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Original Article

Anti-Cancer Potential of Sinensetin, a Bioactive Compound against Human Cervical Carcinoma Cells via its Molecular Mechanism of Apoptosis

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ABSTRACT

Background: Cancer is ranked among the world's leading causes of death in 2020 with an estimated 10 million recorded fatalities attributable to the disease. After breast, colorectal, and lung cancer, cervical cancer is the fourth most frequent disease in women worldwide. Each year, there are 6,00,000 new cases and 3,40,000 fatalities due to cervical cancer. HPV (Human Papilloma Virus), the main cause of cervical cancer, spreads to the cervix's epithelial cells during intercourse. **Aim:** The use of natural products in conjunction with chemotherapy treatments is seen to be a viable alternative to chemotherapy medications. **Materials and Methods:** The purpose of this study was to determine the cytotoxic and apoptotic effects of sinensetin on HepG2 cells. For the determination of cytotoxicity using XXT assay, and LDH release assay, generation of reactive oxygen species was identified by DCFH-DA staining, Nuclear DNA damage was identified by DAPI staining, and apoptotic cell death was detected through AO/EB dual staining, and estimation of pro-apoptotic markers using ELISA method. **Results:** In the current investigation, the potential of Sinensetin as an anticancer agent for cervical cancer was evaluated. The results showed that apoptosis was induced by Sinensetin in HeLa cells by triggering ROS pathway, DNA damage, and caspase pathway. The cell viability of HeLa cells was reduced with increased concentration of Sinensetin. The LDH level was increased with increased concentration of Sinensetin. **Conclusion:** All these results suggest that Sinensetin can be used as a potent anticancer drug for cervical cancer.

Keywords: Cervical cancer, HeLa cells, Sinensetin, Apoptosis, Reactive oxygen species.

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INTRODUCTION

The most prevalent gynecological malignancies in the world are still cervical cancer and ovarian cancer. Cervical cancer is now the fourteenth most common cancer overall and the fourth most common cancer in women globally.[1] Over the past 30 years, young women have been diagnosed with cervical cancer at an accelerating incidence of 10% to 40%. Cervical cancer trails breast cancer in terms of incidence and fatalities under lower Human Development Index (HDI) values. inevitably, 85% of cervical cancer mortality worldwide are recorded in underdeveloped or poor countries, and the death rate is 18 times higher in low- and middle-income countries than it is in wealthy countries.[2]

Cervical cancer has been identified as being mostly induced by high-risk Human Papilloma Virus (HPV) infection. HPV DNA is present in more than 90% of squamous cervical malignancies. The virus is primarily transmitted through sexual contact.[3] Other significant risk factors for cervical cancer include having several sexual partners, having sexual contact before the age of 16 years old, having a high parity, smoking, and having a low socioeconomic status.[4] Cervical cancerogenesis is the process of uncontrolled cell proliferation that involves several cellular alterations, including the incorporation of the HPV gene, as well as epigenetic factors and other cellular modifications. Once the HPV infection progresses, the DNA may experience changes due to cellular and environmental factors, resulting in viral DNA integration and host DNA synthesis machinery function. In order to promote cell growth and suppress cellular apoptotic systems, viruses can thereby evade immunological and cellular defense mechanisms.[5,6]

Radiation therapy, chemotherapy, and surgery including pelvic lymphadenectomy and radical hysterectomy are the key therapies for cervical cancer. Nevertheless, these therapies revealed potential negative effects and issues: Radiation therapy can result in menopause, infertility, discomfort, or pain during sexual activity. Surgery can result in bleeding, injury to the organs nearby, and a threat of blood clots in the deep veins of

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the legs. Chemotherapy side effects can affect both cancer cells and rapidly dividing cells in various body systems. Also, the typical cervical cancer medications exhibited a variety of adverse effects and treatment resistance.[7] As a result, our research has been focused on finding new, effective natural remedies for cervical cancer. Since a very ancient time, plants and their active phyto products have been employed in contemporary medicine and have played a significant role in healthcare. The diverse pharmacological activity and health advantages of the flavonoid class of phytochemicals make them a significant class of natural products in contemporary medicine. Flavonoids have been utilized to treat human diabetes and its accompanying secondary problems. In the field of medicine, flavonoids have the potential to be anti-apoptotic, anti-hyperlipidemic, anti-inflammatory, and antioxidant. *Orthosiphon* spp. contains sinensetin, also known as 3',4',5,6,7-pentametoksiflavon, a colorless component having a molecular weight of 372.37g/mol.[8,9] Sinensetin has pharmacological properties including anti-inflammatory, anticancer, antioxidant, anti-obesity, antibacterial, vasorelaxant, anti-dementia, and antitrypanosomal effects.[10] The current research was aimed at examining the effect of Sinensetin on reactive oxygen species, cell viability, apoptosis, and the number of apoptotic markers.

MATERIALS AND METHODS

Cell line and cell culture

The human cervix epithelial adenocarcinoma cell line HeLa was procured from ATCC, USA. It was grown in 25 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin/streptomycin antibiotics, and 10% fetal bovine serum as a supplement. Cells were cultivated and incubated at room temperature in a humidified CO_2 incubator with 5% CO_2 and 95% air. Prior to subculture, the medium was replaced every 48-72 hr until confluence reached 80-90%. Phosphate-buffered solution, trypsin EDTA, trypan blue dye, and MTS were other materials utilized in cell culture. Trypsin-EDTA (0.25% trypsin with 0.01% EDTA) was applied to the cells for 10 min in order to remove them, and the reaction was subsequently inhibited by the addition of complete media.

Biological activity

Cytotoxicity assay

A 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide salt (XXT)-based cell viability test kit was used to assess the viability of the cells. 100 µL of HeLa cells were seeded in a 96-well microtiter plate. The cells were then allowed to adhere to the bottom of the wells of the plate by being incubated for 24 hr at room temperature and 5% CO_2. Different concentrations of Sinensetin were added to the microtiter plate and incubated. An additional 72 hr were spent incubating the microtiter plate. The Doxorubicin (DOX) was used in the positive control well. The XTT reagent (50 µL) was added to the plate and diluted to a final concentration of 0.3 mg/mL after the 72 hr incubation period. The plate was then incubated for an additional 2 hr. Following incubation, the absorbance of the color complex was measured using a plate reader at 490 nm with a reference wavelength set at 690 nm. In order to determine the IC_{50} of the cell population for each compound, the test was run in triplicate. With the help of the GraphPad Prism 4 application, the findings were analyzed.

LDH Assay

Sinensetin was added to the cells at various concentrations and the supernatants out of each treatment were obtained. The supernatants were added to 50 µL of the reconstituted 2X LDH assay buffer. Shaking the reagent for 30 sec produced a gentle mixing. The assay plate was shielded from light, incubated for 10 to 30 min at temperature (22 to 25°C), and then 50 µL of stop solution was added to each well. For 30 sec, the reagent was shaken to blend it. About 490 to 520 nm were used to measure the absorbance. The Doxorubicin (DOX) was used in the positive control well. The following formula was used to compute the percentage of cytotoxicity:

Percent Cytotoxicity=(Experimental LDH Release-Medium Background) (Maximum LDH Release Control-Medium Background) X 100.[11]

AO/EtBr Dual staining

HeLa cells were plated at a density of 1×10^6 cells per well and allowed to grow for 24 hr. HeLa cells were exposed to Sinensetin at IC_{50} concentrations and positive control DOX for 24 hr following confluence. The monolayer of HeLa cells used in this nuclear analysis investigation was stained for 5 min with 1 mM acridine orange and ethidium bromide. Using fluorescent microscopy, DNA damage, morphological alterations in the apoptotic nuclei, and condensed chromatin were seen after incubation in the labeled cells.

DAPI staining

By using DAPI, a fluorescent nuclear dye, the apoptotic induction caused by Sinensetin was examined. The labelling was performed to demonstrate the nucleic DNA of apoptotic cells fragmentation and condensation. For the purpose of ensuring appropriate adhesion to the 96-well polystyrene culture plate, the cells (1 x 10^4) were seeded in 100 µL complete media in each well of the 96-well culture plate for 24 hr at room temperature and 5% CO_2. A minimal quantity of DMSO was used to dissolve the compound, which was then diluted to the necessary quantities in the medium and administered in triplicate to the wells containing cells that were growing exponentially. The extract with medium was removed after the treatment period of 24 hr. Following a PBS wash, cells were fixed in 4% paraformaldehyde for a duration of 10 min. After that, the cells were stained with 50 µL of DAPI at

a final concentration of 1 µg/mL after being permeabilized using permeabilizing solution (3% paraformaldehyde and 0.5% Triton X-100). After an hour, the cells were examined for fluorescence intensity and cells with fragmented and condensed nuclei. A fluorescent microscope was used to capture the photographs. Apoptotic cells were defined as those with contracted and fragmented nuclei.

Estimation of ROS

The dichloro-dihydrofluorescein diacetate (DCFH-DA) test was used to determine the ROS concentration in treated HeLa cells. Prior to the (DCFH-DA) experiment, HeLa cells were plated at a density of 2×10^5 cells in six-well plates and incubated at room temperature for 24 hr. The 45 µg/mL supernatant was added after 24 hr, and the treatment was maintained for an additional 24 hr. The cells were then stained for 20 min at room temperature with 10 µmol/L DCFH-DA. Subsequently, a multi-wavelength, multifunctional enzyme spectrometer was used to measure the fluorescence intensity, and a fluorescence microscope was used to view the results.

Analysis of Caspase-3, -8, and -9 by ELISA

To further demonstrate the potential of the Sinensetin to cause apoptotic cell death, a number of apoptosis effectors were investigated. Using commercial ELISA test kits, we calculated the protein levels of Caspase-3, -8, and -9 in untreated and Sinensetin treated HeLa cells at dose (IC_{50}) and the Doxorubicin (DOX) was used in the positive control well.

Statistical Analysis

Three distinct triplicate trials means and standard deviations are shown. The significance of differences between the treatment and control groups was assessed using one-way analysis of variance, and the data was analyzed using a student's *t*-test. Statistical significance was defined as a *p*-value less than 0.05.

RESULTS

Effect of Sinensetin on cell viability by XTT assay

In Figure 1A the XTT assay depicts how the Sinensetin affected the viability of the HeLa cells. During incubation with 2.5, 5, 10, 20, 40, and 80 µg/mL of Sinensetin, it was shown that the treatment suppressed cell viability in a concentration-dependent way. The DOX was used in the positive control well. The results show the effect of the Sinensetin IC_{50} value at a concentration of 13.08 µg/mL.

Effect of Sinensetin on Lactate Dehydrogenase (LDH) Release from HeLa Cells

Figure 1B displays the cytotoxicity of Sinensetin at various doses (2.5, 5, 10, 20, 40, and 80 µg/mL) on LDH release from HeLa cells for 24 hr. LDH release is quantified as a percentage compared to cells that have received Triton X-100 treatment. According to our findings, the treatment of HeLa cells with Sinensetin at increasing concentrations caused a considerable release of LDH in a dose-based manner, indicating a breakdown of the cell membrane's structure.

Evaluation of apoptosis by AO/EB dual staining

Figure 2 displays the effect of the Sinensetin on HeLa cell apoptosis using AO/EB dual labelling. Acridine Orange (AO) marks both living and dead cells with green nuclei, whereas Ethidium Bromide (EB) only colors cells with reduced membrane integrity in red. As a result, living cells appear green while early apoptotic cells appear reddish red. Cells that are red suggest late apoptotic cells. The results showed a decrease in the green staining of the nuclei, which indicated cell damage and apoptosis, and a steady increase in the yellowish red and red staining when treated with Sinensetin with IC_{50} and positive control DOX (Figure 2 b, c).

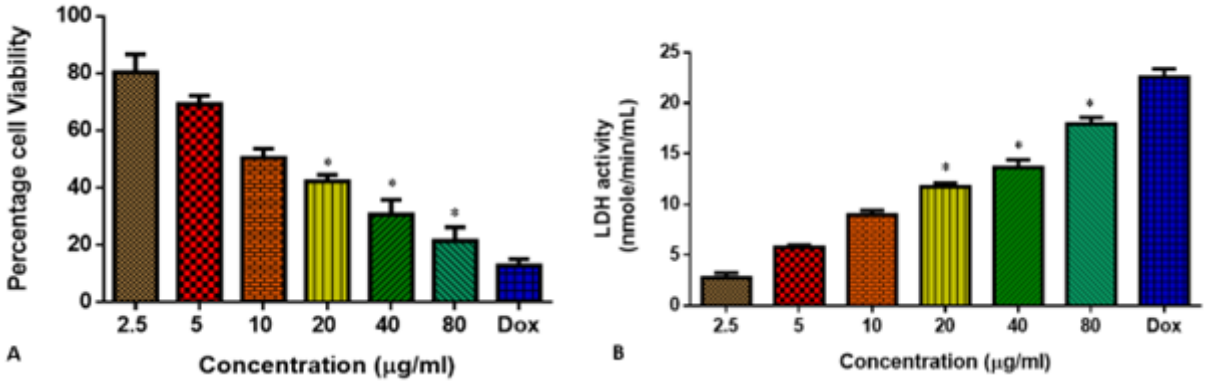

Figure 1: The cytotoxic potential of XTT assay (A) lactate dehydrogenase activities (B) on Sinensetin at different concentrations in HeLa cells. Doxorubicin was employed as the positive control. Results were computed using the mean±standard deviation of three distinct investigations. '*' denotes a significant difference from the control in the data ($p<0.05$).

Estimation of apoptosis by DAPI staining

Figure 3A depicts the potential of Sinensetin to promote apoptosis in HeLa cell lines with the observed morphological nuclear alterations assessed using DAPI labeling. A DNA-binding fluorescent dye called DAPI produces blue fluorescence when it attaches firmly to the AT-rich region of DNA. The morphological alterations caused by nuclear damage brought on by induced apoptosis are clearly seen in the DAPI labeling picture. The cells treated with positive control Dox (c) showed intense blue fluorescence. As compared to the untreated control, the cells treated with Sinensetin with an IC_{50} (b) value display a high level of fluorescence caused by triggered apoptosis with chromatin condensation, cell shrinkage, and nuclear disintegration.

The impact of Sinensetin on the nuclear DNA of HepG2 cells was detected by DAPI staining (A) (top panel), and the generation of reactive oxygen species was detected by DCF-DA staining (B) (bottom panel) in HepG2 cells. The images revealed that the untreated control cells (a) (d), Sinensetin (IC_{50} concentration) administered cells (b) (e), and DOX treated cells (c) (f). Sinensetin-treated cells displayed bright blue colors indicates loss of nuclear integirity and fragmentation of nuclear DNA which leads to apoptotic cell death in HepG2 cells. ROS production in the stained Sinensetin-treated cells indicated by greater green fluorescence than the control cells because of increased ROS production.

Detection of ROS by DCFH-DA staining

Figure 3B depicts the effect of the Sinensetin on the level of ROS in HeLa cells using the DCFH-DA staining method. Control cells showed little fluorescence, however after being exposed to Sinensetin and DCFDA, this fluorescence was considerably enhanced. It was measured how the fluorescence changed. Comparing cells treated Dox-treated HeLa cells had noticeably greater fluorescence. Our findings imply that Sinensetin specifically targets cancer cells, possibly because these cells have higher ROS levels.

Analysis of levels of caspase-3, 8, and 9 by ELISA

To ascertain if the activation/repression of apoptotic markers is involved in the Sinensetin -induced apoptosis on HeLa cells, the parameters were evaluated using the ELISA technique. The findings of the investigation into how the Sinensetin treatment changed the levels of caspase-3, -8, and -9 are shown in Figure 4 Comparing cells treated with DOX to those treated with Sinensetin revealed higher levels of caspase-3, -8, and -9 in the treated cells. The cervical cancer cells had significantly higher levels of caspase-3, -8, and -9 after receiving sinensetin compared to the control cells. This demonstrates the effectiveness of sinensetin as a cancer preventative.

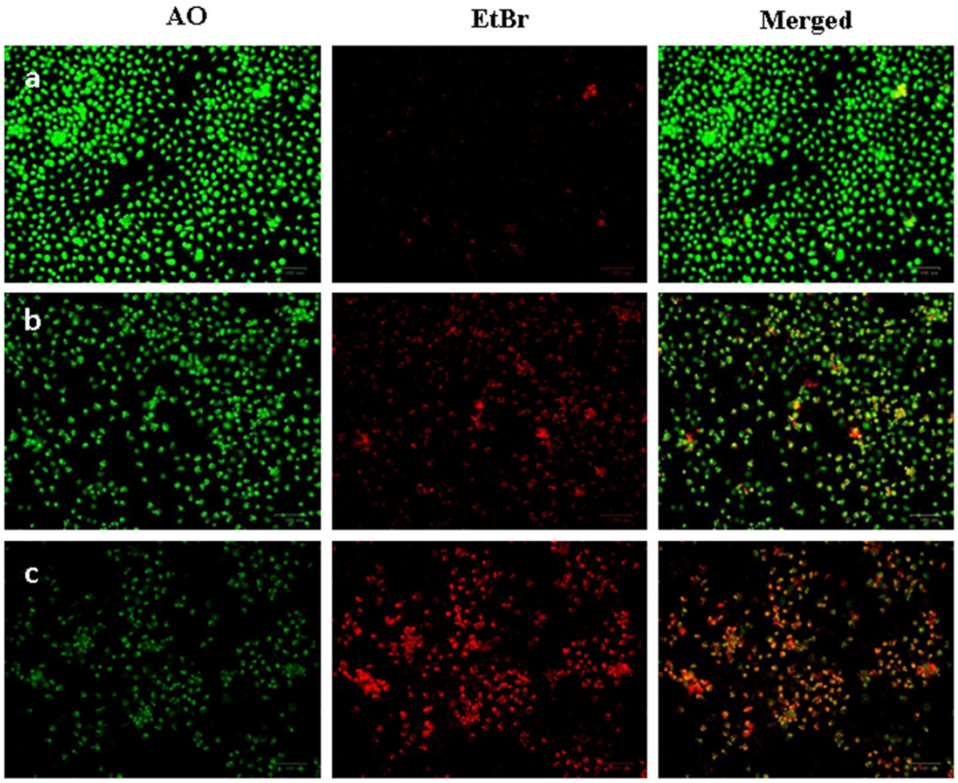

Figure 2: Effect of Sinensetin induces apoptotic induction in HeLa cells. The cells were treated within control (a), Sinensetin (IC_{50}) (b) and DOX (c) for 24 hr, stained with dual dye AO/EB and then analyzed by fluorescence microscopy. This is a representative image of the experiment performed in triplicate with magnification at 20X.

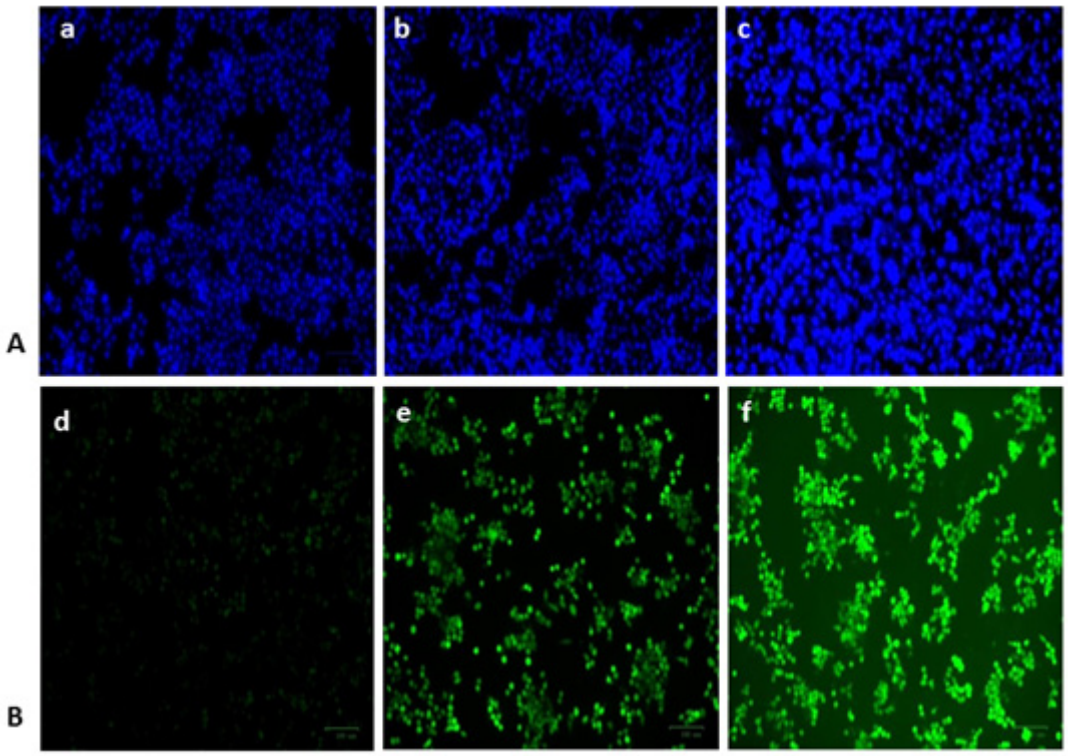

Figure 3: Sinensetin-induced DNA damage and ROS generation in HepG2 cells.

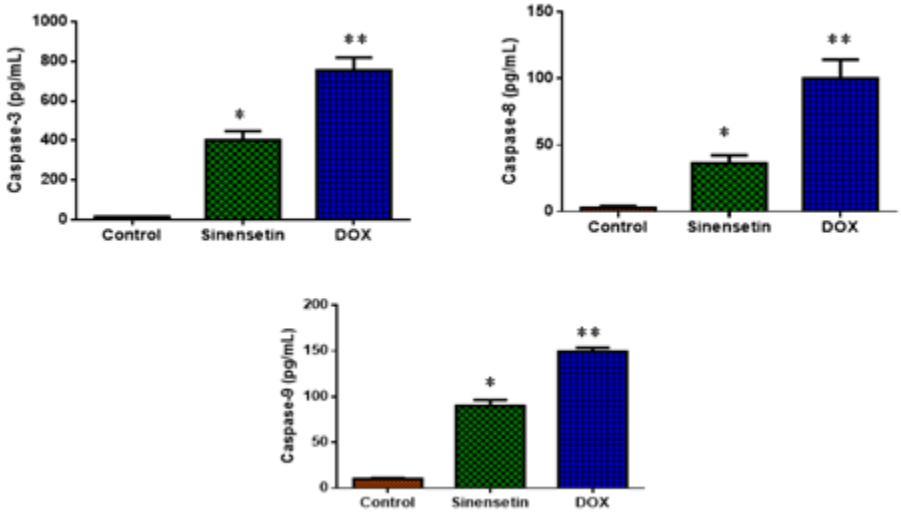

Figure 4: Sinensetin inhibits HeLa cell proliferation and promotes apoptosis. Levels of caspase-3, 8, and 9 in HeLa cells measured via ELISA. Three independent repeated tests were conducted. The results were presented as mean±standard deviation. One-way ANOVA was employed to analyze the data. '*' denotes a significant difference from the control in the data (p<0.05). '**' denotes a significant difference from the control in the data (p<0.01).

DISCUSSION

Around 200,000 fatalities and more than 500,000 newly diagnosed instances of cervical cancer occur each year, making it the second most common malignancy among women globally. Citrus-specific Polymethoxyflavones (PMFs), which have biological effects such as regulating lipoprotein metabolism and having anti-inflammatory and antioxidant properties, are of special interest. One of the Polymethoxyflavones is sinensetin (PMFs). It has been revealed via prior study that sinensetin causes Ca^{2+} mediated apoptosis and antiproliferative action in

breast cancer cells. Sinensetin also prevents gastrointestinal cancer cells from proliferating and stimulates apoptosis in these cells.[12] Sinensetin has a wide range of pharmacological effects, including anti-inflammatory, anti-bacterial, anti-trypanosomal, anti-obesity, anti-dementia, vasorelaxant, and anti-cancer properties. Sinensetin has been shown in several *in vivo* and *in vitro* experiments to exhibit great selectivity, strong efficacy against tumor cells, and low toxicity against normal cells.[13,14] While sinensetin is frequently observed in the treatment of a number of tumors, the impact on cervical cancer has not yet been precisely established, and the probable mechanisms are still unclear. We initially looked at sinensetin's ability to inhibit the proliferation of the HeLa cell line in the current investigation. Cervical cancer cells (HeLa) were significantly cytotoxicity affected by sinensetin.

Typically, PMF's because cancer cells to undergo apoptosis, cell cycle arrest, and autophagy in order to combat cancer. Thus, we estimated the cell viability of HeLa cells by XTT and LDH assay; AO/EtBr, DPAI, ROS, and estimation of caspases for apoptosis. Generally stated, cell viability refers to a cell's potential to fulfil specific physiological processes, such as the capability for metabolism, growth, proliferation, response, and adaptability. Most of the time, a cell's viability is assessed by examining one or two of its properties, such as its physiological state, membrane integrity, and rate of cell proliferation.[15] As it is soluble in the majority of aqueous solutions, XTT can be employed as an alternative to MTT. The XTT test also works with cells that are in suspension, which is a helpful feature. The test relies on XTT's extracellular reduction by NADH, which is produced in the mitochondria through an electron mediator and trans-plasma membrane electron transport. NADH is a type of NAD, or nicotinamide adenine dinucleotide.[16] The XTT assay findings demonstrated that sinensetin has a strong cytotoxic effect. After being treated with sinensetin, HeLa cells were visibly decreased in a dose-based way, which may be caused by cell cycle arrest and the activation of apoptosis. The results obtained above were supported by the effect of sinensetin on HeLa cell lines in which the IC_{50} value was 50 μM.[17] LDH is a cytoplasmic enzyme that is contained by healthy cells with intact plasma membranes but is released by necrotic cells with disrupted membranes. It is well known that when cells are exposed to high concentrations of anticancer drugs, increase in lactate dehydrogenase levels-a sign of necrosis in the cell medium. However, it is a well-known fact that when given in greater doses, cytotoxic medications, which can cause apoptosis, also stimulate necrosis, which can be hazardous to normal cells.[18] Our results showed that increase in concentration of sinensetin also elevated the LDH levels. This proves that sinensetin has the potential of anticancer agent for cervical cancer.

Apoptosis is a controlled and organized cellular process that takes place in both healthy and unhealthy environments. These three basic biochemical changes are indicative of apoptosis: Caspases are activated, proteins and DNA are broken down, membrane modifications occur, and phagocytic cells are alerted to the presence of a target.[19] Numerous clinical problems, such as cancer, autoimmune disorders, ischemia damage, and neurodegenerative diseases, are influenced by inappropriate apoptosis.[20] In apoptotic cells, dual AO/EB fluorescence labelling can identify fundamental morphological alterations. It also permits the differentiation of necrotic cells, early and late apoptotic cells, and normal cells. A qualitative and quantitative approach to identify apoptosis is therefore AO/EB staining.AO emits a green fluorescence when attached to DNA. capable of navigating intact membranes of normal and early apoptotic cells. The only cells that EB could penetrate were those with ruptured membranes, such as late apoptotic and dead cells. Additionally, EB only produced orange-red fluorescence when linked to concentrated DNA fragments or apoptotic bodies. Dual AO/EB staining also has the ability to identify minor DNA damage.[21] In our study we observed that the orange-red fluorescence increases as the concentration of sinensetin increases. This study shows that sinensetin exhibits apoptosis. Unbalanced, abrupt elevations in intracellular ROS can cause aging and tumor cell death. Using fluorescent dyes, such as acetylated versions of 2′,7′-dichlorofluorescein, is one of the most well-liked techniques for studying intracellular ROS production (DCFH-DA). As a result, the amount of peroxide produced by the cells and the fluorescence's intensity are directly correlated.[22] Using the HeLa cell line, the current experiment assessed sinensetin potential to produce ROS. The IC_{50} concentration of sinensetin was observed to increase ROS generation in the treated cells as compared to the control, as seen by increased green fluorescence.

DAPI is a DNA-sensitive and specific stain. By measuring the fluorescence of DAPI-stained nuclei using flow cytometry, it is possible to determine the DNA content and ploidy. With this technique, it is possible to demonstrate the differences between normal and malignant cells in terms of DNA content and DNA ploidy.[23] To distinguish between apoptosis and necrosis, it is important to analyze it. One of the most noticeable aspects of the apoptotic process of cell death is nuclear fragmentation. The fluorescent DNA-binding chemical DAPI was used to investigate apoptosis-related cell death and morphological alterations in cells.[24] Two factors, DNA fragmentation and unequal cell size, which results in the cells shrinking and becoming smaller, are indicators that cancer cells have begun to undergo apoptosis.[25] DAPI was negative in the control group's viable cells, which had intact DNA and seemed hardly active in the fluorescence microscope picture. DAPI-positive cells' intensities and numbers were also elevated when cells were treated with sinensetin. This suggests that the majority of the cells experienced cell death predominantly by apoptosis as a result of sinensetin treatment. The primary agents of apoptosis are the caspase family of proteases, which are unique to aspartic acids. Caspases inhibit the proteolysis of several substrate proteins in order to kill

cells.[26] Increased caspase-3 activity is commonly recognized as a hallmark of apoptosis and a strong sign of the efficacy of cancer treatment. Caspase-9, a cysteine-aspartic protease widely known for its role as an activator of intrinsic apoptosis, regulates both normal and pathological tissue deterioration.[27] Caspase-8 is a major player in death receptor-induced programmed cell death, and its activation is crucial for the efficacy of this pathway. It is a classical cysteine protease necessary for the beginning and completion of apoptosis.[28,29] To ascertain if the apoptosis caused by the sinensetin on HeLa cells includes the activation/repression of apoptotic markers, the parameters were evaluated using the ELISA technique. The levels of caspase-3, -8, and -9 were significantly raised in the cervical cancer cells following administration of sinensetin at IC_{50} values, suggesting its anti-carcinogenic potential.

CONCLUSION

According to our research, the antioxidant and anti-cancer characteristics of sinensetin caused apoptosis in HeLa cells. Cell viability was reduced with increased concentration of sinensetin. With increased concentration sinensetin the level of LDH, apoptosis, and ROS increased. We may thus draw the conclusion that sinensetin can induce apoptosis in HeLa cells and may be proved to be a potent anti-cancer drug.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

PMFs: Polymethoxyflavones; **LDH:** Lactate Dehydrogenase; **AO/EtBr:** Acridine Orange and Ethidium Bromide; **DAPI:** 4',6-diamidino-2-phenylindole; **DCFH-DA:** Dichloro-dihydro-fluorescein diacetate; **HDI:** Human Development Index.

SUMMARY

Citrus-specific Polymethoxyflavones (PMFs), which have biological effects such as regulating lipoprotein metabolism and having anti-inflammatory and antioxidant properties, are of special interest. One of the Polymethoxyflavones is sinensetin (PMFs). It has been revealed via prior study that sinensetin causes Ca2+-mediated apoptosis and antiproliferative action in breast cancer cells. Sinensetin also prevents gastrointestinal cancer cells from proliferating and stimulates apoptosis in these cells. We estimated the cell viability of HeLa cells by XTT and LDH assay; AO/EtBr, DPAI, ROS, and estimation of caspases for apoptosis. LDH is a cytoplasmic enzyme that is contained by healthy cells with intact plasma membranes but is released by necrotic cells with disrupted membranes. It is well known that when cells are exposed to high concentrations of anticancer drugs. Our results showed that increase in concentration of sinensetin also elevated the LDH levels. This proves that sinensetin has the potential of anticancer agent for cervical cancer. Apoptosis is a controlled and organized cellular process that takes place in both healthy and unhealthy environments. In apoptotic cells, dual AO/EB fluorescence labelling can identify fundamental morphological alterations. It also permits the differentiation of necrotic cells, early and late apoptotic cells, and normal cells. A qualitative and quantitative approach to identify apoptosis is therefore AO/EB staining. In our study we observed that the orange-red fluorescence increases as the concentration of sinensetin increases. This study shows that sinensetin exhibits apoptosis. The IC_{50} concentration of sinensetin was observed to increase ROS generation in the treated cells as compared to the control, as seen by increased green fluorescence. We may thus draw the conclusion that sinensetin can induce apoptosis in HeLa cells and may be proved to be a potent anticancer drug.

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