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ORIGINAL RESEARCH

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Luteoloside inhibiting the prostate cancer cells growth and promoting the tumor cells autophagy through AKT/mTOR pathway

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Abstract

Prostate cancer (PC) is one of the prevalent tumors in men causing higher mortality. Luteoloside has been discovered for suppressive role into the progression of some cancers. However, the Luteoloside's regulatory functions regarding PC progression are not well understood. This study is thus designed to investigate the Luteoloside impact on PC progression. In this work, it was demonstrated that the PC cell proliferation was gradually inhibited with the increasing Luteoloside dose (0, 20, 40, 80 μM). Luteoloside also enhanced the PC cell apoptosis. It promoted the autophagy in PC through increasing microtubule-associated protein light chain (LC) 3II/LC3I levels and decreasing p62 levels. Moreover, the Luteoloside reduced phosphorylated-protein kinase B (p-AKT)/AKT and phosphorylated-mechanistic target of rapamycin (p-mTOR)/mTOR levels, indicating that it retarded the AKT/mTOR pathway. In conclusion, the Luteoloside inhibited PC cells growth and promoted tumor cells autophagy through AKT/mTOR pathway. This discovery suggested the Luteoloside as a potential drug in treating PC.

Keywords

Luteoloside; Autophagy; Prostate cancer; AKT/mTOR pathway

1. Introduction

Prostate cancer (PC) is one of the leading causes of cancer-related deaths and impacts men health in the western world [1]. Previous research indicates that ~10% of newly diagnosed patients have metastatic lesions which aggravates PC progression [2, 3]. Prognoses of late stage and metastatic PC patients is challenging despite the recent global efforts of identifying novel treatment strategies [1, 4]. Therapeutic compounds targeting the cellular pathways are therefore important.

Luteoloside (cynaroside or luteolin 7-glucoside) is a naturally abundant flavonoid of plant kingdom [5]. The Luteoloside content has been utilized as quality control index for many Chinese medicinal materials such as honeysuckle, chrysanthemum, and perilla. Luteoloside shows anti-tumor activity, *e.g.*, it modulates the mitogen-activated protein kinas (MAPK) pathway to suppress the cervical cancer cell proliferation and promotes cell apoptosis [6]. Moreover, it retards the AKT/mTOR pathway to attenuate the cell growth, migration, and invasion in gastric cancer [7]. It enhances G0/G1 arrest and autophagy in the non-small cell lung cancer by affecting the Ros-mediated AKT/mTOR/70 kDa ribosomal protein S6 kinase (p70S6K) pathway [8]. Besides, the Luteoloside reduces cell proliferation and metastasis in hepatocellular carcinoma by inhibiting the nucleotide-binding domain and leucine-rich repeat related (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome [9]. However, the Luteoloside's role and its mechanism in PC progression are still ambiguous.

DU145 and PC3 cells are the metastatic PC cell lines and represent "castrate resistant" (androgen independent) PC. Luteoloside targets PC cells that are resistant to androgen deprivation therapy "ADT".

In this work, the regulatory functions and related Luteoloside pathway were investigated regarding the PC progression. This discovery revealed that Luteoloside inhibited the PC cells growth and promoted the tumor cells autophagy through AKT/mTOR pathway. This work may assist in treating PC progression by Luteoloside.

2. Materials and methods

2.1 Cell lines and cell culture

The PC cell lines (DU145 and PC3) were acquired from American Tissue Culture Collection (ATCC, USA). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, 10099-141, Gibco Laboratories, Grand Island, NY, USA) in an incubator with 5% carbon dioxide (CO_2) at 37 °C.

2.2 CCK-8 assay

The cell viability was assessed by cell counting kit-8 (CCK-8; CK04, Dojindo Laboratories, Kumamoto, Japan). PC cells were placed in 96-well plate (1000 cells/well). At 24 h, 36 h, and 48 h, CCK-8 solution (10 μL) was added to each well for another 4 h. The absorbance was recorded by spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The reference wavelength was set as 620 nm.

2.3 Colony formation assay

DU145 and PC3 cells (1000 cells/well) were placed in 6-well plate for 14 days. They were fixed (4% paraformaldehyde) and stained (0.1% crystal violet). The images were taken with a microscope, and colonies were manually counted.

2.4 Flow cytometry

The cell apoptosis was measured with Annexin V-fluoresceine isothiocyanate (FITC) Apoptosis Detection Kit (C1062, Beyotime, Shanghai, China). PC cells were washed with phosphate buffer solution (PBS), fixed by ethanol (70%), and centrifuged. The single cell suspension was prepared as 1×10^6 cells/mL concentration. PC cells were stained (Annexin V-FITC and Propidium Iodide (PI)) in dark. The cell apoptosis was examined by the flow cytometry (BD FACS Aria III, BD Biosciences, San Jose, CA, USA).

2.5 Western blot

The proteins (isolated from PC cells) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and shifted to polyvinylidene fluoride (PVDF) membranes (Beyotime, Shanghai, China). After blocking with skimmed milk, the primary antibodies were added in the membranes and kept for 12 h at 4 °C, followed by adding secondary antibodies (1:2000; ab7090) and incubated for 2 h. Lastly, the bands were analyzed with chemiluminescence detection kit (89880, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein bands intensity was measured using ImageJ.

The primary antibodies: LC3I (1:2000; ab192890; Abcam, Shanghai, China), LC3II (1:2000; ab192890), p62 (1:10000; ab109012), Beclin1 (1:2000; ab207612), p-AKT (1:1000; ab38449), AKT (1:500; ab8805), p-mTOR (1:1000; ab109268), mTOR (1:1000; ab32028), and β-actin (internal reference) (1 μg/mL; ab8226).

2.6 Statistical analysis

The data were presented as mean $\pm$ standard deviation (SD). Each experiment was performed in triplicate. The statistical analysis was made using GraphPad Prism Software 9 (GraphPad Software, San Diego, CA, USA). Student's *t*-test (for two groups) or one-way analysis of variance (ANOVA) (for multiple groups) were conducted for the comparisons. *Post-hoc* test was performed by Least Significant Difference (LSD) method. $p < 0.05$ was set as statistically significant.

3. Results

3.1 Luteoloside inhibiting the cell proliferation in PC

The Luteoloside structure is shown in Fig. 1A. DU145 and PC3 cells viabilities were gradually decreased with the increase in Luteoloside dose (0, 20, 40, 80 μM) (Fig. 1B). The colonies were minimized with the increase in Luteoloside dose (0, 20, 40, 80 μM) as monitored through the colony formation assay (Fig. 1C). The data demonstrated that Luteoloside inhibited the PC cell proliferation.

3.2 Luteoloside promoting the cell apoptosis in PC

DU145 and PC3 cells apoptosis was promoted by the increased Luteoloside dose (0, 20, 40, 80 μM) (Fig. 2). Luteoloside thus strengthened the cell apoptosis in PC.

3.3 Luteoloside enhancing the autophagy in PC

LC3II/LC3I levels were gradually upregulated with the increase in Luteoloside dose (0, 20, 40, 80 μM) as recorded by western blot (Fig. 3A). The p62 protein expression was reduced, and that of Beclin 1 was enhanced with the increased Luteoloside dose (0, 20, 40, 80 μM) (Fig. 3B). These findings indicated that the Luteoloside enhanced the autophagy in PC.

3.4 Luteoloside retarding the AKT/mTOR pathway

The p-AKT/AKT levels were attenuated by the increase in Luteoloside dose (0, 20, 40, 80 μM) (Fig. 4A). Besides, p-mTOR/mTOR levels were reduced with the increase of Luteoloside dose (0, 20, 40, 80 μM) (Fig. 4B). Furthermore, the LC3II/LC3I levels were enhanced after the Luteoloside treatment (80 μM), but this change was attenuated by the MHY1485 (mTOR activator) treatment (Fig. 4C). The p62 protein expression was reduced after Luteoloside treatment, however this effect was reversed by the MHY1485 treatment (Fig. 4D). These outcomes revealed that Luteoloside retarded the AKT/mTOR pathway.

4. Discussion

This work has focused on finding the efficient but less toxic anti-cancer compounds from plants, including for the PC progression. For instance, Matrine enhances Growth arrest and DNA damage-inducible beta (*GADD45B*) expression to retard the PC progression [10]. Vitexicarpin regulates G2/M phase arrest to promote the PC cell apoptosis [11]. Leonurine modulates the miR-18a-5p/Ferroportin (*SLC40A1*) axis to inhibit tumor growth in PC [12]. Triptolide affects histone methylation and heterochromatin formation to aggravate cell senescence in PC [13]. Luteoloside has the anti-tumor activity [6–9], however, its regulatory mechanism in PC is yet not clear. Herein, it was demonstrated that the PC cell proliferation was gradually inhibited by increasing the Luteoloside dose (0, 20, 40, 80 μM). Moreover, Luteoloside was verified to promote

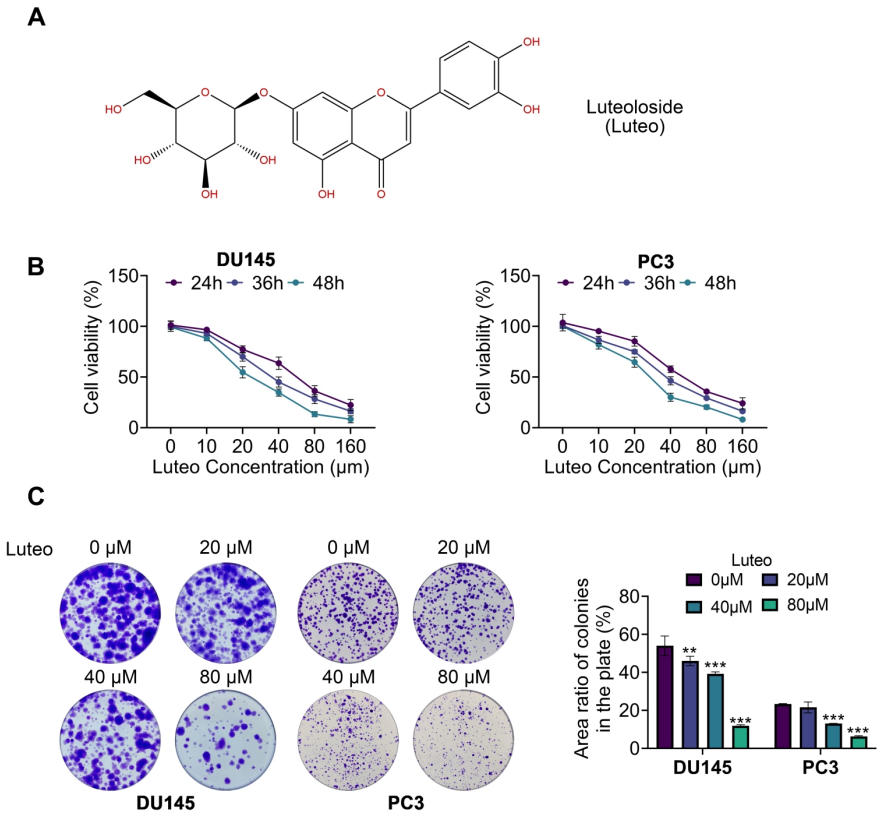

FIGURE 1. Luteoloside inhibiting the cell proliferation in PC. Groups were divided into the Luteoloside (Luteo) 0 μM, Luteo 20 μM, Luteo 40 μM, and Luteo 80 μM groups. (A) The DU145 and PC3 cells viabilities were examined by CCK-8 assay. (B) The DU145 and PC3 cells proliferations were tested *via* the colony formation assay. (C) The cell proliferation abilities of DU145 and PC3 cells were examined by colony formation assay. $^{**}p < 0.01$, $^{***}p < 0.001$. PC: Prostate cancer.

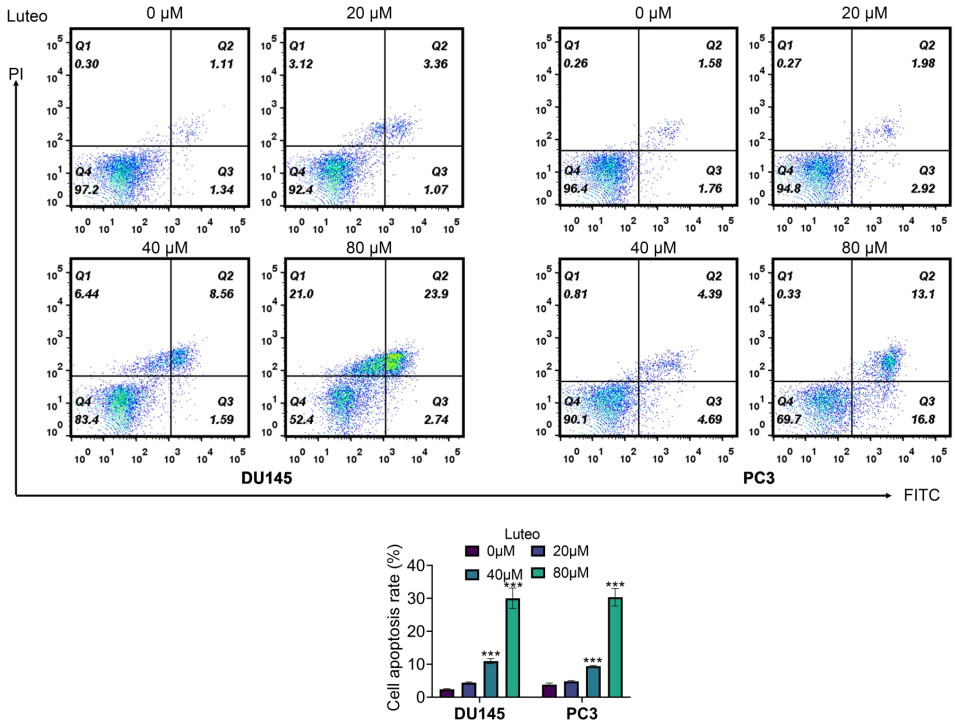

FIGURE 2. Luteoloside strengthening the cell apoptosis in PC. Groups were divided as the Luteo 0 μM, Luteo 20 μM, Luteo 40 μM, and Luteo 80 μM. DU145 and PC3 cells apoptosis was measured by flow cytometry. $^{***}p < 0.001$. PC: Prostate cancer; FITC: fluoresceine isothiocyanate.

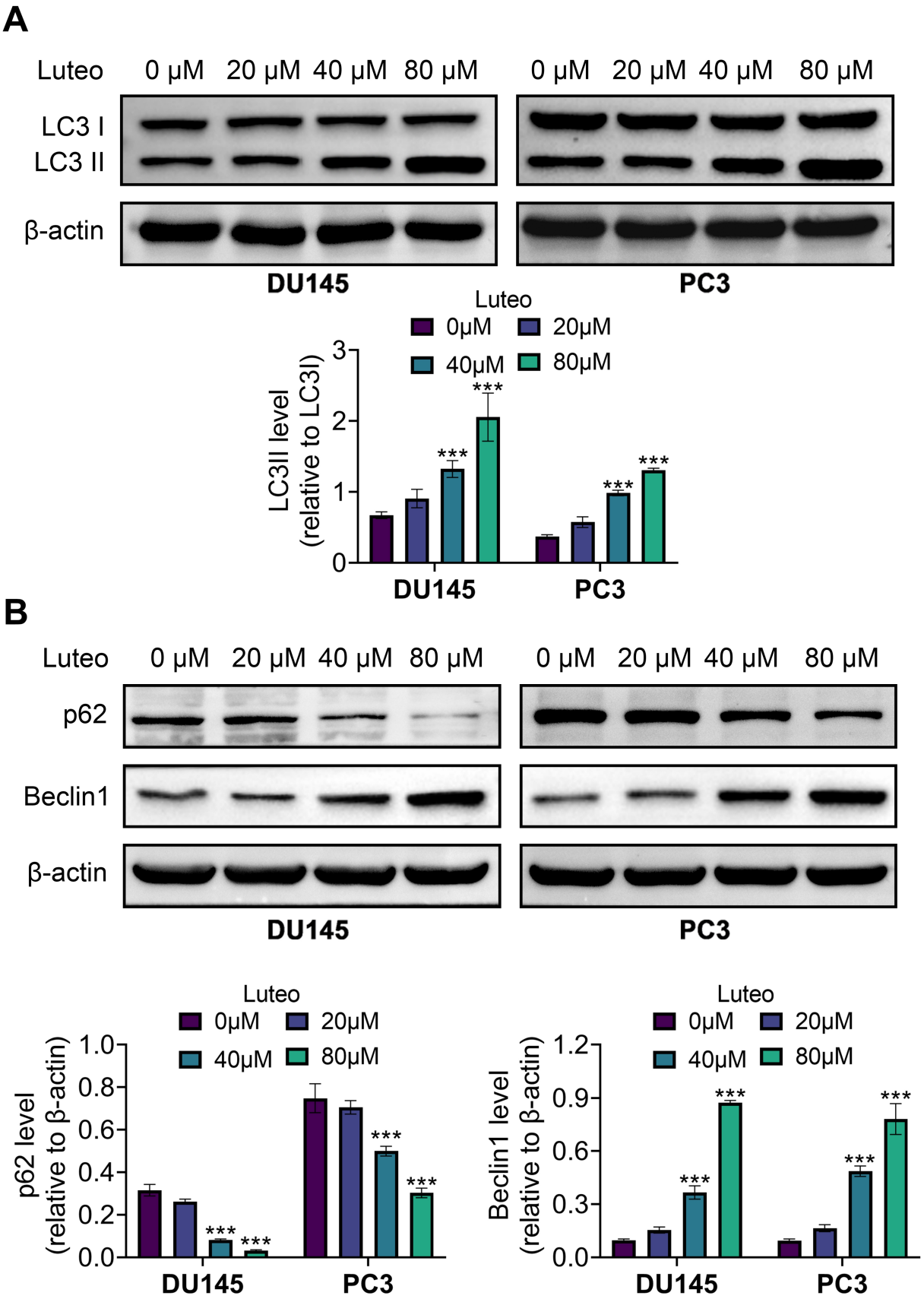

FIGURE 3. Luteoloside enhancing the autophagy in PC. Groups were divided as the Luteo 0 μM, Luteo 20 μM, Luteo 40 μM, and Luteo 80 μM. (A) The LC3I and LC3II protein expressions were confirmed *via* the western blot. (B) The p62 and Beclin 1 protein expressions were verified by western blot. $^{***}p < 0.001$. PC: Prostate cancer; LC3: microtubule-associated protein light chain.

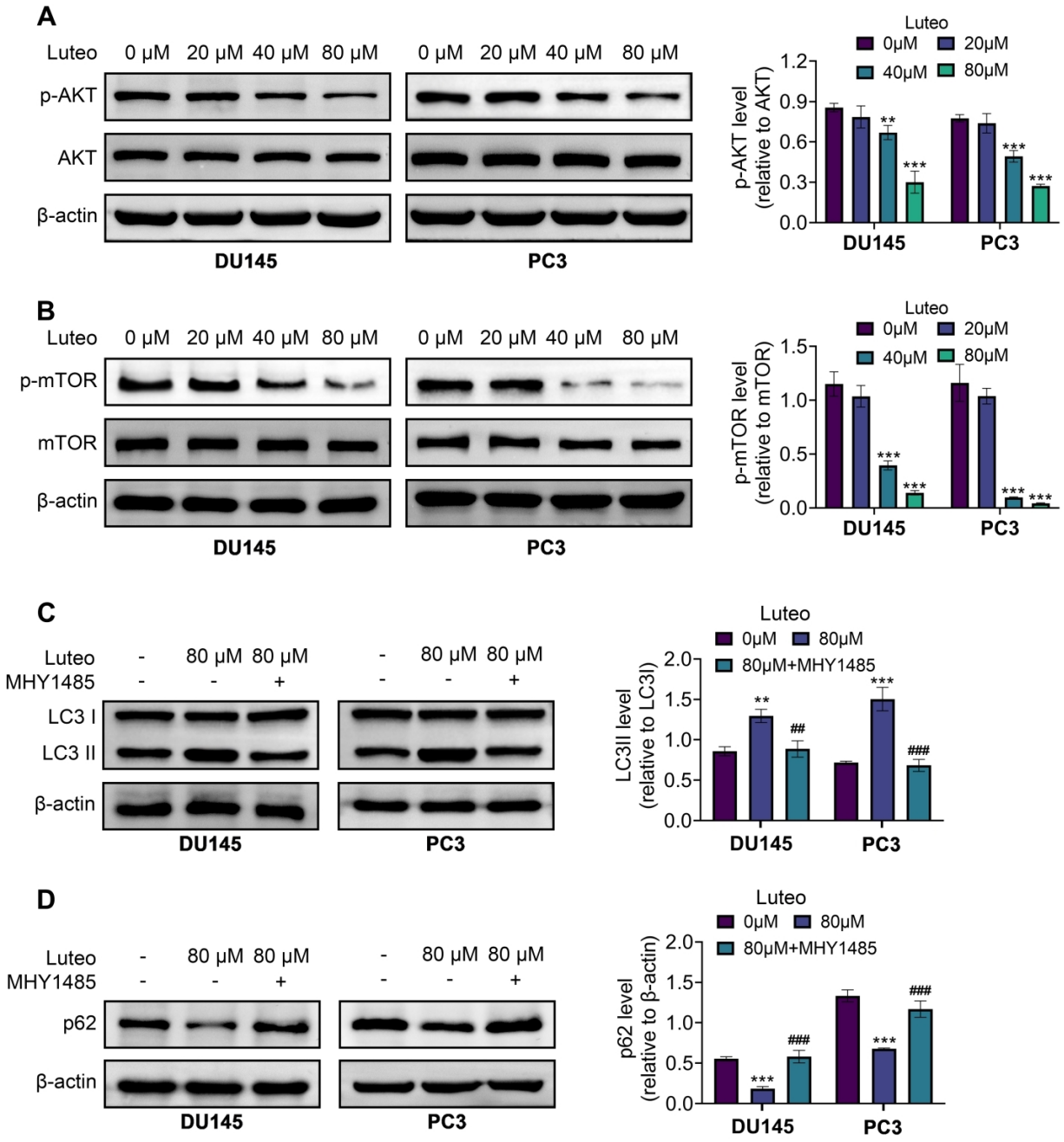

FIGURE 4. Luteoloside retarding the AKT/mTOR pathway. Groups were divided as the Luteo 0 μM, Luteo 20 μM, Luteo 40 μM, and Luteo 80 μM. (A) The p-AKT and AKT protein expressions were examined by western blot. (B) The p-mTOR and mTOR protein expressions were tested *via* the western blot. Groups were divided as the Luteo 0 μM, Luteo 80 μM, and Luteo 80 μM + MHY1485 (mTOR activator). (C) The LC3I and LC3II protein expressions were measured by western blot. (D) The p62 protein expression was tested by western blot. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the Luteo 0 μM group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* the Luteo 80 μM group. PC: Prostate cancer; AKT: protein kinase B; mTOR: mechanistic target of rapamycin; MHY1485 (mTOR activator); LC3: microtubule-associated protein light chain 3.

the PC cell apoptosis.

Autophagy is a damage repair mechanism in intracellular organelles. It has pivotal role in cancers' progression including PC. For example, lncRNA Rhophilin 1-antisense RNA 1 (*RHPN1-AS1*) affects miR-7-5p/epidermal growth factor receptor (EGFR)/phosphoinositide 3 kinase (PI3K)/AKT/mTOR pathway to attenuate autophagy and apoptosis in PC [14]. Suppression of circ_Cyclin B2 (*CCNB2*) targets the miR-30b-5p/Kinesin Family, Member 18A (*KIF18A*) axis to retard autophagy in PC [15]. Mir-139 impacts autophagy to repress the PC progression [16]. The circ-cell migration-inducing protein (*CEMIP*) strengthens protective autophagy to aggravate anoikis-resistance in PC [17]. It is confirmed that the autophagy-related proteins LC3II/LC3I levels and Beclin 1 expression are negatively correlated with p62 expression in PC [18, 19]. In this study, it was shown that the Luteoloside enhanced autophagy by increasing LC3II/LC3I and decreasing p62 protein expression in PC.

Abnormal activation of the AKT/mTOR pathway has been tracked in variety of cancers, including PC [20, 21]. Resultantly, mTOR is phosphorylated and acts as a protein kinase to affect protein synthesis and regulate cell growth, metabolism, and migration by phosphorylating some downstream signaling proteins [22]. Moreover, apoptosis and autophagy are modulated by the AKT/mTOR pathway [23]. In this study, it was found that Luteoloside decreased p-AKT/AKT and p-mTOR/mTOR levels, indicating that Luteoloside retarded the AKT/mTOR pathway.

5. Conclusions

In conclusion, this study manifested that Luteoloside inhibited the PC cells growth and promoted tumor cells autophagy through AKT/mTOR pathway (Fig. 5). Some limitations however existed, such as lacking human samples, *in vivo* experiments, and other progresses (angiogenesis, exosome, and immune response). In future, more experiments can be conducted for PC progression to find other regulatory functions of Luteoloside.

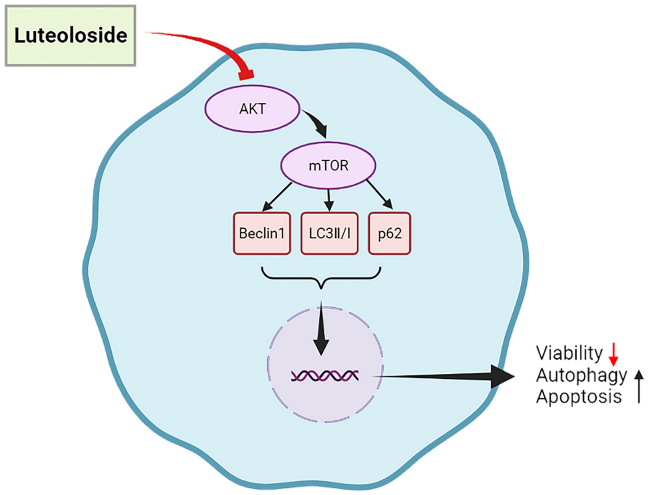

FIGURE 5. Luteoloside inhibiting the AKT/mTOR pathway to reduce cell viability and promote autophagy. Luteoloside decreased the p-AKT/AKT and p-mTOR/mTOR levels to inhibit the AKT/mTOR pathway. Luteoloside increased LC3II/LC3I and Beclin 1 levels, and decreased p62 levels to strengthen the autophagy. AKT: protein kinase B; mTOR: mechanistic target of rapamycin; LC3: microtubule-associated protein light chain 3.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

HYL and LH—designed the study and carried them out; HYL, XW, AYZL, YMP and WZ—supervised the data collection, analyzed the data, interpreted the data; HYL and LH—prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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