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Cytotoxic and apoptotic effect of chalcone 5 on mouse colon cancer cells CT-26

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According to World Health Organization data, various forms of cancer cause around 10 million deaths each year. Nearly 2 million new colon and rectal cancer cases were detected in 2020, making it the second greatest cause of cancer death. Aside from the harmful effects of various chemotherapeutic agents, a special difficulty for treating and eradicating these diseases is tumor resistance to existing anticancer medications and undesired side effects of chemotherapy against healthy cells and tissue. Chalcones are precursors for the synthesis of flavonoids and other compounds with a heterocyclic structure. The biological activities of this category of chemicals include antibacterial, anti-inflammatory, anticancer, antidiabetic, and antiproliferative properties. In the current study, we therefore examined the antitumor and apoptotic effects of chalcone 5 on mouse colon carcinoma cell line CT-26. Chalcone 5 caused the inner mitochondrial apoptotic pathway to be triggered in CT-26 cells by inducing apoptosis, disrupting the potential of the mitochondrial membrane and causing the release of cytochrome *c* into the cytosol. Based on results obtained in this study, chalcone 5 may be recommended as a potential promising anti-cancer candidate for future *in vivo* research studies utilizing experimental animals.

Keywords: Apoptosis, Cell death, Cell line, Chalcones, Colon cancer, Mouse cell line

According to World Health Organization data, various forms of cancer cause around 10 million deaths each year. Nearly 2 million new colon and rectal cancer cases were detected in 2020, making it the second greatest cause of cancer death[1]. Colorectal cancer treatment options currently include surgery, radiation, and chemotherapy. Aside from the harmful effects of various chemotherapeutic agents, a special difficulty for treating and eradicating these diseases is tumor resistance to existing anticancer medications and undesired side effects of chemotherapy against healthy cells and tissue. As a result, the main focus of current research is on the synthesis of novel antitumor drugs. Since many natural substances are known to cause apoptosis in tumor cells, nature might undoubtedly inspire the creation of novel therapeutic medicines. The goal is to develop chemical agents that would demonstrate a strong cytotoxic effect on tumor cells and negligible effects on healthy cells. The prospect of overcoming tumor resistance may be established by integrating additional compounds, substituents, or functional groups into the structure of the known antitumor agent.

Chalcones are precursors for the synthesis of flavonoids and other compounds with a heterocyclic structure. The biological activities of this category of chemicals include antibacterial, anti-inflammatory, anticancer, antidiabetic, and antiproliferative properties[2-4]. Chalcones are composed structurally of two aromatic rings (A and B), joined by a three-carbon unsaturated aliphatic chain. The electrophilic characteristics of chalcones and a variety of pharmacological effects are both caused by the presence of a ketone functional group and conjugated double bonds[5]. Furthermore, the flexible structure of chalcones allows them to interact with a wide range of receptors and enzymes. Chalcones are particularly appealing to researchers because of their relatively simple structure and synthesis, allowing for the incorporation of various substituents into the aromatic rings. The distinctive biological activities of natural and synthetic chalcones are determined by the nature, number, and position of the substituents. Chalcones

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are potent inducers of apoptosis *via* both internal (mitochondrial) and external apoptotic pathways. These effects are primarily achieved by altering the expression of various antiapoptotic and proapoptotic Bcl-2 protein family members. The increase of the expression and the activation of proapoptotic Bcl-2 family proteins like Bax and Bad, together with a decrease in the expression of antiapoptotic proteins like Bcl-2 and Bcl-xL, increases the permeability of the outer mitochondrial membrane. Consequently, the mitochondrial transmembrane potential is lost, mitochondrial content is released including cytochrome *c*, and caspases are activated leading altogether to the execution of apoptosis[6]. Previous research in colon cancer cells *in vitro* has demonstrated that specific chalcones activate pro-caspase 8, indicating that chalcones can potentially initiate the extrinsic apoptosis pathway[7].

We previously demonstrated that specific newly synthesized chalcones exhibited significantly strong antitumor and apoptotic effects in human cancer cell lines. In the current study, we therefore examined the antitumor and apoptotic effects of newly synthesized chalcone derivative (E) -1- (3-methoxy-4) -propoxyphenyl) -5-methylhex-1-en-3-one (chalcone 5) on mouse colon carcinoma cell line CT-26, which is well known to be suitable for both *in vitro* and *in vivo* research and will form tumors in animals following implantation of the cells. Therefore, evaluating the possible anticancer effect of chalcone 5 on CT-26 mouse colon cancer cells represents a development of the current research and a preliminary step toward future research using experimental animals.

Material and Methods

Cell culture, reagents, and compounds

Mouse colon cancer cell line (CT-26) was a gift from the Department of Immunology and microbiology, University of Kragujevac, Faculty of medical sciences; synthesized vanillin based chalcone 5 analogue was a gift from the Faculty of Chemistry; University of Kragujevac. The CT-26 cells were maintained in complete growth RPMI-1640 medium (ATCC 30-2001) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, F7524) in a humidified atmosphere of 5% CO_2.Chalcone 5 was dissolved in DMSO (Sigma Chemical, ST. Lois, Mo) (total DMSO concentration of 0.5%). Annexin V FITC/PI was purchased from BD Pharmingen (Annexin V-FITC Apoptosis; Detection Kit I; BD Pharmingen, US), cytochrome *c* from Promega (G7421, Promega, USA,) and JC-10 Mitochondrial Membrane Potential Assay Kit from Abcam (ab112134).

MTT cell viability assay

To estimate the cytotoxicity of chalcone 5 on both experimental (chalcone 5 treated) and control groups of CT-26 cells – (mouse colon cancer cell line), MTT assay[8-10] was performed during 48 and 72 h period. Briefly, reduction of the MTT reagent by mitochondrial enzymes leads to the production of formazan, which can then be dissolved in DMSO to produce a characteristic absorbance at 595nm. This absorbance reflects the relative metabolic activity, which in turn reflects the percentage of viable cells in each sample. Dilution series 5 (3, 10, 30, 100 and 300 μM) of chalcone 5 were prepared and used for MTT assay. CT-26 cancer cell lines and were seeded at a density of 2×10^4 cells/well and treated with a range of concentrations in triplicate in 96-well cell culture plates for 48 and 72 h, following which cell viability was assessed by MTT assay. In brief, the cells were plated in 96-well plates and allowed to attach overnight. The medium was replaced with different dilutions of chalcone 5, and cells were incubated at 37°C for 48 and 72 h. The medium was then replaced with fresh medium containing 1 mg/mL MTT. Following incubation at 37°C for 2-4 h, the wells were aspirated, the dye was solubilized in DMSO and optical density was measured at 595 nm using the microplate reader (Zenyth 3100, Anthos Labtec Instruments). The percentage of cytotoxic cells was calculated using the formula:

Viability (%) = (ABS experimental) x100/(ABS control group); Cytotoxicity % = 100- viability.

The IC_{50} values for chalcone 5 was calculated using IC_{50} calculator (https://www.aatbio.com/tools/ic50-calculator).

Assessment of CT-26 cells morphology

The morphology of CT-26 cells was studied by phase-contrast microscopy following 48 and 72 h of various concentrations of chalone 5 treatment. Changes in the morphology of the treated CT-26 cells were clearly visible compared to the control cells. Untreated, control CT-26 cells were normally distributed, adherent and fibroblast like on the cultured field with maintained normal morphology. The treatment of CT-26 cells with chalcone 5 induced dose and time dependent disruption in cell

morphology which was presented with loss of cell adherence and loss of cell shape.

Apoptotic Annexin V-FITC assay

Apoptosis was assessed using the Annexin V-FITC/PI apoptosis kit in accordance with the manufacturer's protocol (Annexin V-FITC Apoptosis; Detection Kit I; BD Pharmingen, US). In brief, after the 48 h treatment with IC_{50} value of chalcone 5, CT-26 cells were trypsinized, washed with PBS, and resuspended in 1X binding buffer, and incubated with 5 µL Annexin V-FITC and 5 µL PI for 10 min in the dark, and then analyzed by Flow cytometer. The results were analyzed by The FlowJo V10 software.

Immunofluorescence analysis of cytochrome *c* distribution and expression

CT-26 cells were grown on glass coverslips and treated with IC_{50} value of chalcone 5 during 48 h period. Following the treatment, cells were washed with PBS, fixed with 4% formaldehyde for 20 min, followed by permeabilization with 0.2% Tween-20 for 30 min. After washing in PBS, cells were first blocked with 0.1% Tween-20, 10% FBS buffer for 30 min and then incubated with monoclonal anti-mouse cytochrome *c* antibody (G7421, Promega, USA, 1:100) for 1 h at RT. Finally, cells were cells washed with 1×PBS, then incubated with goat anti-mouse FITC (1:200) diluted in blocking buffer for 30 min, then washed with PBS and analyzed by (Olympus IX50). Images were captured and then analyzed by ImageJ software on an Olympus IX50 inverted fluorescence microscope.

Measurement of mitochondrial membrane potential

In normal cells, JC-10 is localized in the mitochondria where it forms red fluorescent aggregates. However, in apoptotic and necrotic cells, JC-10 exists in monomeric form and stains cells green. The dye JC-10 (Enzo Life Sciences, Farmingdale, NY, USA) was used to evaluate mitochondrial membrane potential. CT-26 cells were cultured in 24-well cell culture plate a density around 3×10^4 cells per well overnight in incubator (5% CO_2, 37°C). Cells were treated with IC_{50} value of chalcone 5 for 48 h. After the treatment, cell culture medium was removed, washed once with warm 1xPBS, and then incubated with 2.5 mM JC-10 dye in warm PBS at 5% CO_2, 37°C for 20 min. Cells were rinsed with warm 1xPBS and then observed on an Olympus IX50 inverted fluorescence microscope. Images were captured and then analyzed by ImageJ software. The ratio of fluorescence emissions at 525 and 590 nm was used for quantification analysis.

Results

Chalcone 5 induce cytotoxicity in mouse colon cancer cells CT-26

The potential cytotoxicity of (E) -1- (3-methoxy-4) -propoxyphenyl) -5-methylhex-1-en-3-one (chalcone 5) was investigated by MTT assay. CT-26 and control cells were plated and treated with 3-300 µM of chalcone 5 for 48 or 72 h. The results obtained by MTT assay (Fig. 1) indicated that chalcone 5 clearly displayed both time and dose dependent enhanced cytotoxicity in CT-26 cells compared to control cells.In comparison to control cells, the percentage of cytotoxic CT-26 cells increased at the lowest chalcone dose (3 µM) after 48 and 72 h (by 2.9 and 3.08-fold, respectively). All administered chalcone doses showed an exponential trend in increasing percentage of cytotoxic CT-26 cells that persisted over the course of 48 h (10 µM- 19.09%; 30 µM- 44.80%; 100 µM- 73.41% and 300 µM- 76.79%) and 72 h (10 µM- 26.1%; 30 µM- 64.72%; 100 µM- 86.53% and 300 µM- 86.68%).

Calculated IC_{50} values for chalcone 5 for 48 and 72 h were 31.73 M and 25.19 M, respectively. However, the highest applied dose of chalone 5 (300 µM), in contrast to the dose of 100 µM, lost the exponential trend in the increase of cytotoxicity in cells. Therefore, we could assume from this observation that chalcone 5's cytotoxicity peaked at a dose of 100 µM.

Chalcone 5 induce distinct morphological changes in CT-26

After demonstrating that chalcone 5 inhibited growth of CT-26 cells, we then used a phase-contrast microscope to asses if these lethal concentrations (3 -

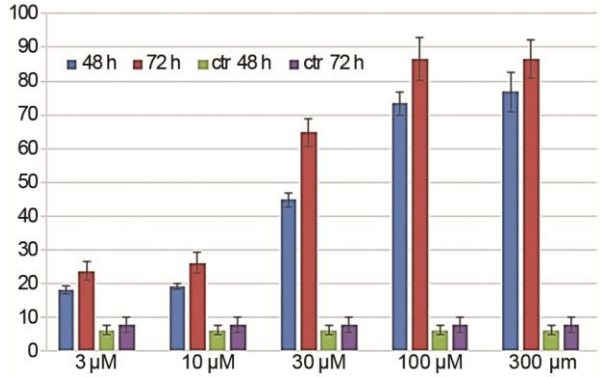

Fig. 1 — Selective cytotoxicity of different doses of chalcone 5 in CT-26 cells following 48 and 72 h of treatment

300 µM) of chalcone 5 caused morphological alterations in CT-26 following 48 and 72 h of chalcone treatment. Changes in the morphology of CT-26 cells treated with chalcone 5 were clearly detectable compared to the control untreated cells (Fig. 2A & B). Untreated control CT-26 cells were evenly distributed, adherent and fibroblast like on the cultivated field, with retained normal morphological shape. Morphological alterations in chalcone 5 treated CT-26 cells displayed as lower cell density (lower cell number), loss of cell-to-cell communication, loss of fibroblast-like cell shape, and ultimately total loss of cell integrity and normal morphology. Chalcone 5 application doses (in a dose-dependent manner) favourably correlated with all observed morphological changes in CT-26 cells.

Chalcone 5 induce apoptosis in CT-26 cells

Next, we applied Annexin V-FITC and PI staining assay to examine whether the inhibitory effect of chalcone 5 on CT-26 cell ability was related to the induction of apoptosis. The assay differentiates between live cells, dead cells, and cells undergoing apoptosis or necrosis through concurrent staining with propidium iodide (a dead-cell-permeable dye) and Annexin-V/PI (an apoptotic-cell permeable dye) detected by Flow cytometer. Untreated control CT-26 cells and CT-26 cells treated with IC_{50} value (32 µM) of chalcone 5 for 48 h were assessed for apoptosis. As shown in Figure 3A & B, the apoptotic CT-26 cells were detected after treatment with IC_{50} value (32 µM) of chalcone 5, whereas necrotic population was almost negligible. The early and late apoptotic treated cell populations were 42.8% and 12.3%, respectively, compared to early and late apoptotic untreated control cells of 16.58% and 1.61%, respectively. These findings suggested that chalcone 5 caused a 3-fold increase in apoptotic cell death in mice colon cancer CT-26 cells when compared to control cells.

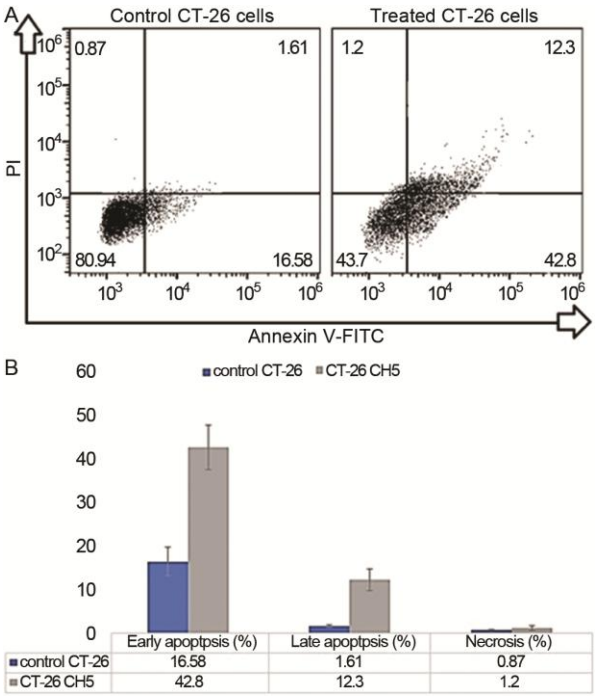

Fig. 3 — (A) Apoptotic cell death in CT-26 cells determined by Annexin V-FITC/PI double staining and flow cytometry. CT-26 cells were treated with IC_{50} value (32 µM) of chalcone 5 for 48 h, evaluated for apoptotic cell death and compared to untreated control CT-26 cells. Lower left square – healthy cells (%), Upper left square- necrosis (%); bottom right square- early apoptosis (%); upper right square- late apoptosis; and (B) Summary of the apoptosis data presented in bar chart. Data are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments

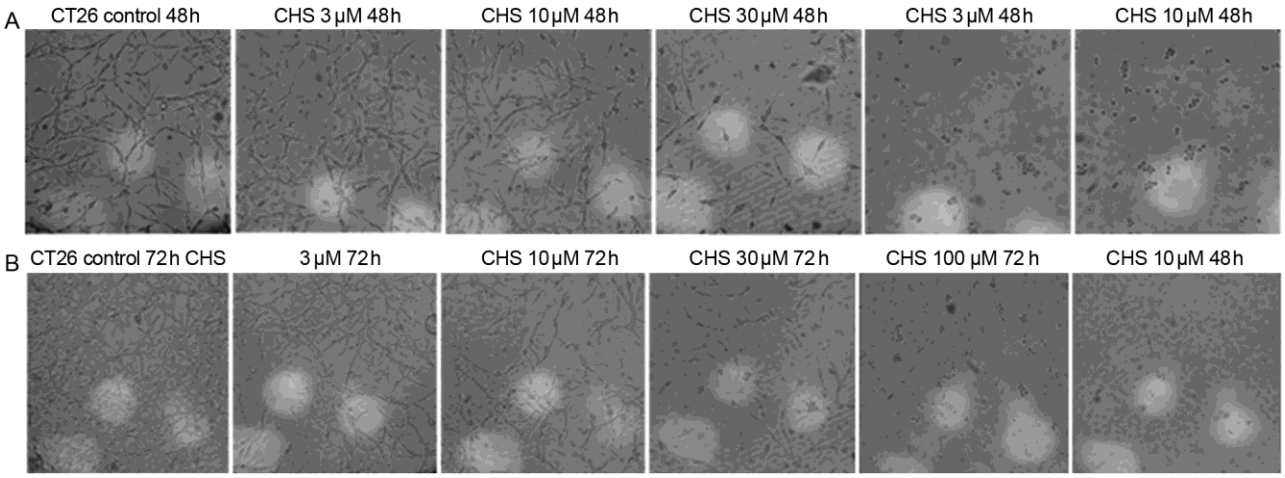

Fig. 2 — Morphological changes of the CT-26 cells following treatment with different doses of chalcone 5 after (A) 48; and (B) 72 h

Chalcone 5 induces cytochrome *c* release from mitochondria in CT-26 cells

Upon induction of mitochondrial apoptosis, cytochrome *c* is released from the mitochondria into the cytoplasm. To examine the possibility of the activation of mitochondrial apoptotic pathway, the cellular localization of cytochrome *c* protein was examined by immunofluorescence microscopy in CT-26 cells treated with IC_{50} of chalcone 5 for 48 h and compared to control untreated cells. Cytochrome *c* showed mainly punctate pattern in untreated cells, which is consistent with its mitochondrial location (70%). Following chalcone 5 treatment, there was release of cytochrome *c* from the mitochondria to the cytosol (60%), manifested by a diffuse staining across CT-26 cells and a less punctate pattern (Fig. 4).

Chalcone 5 induced changes in mitochondrial membrane potential (ΔΨM) in CT26 cells

Once we had determined translocation of cytochrome *c* into cytoplasm of treated CT-26 cells, our next objective was to investigate the changes in the mitochondrial membrane potential produced by chalcone 5 using the JC-10 Mitochondrial Membrane Potential Assay Kit. A membrane-permeable fluorescent dye called JC-10 is employed to detect ΔΨM. JC-10 gathers in the mitochondrial matrix of intact cells, where it creates red fluorescent aggregates. However, JC-10 diffuses from mitochondria in damaged apoptotic cells, transforms into a monomeric form, and stains cells to produce green fluorescence. Treatment of CT-26 cells with IC_{50} of chalcone 5 for 48 h induced increase in JC-10 monomeric form and increased the ratio of JC-10 monomer/ JC-10 aggregate more than 1.5-fold the levels observed in control untreated cells, indicating that chalcone 5 caused changes in ΔΨM and mitochondrial damage in CT-26 treated cells (Fig. 5).

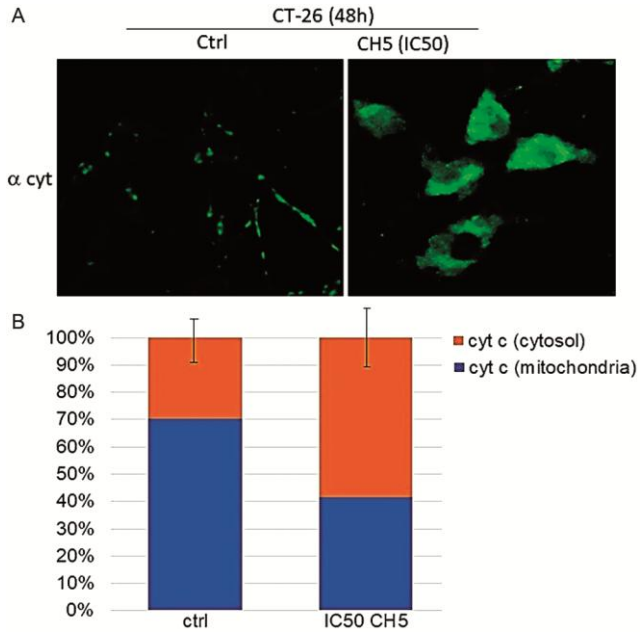

Fig. 4 — (A) Cellular localization of cytochrome *c* in untreated and chalcone 5 treated (IC_{50}) CT-26 cells; and (B) Bar graph bar showing the percentage mitochondrial and cytosolic localization of cytochrome *c* in treated and untreated CT-26 cells

Discussion

A quest for the ideal anticancer therapeutical agent represents a highlight of research for the past two decades. The ideal anticancer drug should be cytotoxic to cancer cells while also having positive impacts on healthy cells and being free of unfavorable side effects[11]. These benefits are demonstrated by their antibacterial, antifungal, anti-inflammatory, antimalarial, antiprotozoal, and anticancer

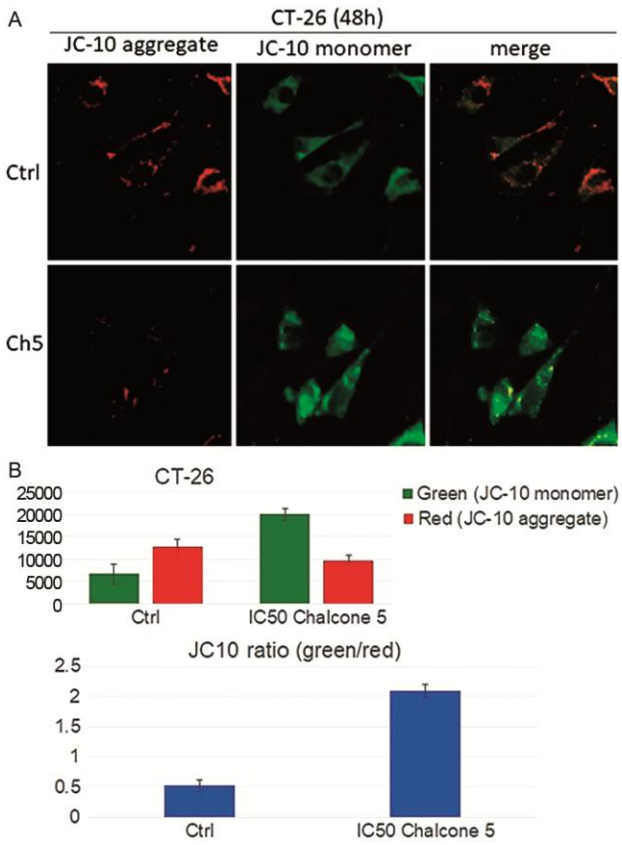

Fig. 5 — Effects of chalcone 5 on modifications in the mitochondrial membrane potential (ΔΨM) in CT-26 cells, as shown by the mitochondrial fluorescence probe JC-10. (A) Immunofluorescence observations of JC-10 in control and CT-26 cells treated with IC_{50} of chalcone 5; and (B) Quantitative results of monomer/aggregate ratio were assumed to be proportional to ΔΨM intensity

capabilities[8-12]. Natural products, their predecessors, analogues, or unique synthetic agents could all be potential antitumor agents that satisfy this golden standard. Chalcones and their derivatives are one of these substances with a high rate of activity in anticancer research. As direct precursors in the biosynthesis of flavonoids, chalcones are direct precursors in the biosynthesis of flavonoids who have α, β-unsaturated carbonyl system which offers them numerous biological properties. Chalcones can be altered in a variety of chemical processes and are also utilized to create heterocyclic compounds and chalcone derivates because of their chemical structure[13-14]. The sole presence of α, β-unsaturated carbonyl system of chalcone gives them biological activity. Chalcones demonstrate a wide range of biological effects due to the inclusion of different functional groups (hydroxyl, methyl, aryls, carboxyl, amino acid, *etc.*), which allow chalcones to bind to molecular targets[15]. Therefore, chalcones are useful templates for the development of novel anticancer agents.

As indicated by previous research converting chalcones' alpha to methoxy groups had a significant impact on the treatment of leukemia[16]; heterocyclic substituted chalcones were introduced for the treatment of breast cancer and osteoporosis[17]; and beta hydroxy chalcone with fluoro substitution had a significant impact on the treatment of HIV[18-19]. According published results in review article[2], the chalcone family has also demonstrated a high potential *in vitro* and *in vivo* activity against cancers, implicating several mechanisms including cell cycle disruption, autophagy regulation, apoptosis induction, and immunomodulatory and inflammatory mediators. Additionally, in our previous research, we successfully demonstrated that chalcone analogs exhibited anticancer effects against a variety of human cancer cell lines, including HeLa, HCT-116, and MDA-MB-231[12-13]. Based on our findings, chalcone analogs could be employed as novel anticancer agents since they exhibit greater cytotoxicity in tumor cells with a lower applied dose than conventional cisplatin.

Moreover, researchers reported that chalcones induced cell death in human colon cancer cells HCT-116, CaCO2 as well as in mouse colon cancer cell line CT-26.WT[1-20]. In a related study, scientists determined that retinoic acid chalcone promoted apoptosis in CT-26.WT and HCT-15 cancer cell lines[21]. Similar results observed significant cytotoxic effects of about 20 chalcones and their derivatives on several cancer cell lines, with average IC_{50} values < 50 μM[16]. The natural polyphenolic flavonoid quercetin, which is widely present in fruits and vegetables such as apples, red grapes, onions, *etc.*, strongly triggered apoptosis and had cytotoxic effects against 9 different cancer cell lines, including the mouse colon CT-26 cell line[22]. Therefore, based on these past investigations, our purpose in this study was to determine the cytotoxic and apoptotic effects of chalone 5 in mouse colon cancer CT-26 cells as a preliminary step toward future research utilizing experimental animals.

Cell morphology is frequently altered by cytotoxic agents, leading to aberrant cell morphology, an increase in cell debris, and a decrease in cell number. Contrary to healthy cells, cancer cells exhibit distinctive morphological characteristics such as atypical cell shape, a small cytoplasmic volume, uncontrolled growth, numerous, enlarged nucleoli, aggregated chromatin, *etc.* Anti-cancer agents can alter the morphology of cancer cells, which can cause the cells to lose their shape, and adherence, and eventually die.

Chalcones derived from natural precursors, chalcone derivatives substituted with nitro, trifluomethyl and cyano chalcone and newly synthesized pyrazolinone all induced morphological changes and had a cytotoxic effect on MCF-7, A549, MDA-231, Caco, and PCL cells. Authors characterized these changes as apoptotic alterations. The authors reported that their ferrocenyl flavonoid caused endothelial cells (EAhy 926) to undergo morphological modifications that are associated with antivascular changes[8]. In the current investigation, CT-26 cells treated with chalcone 5 displayed apparent morphological features indicative of apoptosis, including cellular shrinkage, cell rounding, cytoplasmic condensation, decreased cell number, and cell detachment. These indicated changes in CT-26 cells following chalcone 5 treatment correspond to apoptotic changes in the cell.

There are several types of cell death. Finding out what type of cell death is induced by the investigated drug is one of the key objectives of drug research. Chalcones, their derivatives, analogs, or newly synthesized compounds have been shown to induce cancer cell death *via* several types of mechanisms[6-10]. The majority of research studies indicated apoptosis

induction as the main cause of cell death in various human cancer cells[11-14] and mouse CT-26 cancer cell lines[1,4,15,16].

In this study, in order to comprehend how chalcone 5 exerted cytotoxic effects in CT-26 cell line, we investigated the type of cell death using Annexin-V-FITC/7AAD staining assay. Chalcone 5 caused the CT-26 cell line to undergo apoptosis, which is consistent with the findings of other researchers. Early apoptosis was found in 42.8% of the treated CT-26 cells, which is a 2.5-fold increase in apoptotic cells compared to control cells (16.58%). When compared to control cells, the proportion of treated cells (12.3%) that were in late apoptosis was seven times higher.

Apoptotic regulatory proteins control the process of apoptosis. Cytochrome *c*, which is normally located between the inner and outer mitochondrial membranes in healthy cells, is one of these proteins. Cytochrome *c* functions primarily as an electron carrier in the mitochondrial respiratory chain, both physiologically and pathologically. Cytochrome *c* translocases from its normal mitochondria localization to the cytosol when a cell undergoes apoptosis. Once in the cytoplasm, cytochrome *c* interacts with other proteins to create an apoptosome, which in turn activates caspases and causes irreversible cell death[17]. The release of cytochrome *c* from the intermembrane space of mitochondria into the cytosol is one of the hallmarks of the mitochondrial (inner) apoptotic pathway[22-23]. According to a number of studies, several natural and synthetic chalcones activated apoptosis and induces the release of cytochrome *c* into the cytosol in a variety of human cancer cell lines, including the A2058, BLM, SK-MEL-1, and MEL-HO melanoma cells, the H292-lung cancer cell line, the HepG2, SK-HEP-1, and Huh-7 human hepatocarcinoma cells[22,24]. In addition, the researchers demonstrated that the chalcone derivate L2h17, barbigerone, and chalcone 9X molecules induced apoptosis and the release of cytochrome *c* in mouse cancer cell lines (CT-26, HepG2, and H460 cells)[25,26]. The results obtained in our study correspond with those previous findings. Specifically, our results demonstrated that chalcone 5 initiated the inner mitochondrial apoptotic pathway and induced delocalization of cytochrome *c* from mitochondria to cytosol in CT-26 cells.

Mitochondrial membrane potential ($\Delta\Psi m$) represents key feature in maintaining the physiological function of the respiratory chain and regulates mitochondrial permeability transition pore (MPTP). Disruption of MPTP leads to the collapse of the $\Delta\Psi m$, translocation of cytochrome *c* into the cytosol and consequently caspase activation[27]. Even if the subsequent apoptotic effects are prevented, mitochondrial membrane potential disruption might even result in cell death. Several authors have described this Bcl-2-regulated, caspase-independent cell death[27,28]. Several studies showed that chalcones might cause the disruption of mitochondrial membrane potential in numerous human cancer cell lines. Regarding licochalcone A's apoptotic effect in MCF-7 and MDA MB-23 cells, results demonstrated evidence of apoptotic induction, which was followed by alterations in MMP that caused the release of cytochrome *c*[29,30]. In other studies, apoptosis was induced by Xantohumol and Butein in a variety of human cancer cell lines (A594, H1563, H1975, H23, and HCC827) together with alterations in mitochondrial membrane potential[31-34]. Here, we also observed similar results. Chalcone 5 caused the inner mitochondrial apoptotic pathway to be triggered in CT-26 cells by inducing apoptosis, disrupting the potential of the mitochondrial membrane and causing the release of cytochrome *c* into the cytosol.

Conclusion

Therefore, based on results obtained in this study, chalcone 5 may be recommended as a potential promising anti-cancer candidate for future *in vivo* research studies utilizing experimental animals.

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Conflict of interest

All authors declare no conflict of interest.

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