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Luteolin Induces Growth Arrest in Colon Cancer Cells Through Involvement of Wnt/β-Catenin/GSK-3β Signaling

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ABSTRACT: Cancer is a multistep process that typically occurrs over an extended period of time, beginning with initiation followed by promotion and progression. Colon cancer is the leading cause of morbidity and mortality worldwide. For a variety of reasons, patients prefer naturally occurring dietary substances over synthetic agents to prevent cancer. Luteolin, a bioflavonoid, possesses antioxidant, anti-inflammatory, and antiproliferative effects. We analyzed the *in vitro* anticancer and apoptosis-inducing property of luteolin using HCT-15 colon adenocarcinoma cells. Cell viability was assessed using trypan blue assay at different concentrations. Luteolin at a concentration of 100μM (IC50) decreased the expressions of non-P-β-catenin, phosphorylated (inactive) glycogen synthase kinase-3β, and cyclin D1 expressions in HCT-15 cells, which were confirmed by Western blot analysis. Luteolin also promoted substantial cell cycle arrest at the G2/M phase in HCT-15 cells, and it induces apoptosis in HCT-15 cells, as revealed by flow cytometric analysis. Furthermore, Western blot analysis showed that luteolin treatment enhanced the expression of Bax and caspase-3, whereas the expression of Bcl-2 was suppressed. Together, the results of this study revealed that luteolin can act as a potent inhibitor of HCT-15 proliferation and can be used as an agent against colon cancer.

KEY WORDS: colon cancer, HCT-15, luteolin, β-catenin, apoptosis

I. INTRODUCTION

The global variation in cancer incidence reflects a relation between population dietary habits and the relative risk of cancer development; thus the role of diet in the control of cancer risk has drawn widespread attention.[1,2] Colon carcinogenesis is considered to be linked with dietary habits like high intake of animal fats and consumption of a low-fiber diet.[3] In contrast, a number of studies have suggested that high consumption of fruits and vegetables decreases the risk of colon cancer.[4,5]

Luteolin (3′,4′,5,7-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits and vegetables. Luteolin in artichoke leaf extract is reported to exhibit antioxidant activity in human leucocytes.[6] Luteolin is also reported to have anti-inflammatory property by inhibiting nitric oxide production.[7] Furthermore, luteolin is reported to inhibit the formation of aberrant crypt foci,[8] reduce the levels of glycoproteins,[9] and exert antiallergic,[10] antiproliferative, and antitumorigenic properties.[8,11]

The Wnt/β-catenin signaling pathway is tightly regulated and has an important role in development, tissue homeostasis, and tissue regeneration. Oncogenic activation of the Wnt signaling pathway by mutations in adenomatous polyposis coli (*APC*) or *β-catenin* gene results in the accumulation and nuclear translocation of β-catenin and β-catenin/T-cell factor (TCF)-4–regulated transcription of TCF target genes such as *cyclin D1* and *c-Myc*, which is mandatory for the initial neoplastic transformation of intestinal epithelium.[12] Thus, Wnt/β-catenin signaling is becoming a promising target for cancer chemoprevention and therapy.[13,14]

In this study we analyzed the effects of luteolin on HCT-15 human colorectal cancer cells. Our results demonstrated that luteolin inhibited HCT-15 cell proliferation, which was associated with sup-

pression of non-phospho (p)-β-catenin expression, thereby decreasing its transcriptional activities and the transcription of its target gene *cyclin D1*. Inhibition of the Wnt/β-catenin pathway is mediated by downregulation of the inactive form of glycogen synthase kinase (GSK)-3β. Furthermore, luteolin-induced apoptosis in HCT-15 cells was mainly associated with the involvement of *Bcl-2*, *Bax*, and *caspase-3*.

II. MATERIALS AND METHODS

A. Cell Line and Culture Conditions

The HCT-15 colon cancer cell line was purchased from the National Center for Cell Sciences, Pune, India, and maintained on Roswell Park Memorial Institute medium (Himedia, Mumbai) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Himedia, Mumbai) in an atmosphere of 95% air and 5% carbon dioxide.

B. Cell Viability Assay

HCT-15 cells were plated in 96-well plates at a density of 5×10^5 cells/well and grown until they reached confluence. Luteolin at concentrations of 5, 10, 20, 40, 60, 80, and 100 μM was added to the plates while dimethyl sulfoxide (solvent) was added for the control regimen and grown at 37°C, 5% carbon dioxide, for 0–24 hours. To determine cell viability, the trypan blue exclusion assay was performed. In brief, about 10 μL of cell suspension in phosphate-buffered saline (PBS) was mixed with trypan blue (0.05%), and the number of stained (dead) and unstained (live) cells were counted using a hemocytometer.

C. Cell Cycle Analysis

Cell cycle analysis was performed using the method described by Rasola and Geuna.[15] HCT-15 cells were incubated with 100 μM of luteolin at 37°C for 6 and 12 hours. Cells were harvested in cold PBS, fixed in 70% ethanol, and stored at 4°C for subsequent cell cycle analysis. Fixed cells were washed with PBS once and resuspended in propidium iodide (PI; 100 μg/mL) staining. Samples were incubated in the dark for 30 minutes before cell cycle analysis. The distribution of cells the different phases of the cell cycle was measured using a Becton Dickinson fluorescence-activated cell sorting analysis system, and quantitation of cell cycle distribution was performed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

D. Flow Cytometric Analysis of Annexin V–Fluorescein Isothiocyanate Binding

HCT-15 cells (1×10^6) were incubated with 100 μM of luteolin for 0, 12, and 24 hours and then harvested. Specific binding of annexin V–fluorescein isothiocyanate (FITC) was performed by incubating the cells for 15 minutes at room temperature in a binding buffer (10 mM HEPES, 140 mM sodium chloride, 2.5 mM calcium chloride [pH 7.4]) containing a saturated concentration of annexin V-FITC and PI. After incubation, the cells were pelleted and analyzed in a FACScan analyzer (Becton Dickinson). Annexin$^+$/PI$^+$ cells were defined as necrotic (or late apoptotic), whereas annexin$^+$/PI$^-$ cells were defined as apoptotic.[16]

E. Preparation of Cell Lysates

HCT-15 cells were harvested by scraping and rinsed twice in ice-cold PBS. The cells then were swollen in ice-cold hypotonic lysis buffer (20 mM HEPES [pH 7.1], 5 mM potassium chloride, 1 mM magnesium chloride, 10 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL pepstatin A, 2 μg/mL chymostatin, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 5 μg/mL antipain) for 10 minutes. Then the cells were lysed by 20 strokes in a homogenizer, and the nuclei were cleared by centrifugation (2000 rpm for 10 minutes). After this step, the supernatant was concentrated and stored at -80°C.

F. Immunoblot Analysis

Immunoblotting was performed as described previously,[11] with little modification. Signals were visualized using the Diamino-benzidine (Sigma) and quantified by densitometry using ImageJ software (National Institutes of Health).

G. Statistical Analysis

Values are expressed as mean ± standard deviation. Comparisons between control and treated cells

were made using SPSS version 10 software. $P < 0.05$ was considered statistically significant.

III. RESULTS

A. Luteolin Treatment Decreases Cell Viability

For the control group, the data indicated that a negligible percentage of HCT-15 cells were stained when they were incubated in medium containing only 10% FBS. In the presence of luteolin (10–200 μM), the cells exhibited increased staining in both a time- and concentration-dependent manner, suggesting that luteolin induces cell death in HCT-15 cells. Further increasing in the concentration of luteolin resulted in a greater decrease in the viable number of HCT-15 cells (Fig. 1A).

B. Luteolin Induces G2/M-Phase Cell Cycle Arrest

Because of luteolin-induced DNA damage (data not shown), we investigated the effect of luteolin on the cell cycle in HCT-15 cells. Flow cytometric analysis was performed after treating the cells with 25, 50, and 75 μM luteolin for 24 hours. Cell cycle analysis revealed a concentration-dependent S-phase arrest after treatment with luteolin. A significantly small proportion of cells was arrested in the G2/M phase (Fig. 1B).

C. Luteolin Decreases the Expression of Non-P-β-Catenin and Cyclin D1

Immunoblot blot analysis of non-p-β-catenin and cyclin D1 are represented in Fig. 2A. Whole-cell lysates of HCT-15 cells treated with 100 μM of luteolin for 12 and 24 hours were used as markers of colon carcinogenesis in the immunoblot analysis of non-p-β-catenin and cyclin D1. Luteolin treatment reduced the expression of non-p-β-catenin and its downstream target *cyclin D1*. We also show the dose-dependent inhibition of non-p-β-catenin in HCT-15 cells (Fig. 2C). At concentrations of 50, 75 and 100 μM luteolin showed a decrease in non-p-β-catenin in both the total and nuclear fraction of lysates; a nuclear location of non-p-β-catenin contributes to the development of cancer. The quantifications of the respective blots are shown in Fig. 2B and D, demonstrating that luteolin kills cancer cells by down-regulating the Wnt/β-catenin pathway.

D. Suppression of Wnt Signaling by Luteolin in a GSK-3β-Dependent Manner

Since GSK-3β is a key enzyme involved in targeting β-catenin for proteosomal degradation, inactivation of this enzyme facilitates nuclear translocation of β-catenin with consequent activation of transcription and cellular proliferation.[17–19]

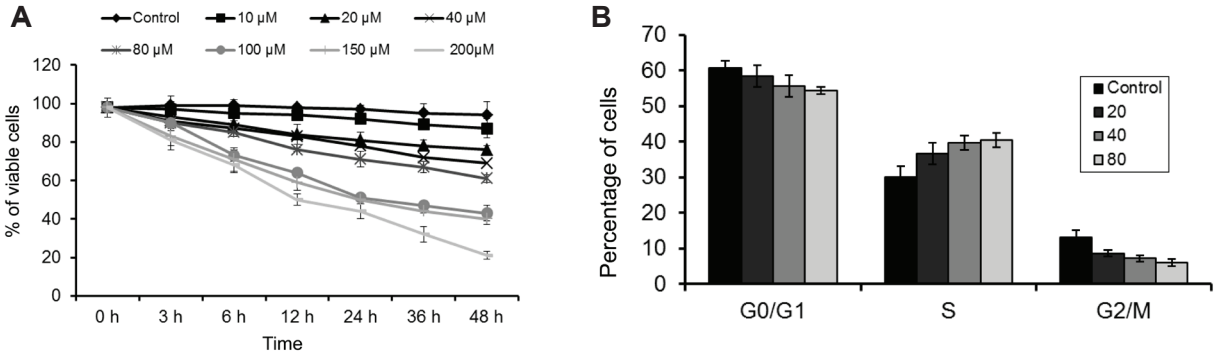

FIG. 1: Luteolin treatment decreases the cell viability and induces G2/M-phase cell cycle arrest. HCT-15 cells were treated with luteolin at different concentration for 48 hours, and cell viability was measured using trypan blue. **A:** At the concentration of more than 100 μM of luteolin, a swift drop in viability occurs with a steady increase in time. Each point represents the mean of triplicate assays of incubations of cells. **B:** HCT-15 cells following treatment with 0, 40, 80, and 100 μM luteolin for 24 hours were fixed and stained with propidium iodide, and cell cycle distribution was then analyzed by flow cytometry. Each value is the mean ± standard deviation of 3 determinations ($P < 0.05$).

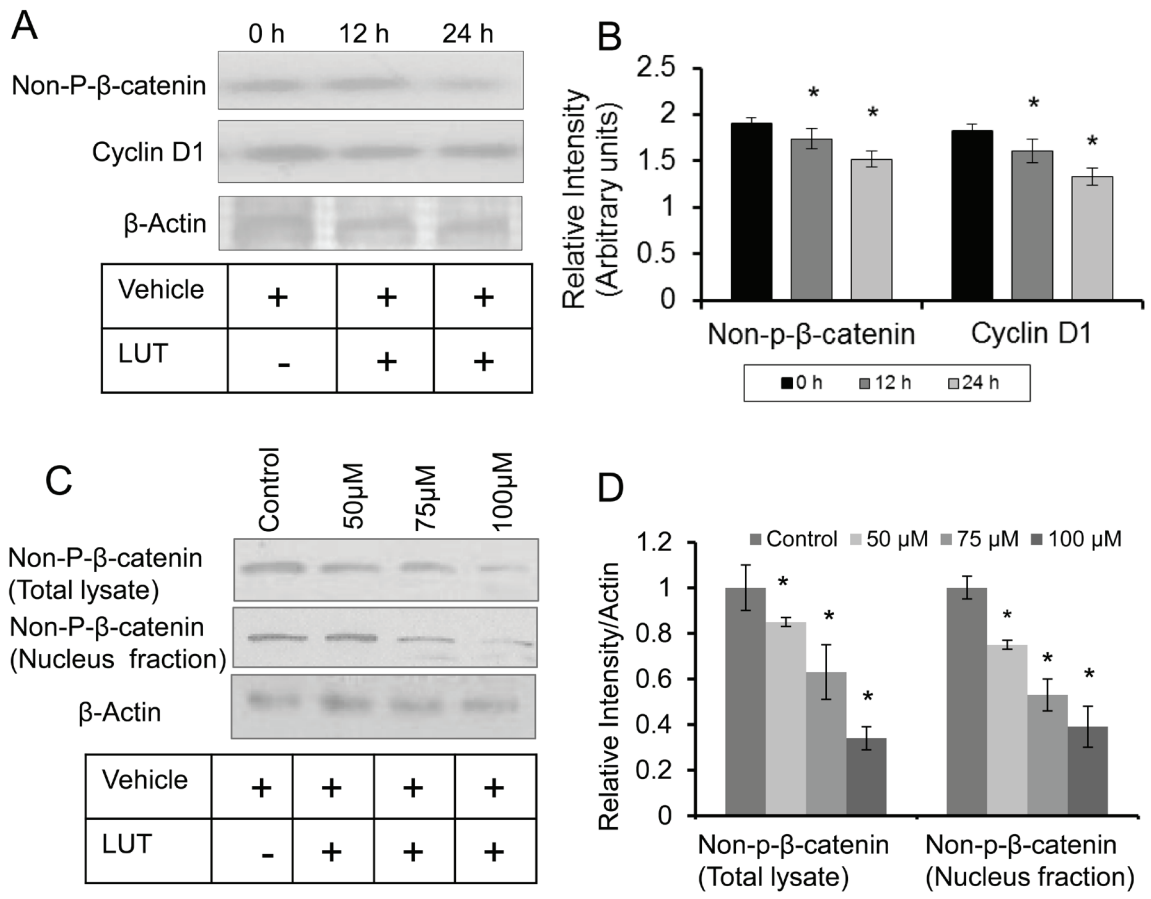

FIG. 2: Luteolin decreases the expressions of non-P-β-catenin and cyclin D1. **A:** Effect of luteolin on the expression of non-P-β-catenin (92 kDa) and cyclin D1 (36 kDa). Total cell lysates of HCT-15 cells treated with or without luteolin for the indicated time were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; they were subsequently immunoblotted with antisera against β-catenin, cyclin D1, and β-actin. **B:** The representative blots in **A** were quantified by ImageJ software. Each value is the mean ± standard deviation of 3 determinations ($P < 0.05$). **C:** The dose-dependent (25, 50, 75, 100 μM) inhibition of non-p-β-catenin showed a decrease in the expression of non-p-β-catenin increase in the concentration of luteolin. Expression of non-p-β-catenin decreased with an increase in the dose of luteolin in the nuclear fraction of HCT-15 cells. **D:** The representative blots in **C** were quantified by ImageJ software. Each value is the mean ± standard deviation of 3 determinations ($P < 0.05$).

The phosphorylation of GSK-3β is mediated by pAkt and is inactivated during colon carcinogenesis. Western blot analysis of p-GSK-3β is shown in Fig. 3A. Luteolin decreases the expression of p-GSK-3β in 12- and 24-hour treatment. Furthermore, inhibition of GSK-3β by lithium chloride attenuated the luteolin-induced reduction in active β-catenin (Fig. 3B). These findings indicate that luteolin suppress the Wnt signaling pathway through a GSK-3β-mediated mechanism that involves phosphorylation of β-catenin, leading to a reduction in the nuclear and cellular levels of active non-p-β-catenin.

E. Luteolin Induces Apoptosis in Colon Cancer Cells

Redistribution of membrane phosphatidylserine from the inner leaflet of the plasma membrane to the outer surface is a common feature of apoptotic cells. HCT-15 cells were treated with 60 μM luteolin for 12 and 24 hours and costained with annexin V-FITC and PI. As shown in Fig. 4A and B, treatment with luteolin resulted in an increase of the cell population that was positive for annexin V staining. A substantial number of the annexin V-positive cells were not stained by PI, indicating

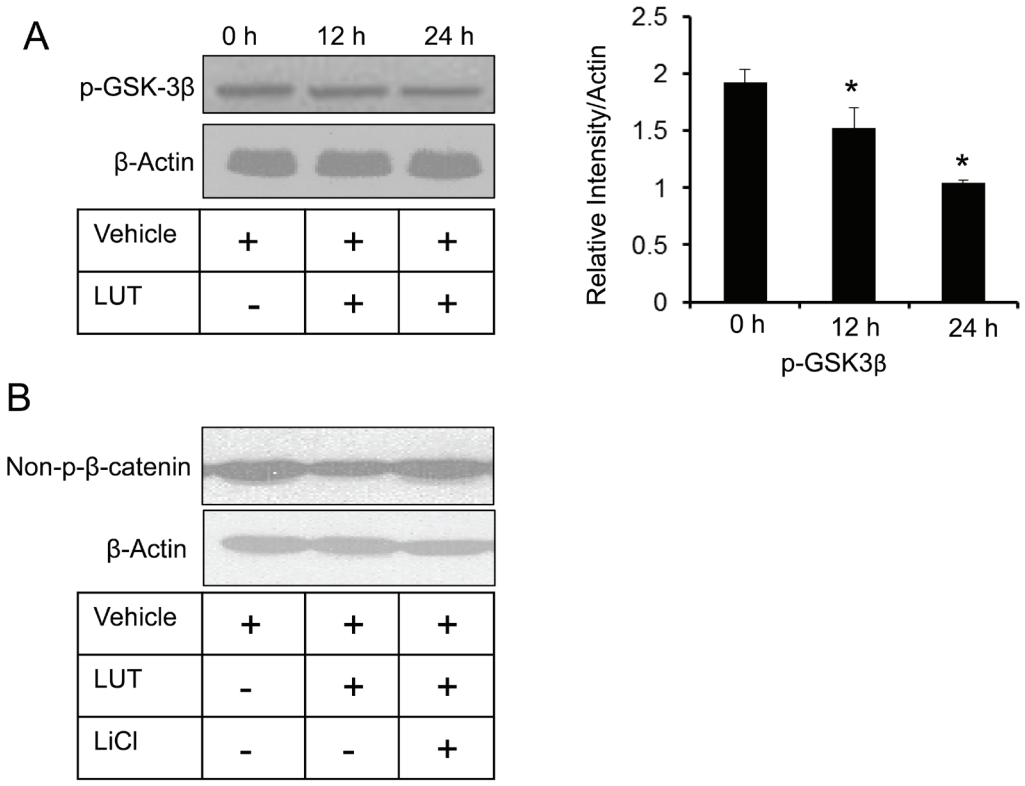

FIG. 3: Suppression of Wnt signaling by luteolin is mediated through glycogen synthase kinase (GSK)-3β. **A:** HCT-15 cells were treated with vehicle or 100 μM of luteolin (LUT) at 12 and 24 hours. After the incubation period the cells were harvested and the protein levels of pGSK-3β and actin were determined by immunoblotting. **B:** HCT-15 cells were treated with vehicle or 100 μM of luteolin in the presence or absence of lithium chloride (LiCl; 20 mM) for 3 hours. Cells were harvested and protein levels of non-p-β-catenin and β-actin were determined by immunoblotting.

that they were viable under this specific experimental condition.

F. Expressions of *Bcl-2*, *Bax*, and *Caspase-3* in HCT-15 Cells Treated with Luteolin

Activation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins.[20] The Bcl-2 family of proteins play vital roles in the regulation of cell death mechanisms.[21] Antiapoptotic Bcl-2 family members (Bcl-2, Bcl-xL) can block these mitochondrial events, whereas proapoptotic Bcl-2 family members (Bax, Bak, Bad) can trigger these changes. It is well known that caspases play a central role in the terminal execution of apoptosis induced by various stimulations.[22] To understand the induction mechanism of apoptosis by luteolin, we examined the expressions of *Bcl-2*, *Bax*, and *caspase-3* by immunoblot analysis. The treatment of HCT-15 cells with luteolin resulted in a marked decrease in Bcl-2 protein levels in a time-dependent manner (Fig. 4C). In contrast, the Bax and caspase-3 protein expression levels were increased compared to the vehicle control. The densitometric quantification of the respective blots is shown in Fig. 4D.

IV. DISCUSSION

Luteolin exhibits a wide spectrum of pharmacological properties, but little is known about its anticancer mechanisms. HCT-15, a colon adenocarcinoma cell line, is a reliable model with which to assess the effect of various drugs to check antiproliferative properties. Wnt/β-catenin signaling is important for both the initiation and progression of cancers in different tissues/organs and is becoming a promising target for chemoprevention and chemotherapy.[12] β-Catenin is a cytoplasmic

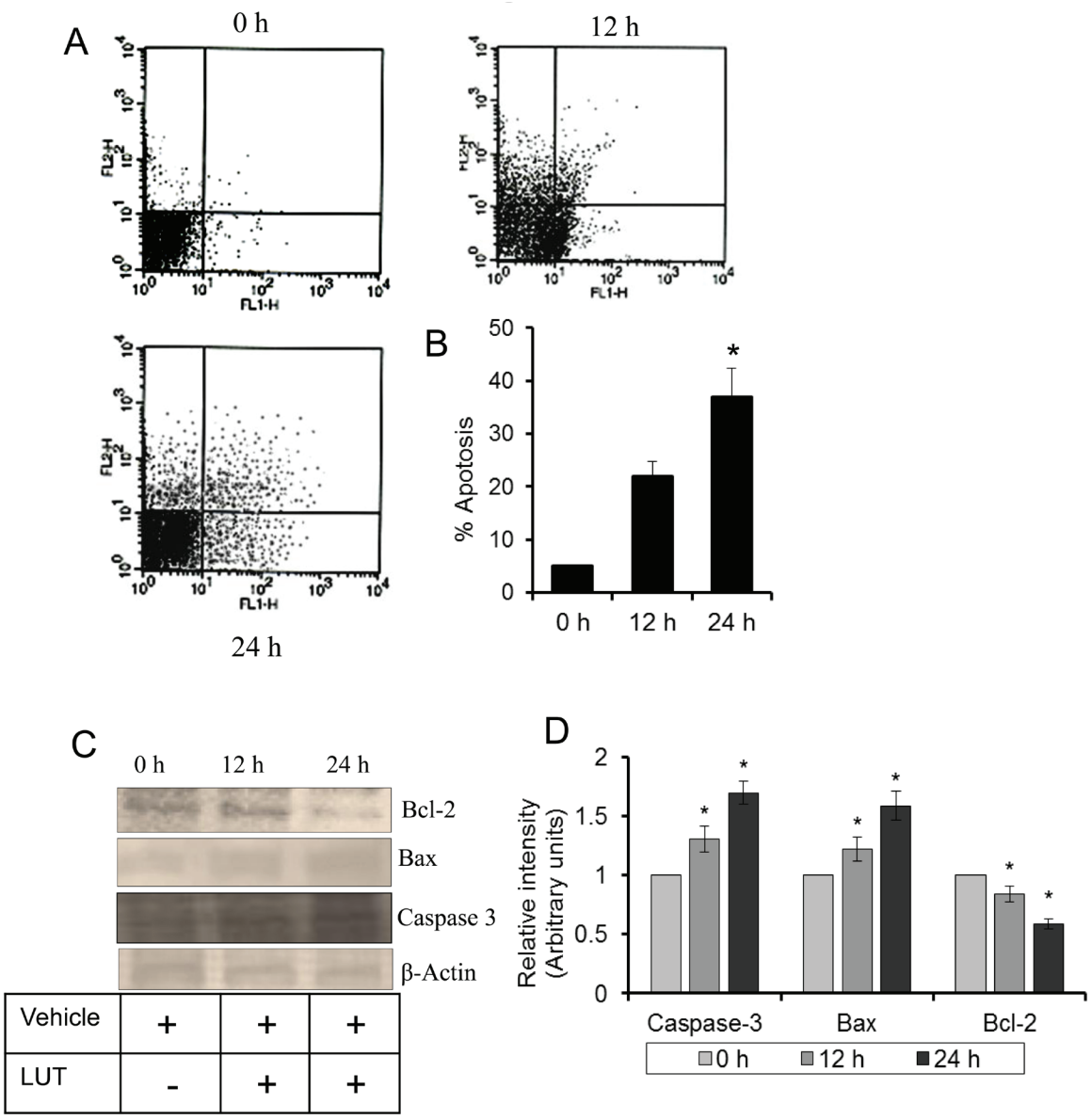

FIG. 4: Luteolin induces apoptosis. **A:** Assessment of apoptosis in HCT-15 cells using flow cytometry and phosphatidyl serine exposure. HCT-15 cells were incubated with luteolin (LUT) for 12 and 24 hours. The cells were stained with annexin V conjugate and propidium iodide (PI) for flow cytometric analysis. Values are represented as the mean of 3 independent experiments. **B:** Values identified in **A** are statistically significant at $P < 0.05$ compared with control. **C:** Total cell lysates of HCT-15 cells treated with or without LUT for the different times were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; they were subsequently immunoblotted with antisera against Bcl-2, Bax, and caspase-3. **D:** The representative blots in **C** were quantified by ImageJ software.

signaling transducer that normally binds to the cytoplasmic domain of transmembrane cadherins and is involved in cell-cell adhesion.[23–26] β-Catenin turnover usually is regulated in cytosol by a large protein complex involving axin, β-TrCP, the tumor suppressor gene product APC, protein phosphatase 2A, casein kinase 1, and GSK-3β. Phosphorylation of β-catenin by GSK-3β prevents the nuclear translocation of β-catenin and promotes its ubiquitination and subsequent degradation by proteasome. Upon stimulation by the canonical Wnt signaling pathway or following mutations in APC,[25]

β-catenin accumulates in the cytosol and translocates to the nucleus, where it may associate to Lef-1/Tcf, thereby activating target gene transcription and biological responses.[25,27] The Wnt signaling pathway regulates cell growth and differentiation during embryonic development and, when aberrantly activated, plays a role in tumor formation.[27,28]

Recent reports state that there is increased expression of β-catenin in colon cancer *in vitro*.[29,30] β-Catenin was overexpressed in HCT-116 colon adenocarcinoma cell lines, and overexpression in the nucleus was decreased on treatment with caffeic acid.[31] Quercetin acts as potent inhibitor of the β-catenin/TCF pathway via suppression of nuclear β-catenin.[32] Epigallocatechin-3-gallate and curcumin inhibit the β-catenin/TCF pathway in HEK-293 and HCT-116 cell lines, respectively.[33–35] Earlier we reported that luteolin modulated Wnt signaling *in vivo* during azoxymethane-induced colon cancer.[11] In this study, luteolin inhibited the expression of β-catenin, as confirmed by immunoblot blot analysis. *Cyclin D1* is a regulator of the cell cycle, which acts with cyclin-dependent kinase (CDK)-4, and its overexpression has been reported in a variety of human cancers, including colon cancer,[36–38] and antisense to *cyclin D1* inhibits the growth and tumorigenicity of human colon cancer cells.[38] Since *cyclin D1* is essential for controlling G1 phase progression and the onset of DNA replication, overexpression of this gene may cause abnormalities in the control of cell growth and the rate of proliferation.[39,40] In this study, treatment with luteolin at the time periods of 12 and 24 hours decreased the expression of *cyclin D1*.

The Bcl-2 family of proteins plays a central role in regulating apoptosis.[41] Bcl-2 acts as a prominent antiapoptotic member of the Bcl-2 family that inhibits the release of proapoptotic molecules and cytochrome c from the mitochondria, thereby inhibiting apoptosis and permitting the persistence of tumor cells. In addition, overexpression of *Bcl-2* has been reported to inhibit transcriptional activation of the *Bax* gene.[42]

Components of the apoptosis signaling cascade, including caspases[43,44] and several other triggers and regulators such as the Fas ligand[45] and Bcl-2 family members, are among the most promising targets for pharmacological modulation of cell death.[46,47] Caspases can be regarded as the central executors of the apoptotic pathway. Among the family of caspases, caspase-3 has been reported to be the most frequently activated caspase protease in apoptotic cells, indicating its crucial role in the cell death process.[48]

In this study, treatment with luteolin decreased the expression of antiapoptotic *Bcl-2* and increased the expression of proapoptotic *Bax* and *caspase-3*. Lee et al.[49,50] reported that luteolin induces apoptosis via suppressing *Bcl-2* expression in $HepG_2$ cells and pancreatic cells. We recently reported that luteolin induces apoptosis by modulating the expression of *Bax*, *Bcl-2*, and *caspase 3* and the status of adenosine triphosphatases in azoxymethane-induced colon cancer in mice.[51,52] In the current study, luteolin treatment to HCT-15 cells results in a significant increase in the proapoptotic Bax protein and a decrease in the level of the antiapoptotic Bcl-2 protein, thus shifting the Bax-to-Bcl-2 ratio in favor of apoptosis. Furthermore, luteolin treatment also demonstrated a significant induction of the executioner protease of apoptosis, caspase-3, in HCT-15 cells.

V. CONCLUSION

The *in vitro* anticancer activity of luteolin in HCT-15 human colon cancer cells was high. The results of our study demonstrate that luteolin has strong antiproliferative effects by inhibiting Wnt/β-catenin signaling, inducing apoptotic cell death, and causing G2/M phase arrest in HCT-15 cancer cells. Since the modes of action of these bioactive compounds are unclear, a greater understanding of their mechanisms of action will help to provide useful information for their possible application in cancer prevention and perhaps therapies for cancer and various other ailments.

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