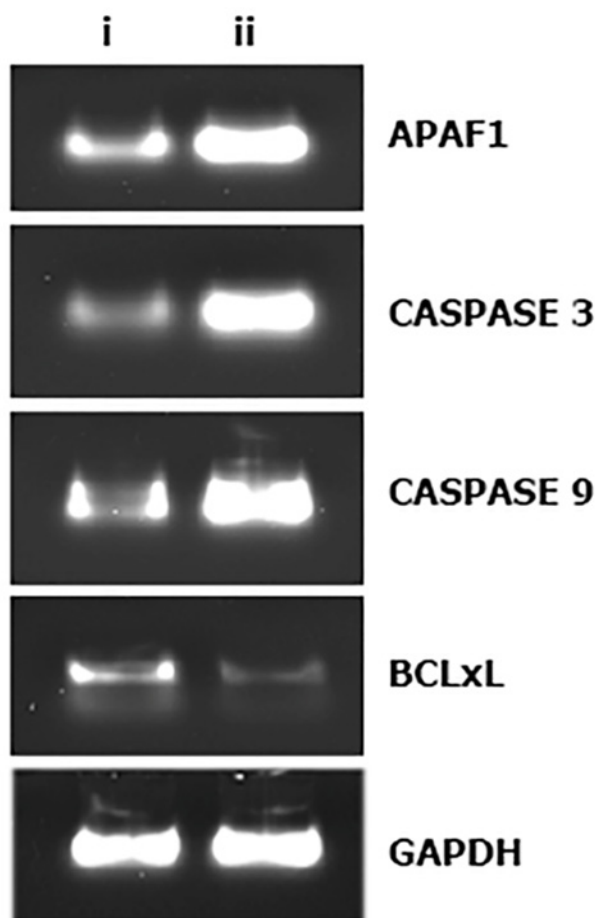


Figure 5: Qualitative confirmation of qPCR by Agarose gel electrophoresis experiment. The gel image showed the bands obtained for Apaf-1, Cas3, Cas9, BCLxL, and GAPDH (Housekeeping gene). Increased band width for apoptotic genes (Apaf-1, Cas3, Cas9) and lower intensity band for Anti-Apoptotic Gene (BCLxL) were observed in the treated group compared with the control.

leads to the cytochrome C release, which directly binds to the APAF-1 protein.²⁷

APAF 1 protein (Apoptosis protease activating factor -1) plays a pivotal role in the caspase cascade activation. APAF-1 protein has three major domains: the Caspase-Recruiting N-Terminal Domain (CARD), the Nucleotide-Binding Domain (CED-4), and the aspartate-tryptophan-containing C-terminal Domain (WD repeats - for protein-protein interaction). Cytochrome C released from mitochondria directly binds with the WD site of APAF-1, which will open the CARD domain for procaspase binding. APAF-1 protein oligomerization through the CED-4 domain will generate a wheel structure called apoptosome, which contains seven spikes, leading to the procaspase 9 cleavage and simultaneously activating the caspase cascade (cas3, cas6, and cas7). These processes all together pave the way to intrinsic apoptosis of breast cancer cell line.²⁸ Anti-proliferative, anti-cancer potency of Conessine was demonstrated in liver cancer cell lines such as Huh7 and Mahlavu, and it was further reported that Conessine induced apoptosis and the EMT mechanism.¹⁷ Other steroidal alkaloids, Solanine and Solasonine, exhibited antiproliferative properties, loss of MMP, downregulated gene expression of Bcl2 and BCLxL, and upregulated levels of Cas 3 and 9 in MCF-7 and BCap-37 (BC) cell lines, respectively, leading to intrinsic/mitochondria-mediated apoptosis signalling pathway.^{29,30} Solamargine, a steroidal alkaloid from the Solanaceae family, exhibited an elevation of the Bax gene expression and downregulated expression of the BCLxL gene in various breast cancer cell lines, HBL-100, ZR-75-1, and SK-BR-3.³¹ Conessine is an unexplored steroidal alkaloid in breast cancer cell lines. Conclusively, the results obtained supported Conessine's anti-cancer and anti-proliferative efficacy. Since very few studies explored its anti-cancer potency, further evaluation is necessary to check for other cancer types, as well as molecular-mechanical

insights, and the pharmacokinetic profile should be explored in-depth to validate Conessine as a future therapeutic candidate for cancer.

CONCLUSION

The present study provided firm evidence of the anti-cancer property of Conessine against the human adenocarcinoma cell line, MCF-7, which is unexplored. Morphological alterations, apoptosis execution, mitochondrial-mediated intrinsic signaling, and the apoptosis mechanism were confirmed by the experiments. Since this study is limited to the MCF-7 cell line, *in vitro* and *in vivo* analyses with other cancer cell lines should be conducted. Understanding the pharmacokinetic and toxicological profile of Conessine is also essential for validating it as a promising therapeutic candidate for cancer in the future.

ABBREVIATIONS

7-AAD: 7-Aminoactinomycin D; **ANOVA:** Analysis of Variance; **AO:** Acridine Orange; **APAF-1:** Apoptotic Protease Activating Factor-1; **ATCC:** American Type Culture Collection; **BBB:** Blood-Brain Barrier; **BC:** Breast Cancer; **BCLxL:** B-cell Lymphoma Extra-Large; **CARD:** Caspase Recruitment Domain; **Cas3:** Caspase-3; **Cas9:** Caspase-9; **CED-4:** Cell Death Abnormality-4; **CNS:** Central Nervous System; **DMEM:** Dulbecco's Modified Eagle's Medium; **DMSO:** Dimethyl Sulfoxide; **DNA:** Deoxyribonucleic Acid; **ED₅₀:** Effective Dose 50; **EMT:** Epithelial-Mesenchymal Transition; **ER:** Estrogen Receptor; **EtBr:** Ethidium Bromide; **FBS:** Fetal Bovine Serum; **GAPDH:** Glyceraldehyde-3-Phosphate Dehydrogenase; **HC9:** Polyherbal Formulation HC9; **H3:** Histamine H3 Receptor; **IC₅₀:** Inhibitory Concentration 50; **mPTP:** Mitochondrial Permeability Transition Pore; **MMP:** Mitochondrial Membrane Potential; **MPD:** Mito Potential Dye; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **OD:** Optical Density; **PBS:** Phosphate-Buffered Saline; **PCR:** Polymerase Chain Reaction; **PR:** Progesterone Receptor; **qPCR:** Quantitative Polymerase Chain Reaction; **RNA:** Ribonucleic Acid; **ROS:** Reactive Oxygen Species; **RT:** Room Temperature; **SA:** Steroidal Alkaloid; **SD:** Standard Deviation; **SYBR:** SYBR Green Dye; **WHO:** World Health Organization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Ahad Amer Alsaieri: Conceptualized and designed the study, supervised the research work, performed data analysis and interpretation, and drafted the original manuscript. **Hanadi M. Zbrmawi:** Contributed to methodology development, experimental execution, and data acquisition. **Jawaher Amer Alsaieri:** Assisted in data analysis, validation of results, and

manuscript review and editing. **Mariam M. Jaddah:** Participated in the investigation, data curation, and preparation of figures and tables. **Aisha Abdullah Alshantiti:** Contributed to literature review, formal analysis, and manuscript editing. **Hanan Salem Alghamdi and Amal Saeed Al Ayed:** Provided critical revision of the manuscript, contributed to the interpretation of results, and approved the final version for submission. All authors have read and approved the final manuscript.

SUMMARY

This study investigates the anticancer potential of the steroidal alkaloid conessine in human breast cancer MCF-7 cells, with a focus on mitochondrial-mediated apoptosis. Conessine exhibited dose-dependent cytotoxicity with an IC₅₀ of 123.6 µg/mL, accompanied by marked morphological alterations and apoptotic features confirmed by AO/EtBr staining. Mitochondrial membrane potential analysis revealed significant membrane depolarization following treatment. Quantitative PCR demonstrated upregulation of apoptotic genes APAF-1, caspase-3, and caspase-9, alongside downregulation of the anti-apoptotic gene BCLxL. Collectively, the findings indicate that conessine induces intrinsic apoptosis via the mitochondrial pathway, highlighting its potential as a effective anticancer agent.

REFERENCES

1. Jin S, Zhang QY, Kang XM, Wang JX, Zhao WH. Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway. *Ann Oncol.* 2010;21(2):263-8. doi: 10.1093/annonc/mdp499, PMID 19889614.
2. Bate J, Rasmussen E. Key facts. In: Bate J, Rasmussen E, editors. *Macbeth*. London: Macmillan Education UK; 2009. p. 21-2. doi: 10.1007/978-1-137-00443-7_3.
3. Lillienfeld AM. The epidemiology of breast cancer. *Cancer Res.* 1963;23:1503-13. doi: 10.3322/canjclin.41.3.146, PMID 14072689.
4. Kim J, Harper A, McCormack V, Sung H, Houssami N, Morgan E, et al. Global patterns and trends in breast cancer incidence and mortality across 185 countries. *Nat Med.* 2025;31(4):1154-62. doi: 10.1038/s41591-025-03502-3, PMID 39994475.
5. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, et al. Current and future burden of breast cancer: global statistics for 2020 and 2040. *Breast.* 2022;66:15-23. doi: 10.1016/j.breast.2022.08.010, PMID 36084384.
6. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024;74(3):229-63. doi: 10.3322/caac.21834, PMID 38572751.
7. Jan H, Khan NU, Al-Qaane AM, Tasleem M, Almutairi MH, Ali I. BRCA2 polymorphisms and breast cancer susceptibility: a multi-tools bioinformatics approach. *Cell Physiol Biochem.* 2024;58(2):128-43. doi: 10.33594/000000689, PMID 38623065.
8. Tseng HS, Wang YF, Tzeng YM, Chen DR, Liao YF, Chiu HY, et al. Aloe-emodin enhances tamoxifen cytotoxicity by suppressing Ras/ERK and PI3K/MTOR in breast cancer cells. *Am J Chin Med.* 2017;45(2):337-50. doi: 10.1142/S0192415X17500215, PMID 28231748.
9. *In vitro* cytotoxic activity of leaves extracts of *Holarrhena Antidysenterica* against some human cancer cell lines – PubMed. PubMed. [cited 2025 November 5]. Available from: <https://pubmed.ncbi.nlm.nih.gov/24791416/>.
10. Pawar G, Pandey V, Saxena HO, Yadav AK, Dabral A. *Holarrhena* species: a review of the traditional uses, active constituents and pharmacological properties. *Discov Plants.* 2024;1(1):1-21. doi: 10.1007/S44372-024-00052-9.
11. Abd Karim HA, Ismail NH, Osman CP. Steroidal alkaloids from the Apocynaceae Family: their isolation and biological activity. *Nat Prod Commun.* 2022;17(11), 1934578X221141265. doi: 10.1177/1934578X221141265.
12. Siritong T, Voravuthikunchai SP, Coote PJ. Steroidal alkaloids and conessine from the medicinal plant *Holarrhena Antidysenterica* restore antibiotic efficacy in a *Galleria mellonella* Model of multidrug-resistant *Pseudomonas aeruginosa* infection. *BMC Complement Altern Med.* 2018;18(1):285. doi: 10.1186/s12906-018-2348-9, PMID 30340578.
13. Naka I, Patarapotikul J, Tokunaga K, Hananantachai H, Tsuchiya N, Ohashi J. A replication study of the association between the IL12B promoter allele CTCTAA and

- susceptibility to cerebral malaria in Thai population. *Malar J.* 2009;8(1):290. doi: 10.1186/1475-2875-8-290, PMID 20003322.
14. Kumar D, Kumar R, Singh B, Agrawal V. Modulation in the enzymatic antioxidants, MDA level and elicitation in conessine biomolecule in *Holarrhena pubescens* (medicinal tree) cultures exposed to different heavy metals: Ni, Co, Cr and As. 3 *Biotech.* 2023;13(9):307. doi: 10.1007/s13205-023-03697-7, PMID 37608912.
15. Zhao C, Sun M, Bennani YL, Gopalakrishnan SM, Witte DG, Miller TR, *et al.* The alkaloid conessine and analogues as potent histamine H3 receptor antagonists. *J Med Chem.* 2008;51(17):5423-30. doi: 10.1021/jm8003625, PMID 18683917.
16. Suryavanshi S, Zanwar A, Hegde M, Kaul-Ghanekar R. Standardization of a polyherbal formulation (HC9) and comparative analysis of its cytotoxic activity with the individual herbs present in the composition in breast cancer cell lines. *Pharmacogn J.* 2014;6(2):8-16. doi: 10.5530/pj.2014.2.2.
17. Li CY, Lin YS. Abstract 1821: Conessine inhibits cell proliferation, migration and invasion, and induces apoptosis in liver cancer cells. *Cancer Res.* 2024; 84(6_Supplement):1821-Abstract 1821. doi: 10.1158/1538-7445.AM2024-1821.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65(1-2):55-63. doi: 10.1016/0022-1759(83)90303-4, PMID 6606682.
19. Dey P, Kundu A, Chakraborty HJ, Kar B, Choi WS, Lee BM, *et al.* Therapeutic value of steroidal alkaloids in cancer: current trends and future perspectives. *Int J Cancer.* 2019;145(7):1731-44. doi: 10.1002/ijc.31965, PMID 30387881.
20. Huang Y, Li G, Hong C, Zheng X, Yu H, Zhang Y. Potential of steroidal alkaloids in cancer: perspective insight into structure-activity relationships. *Front Oncol.* 2021;11:733369. doi: 10.3389/fonc.2021.733369, PMID 34616681.
21. Xiang ML, Hu BY, Qi ZH, Wang XN, Xie TZ, Wang ZJ, *et al.* Chemistry and bioactivities of natural steroidal alkaloids. *Nat Prod Bioprospect.* 2022;12(1):23. doi: 10.1007/S13659-022-00345-0, PMID 35701630.
22. Kim H, Lee KI, Jang M, Namkoong S, Park R, Ju H, *et al.* Conessine interferes with oxidative stress-induced C2C12 myoblast cell death through inhibition of autophagic flux. *PLOS One.* 2016;11(6):e0157096. doi: 10.1371/journal.pone.0157096, PMID 27257813.
23. Dua VK, Verma G, Singh B, Rajan A, Bagai U, Agarwal DD, *et al.* Anti-malarial property of steroidal alkaloid conessine isolated from the bark of *Holarrhena Antidysenterica*. *Malar J.* 2013;12(1):194. doi: 10.1186/1475-2875-12-194, PMID 23758861.
24. Badmus JA, Ekpo OE, Hussein AA, Meyer M, Hiss DC. Cytotoxic and cell cycle arrest properties of two steroidal alkaloids isolated from *Holarrhena floribunda* (G. Don) T. Durand & Schinz leaves. *BMC Complement Altern Med.* 2019;19(1):112. doi: 10.1186/s12906-019-2521-9, PMID 31151442.
25. Lee SO, Joo SH, Kwak AW, Lee MH, Seo JH, Cho SS, *et al.* Podophyllotoxin induces Ros-mediated apoptosis and cell cycle arrest in human colorectal cancer cells via P38 Mapk signaling. *Biomol Ther (Seoul).* 2021;29(6):658-66. doi: 10.4062/BIOMOLTHER.2021.143, PMID 34642263.
26. He H, Li DW, Yang LY, Fu L, Zhu XJ, Wong WK, *et al.* A novel bifunctional mitochondria-targeted anticancer agent with high selectivity for cancer cells. *Sci Rep.* 2015;5(1):13543. doi: 10.1038/srep13543, PMID 26337336.
27. Chang JC, Chang HS, Wu YC, Cheng WL, Lin TT, Chang HJ, *et al.* Mitochondrial transplantation regulates antitumour activity, chemoresistance and mitochondrial dynamics in breast cancer. *J Exp Clin Cancer Res.* 2019;38(1):30. doi: 10.1186/s13046-019-1028-z, PMID 30674338.
28. Zlobec I, Minoo P, Baker K, Haegert D, Khetani K, Tornillo L, *et al.* Loss of APAF-1 expression is associated with tumour progression and adverse prognosis in colorectal cancer. *Eur J Cancer.* 2007;43(6):1101-7. doi: 10.1016/j.ejca.2007.01.029, PMID 17350821.
29. Zhang XH, Zhu J, Xu SL. Solanine induces human breast cancer MCF-7 apoptosis through mitochondrial pathway. *Chin Pharm J.* 2014; 49. doi: 10.11669/cpj.2014.1.6.010.
30. Li N, Cao L, Wang Y, Tao X, Ding G, Wang Z *et al.* Induction of solasonine on apoptosis of human breast cancer Bcap-37 cells through mitochondria-mediated pathway. *Chin Herb Med.* 2016;8(2):164-72. doi: 10.1016/s1674-6384(16)60026-6.
31. Shiu LY, Chang LC, Liang CH, Huang YS, Sheu HM, Kuo KW. Solamargine induces apoptosis and sensitizes breast cancer cells to cisplatin. *Food Chem Toxicol.* 2007;45(11):2155-64. doi: 10.1016/j.fct.2007.05.009, PMID 17619073.

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