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The antiandrogenic effect of neferine, liensinine, and isoliensinine by inhibiting 5-α-reductase and androgen receptor expression via PI3K/AKT signaling pathway in prostate cancer

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Neferine, liensinine, and isoliensinine are bisbenzylisoquinoline alkaloids extracted from seed-embryos of *Nelumbo nucifera* Gaertn. In this study, we evaluated the anticancer activities and mechanism of action of these natural products in prostate cancer cells by MTT, wound healing, ELISA and Western blotting. Neferine, liensinine, and isoliensinine showed growth inhibition and displayed a significant anti-migration activity in prostate cancer cells. They induced apoptosis and autophagy by activating cleaved caspase-9, cleaved PAPR, Bax, LC3B-II, but decreased Bcl-2 and PARP protein expression in LNCaP cells 24 h after treatments. The apoptotic and cytotoxic effects of neferine, liensinine, and isoliensinine were significantly attenuated in the presence of the caspase inhibitor, Z-VAD-FMK. However, the effects were enhanced in the presence of Akt inhibitor (MK2206) and PI3K inhibitor (LY294002). Moreover, neferine, liensinine, and isoliensinine also downregulated the protein expression of androgen receptor, prostate-specific antigen, and type II 5-α-reductase. These results demonstrated that these bisbenzylisoquinoline alkaloids have the potential as promising therapeutics agents. They induced apoptosis *via* inactivation with the PI3K/AKT signal pathway.

1. Introduction

Prostate cancer is a malignant tumor that has a high mortality rate in Western countries. Chemotherapy, radiotherapy, and surgery are therapeutic strategies that are used for the treatment of prostate cancer (Nguyen-Nielsen and Borre 2016). At initial stage, prostate cancer is sensitive to androgen-deprivation therapy which blocks androgen activity (Komura et al. 2018). However, many patients become resistant to hormone therapy. Therefore, it is essential to develop chemotherapeutic agents that can be used to treat prostate cancer with only few side effects.

PSA (prostate-specific antigen) is a glycoprotein produced by the prostate epithelial cell. Its serum level is used as a marker for the diagnosis and progression of prostate cancer (Feng and Huang 2019). The 5-α-reductase is an enzyme that is responsible for converting testosterone into dihydrotestosterone. Previous studies indicated that the 5-α-reductase inhibitor, finasteride, has a growth inhibition activity in androgen-dependent- and PSA- positive LNCaP cells (Bologna et al. 1995; Wu et al. 2011). Recently, 5-α-reductase inhibitors were considered as chemopreventive agents for prostate cancer (Chau and Fig. 2018; Rivero et al. 2018). Autophagy and apoptosis are two forms of cell deaths. Autophagy is involved in the degradation or recycling of harmful and damaged cellular components and its activation can result in cell survival or death (Levy et al. 2017). Moreover, phytochemicals can trigger apoptosis or autophagy in cancer cells (Ramadan et al. 2019; Patra et al. 2020). The PI3K/AKT signaling pathway is associated with cancer progression and it has been shown that autophagy and apoptosis are involved in the regulation of the PI3K/AKT signaling pathway in cancer cells (Alzahrani 2019).

Nelumbo nucifera Gaertn, also called Indian lotus or Chinese water lily, is a source of 249 phytochemicals with pharmacological activities that are isolated from different parts of this plant, including alkaloids, flavonoids, and terpenoids (Sharma et al. 2017; Chen et al. 2019). Neferine, liensinine, and isoliensinine are bisbenzylisoquinoline alkaloids extracted from seed-embryos of *Nelumbo nucifera* Gaertn (Chen et al. 2019). Previous studies have reported that these natural compounds have pharmacological activities, including anticancer, antioxidation, and antiinflammation (Guolan et al. 2018; Marthandam Asokan et al. 2018; Manogaran et al. 2019). A recent study indicated that the combination of neferine and isoliensinine with cisplatin increases the production of reactive oxygen species (ROS), resulting in apoptosis (Manogaran et al. 2019). Neferine, liensinine, and isoliensinine can inhibit cancer cell growth through several signaling pathways, including the Wnt/β-catenin, PI3K/AKT/mTOR and p38 MAPK/JNK signaling pathways (Poornima et al. 2013; Liu et al. 2015; Xu et al. 2016).

Neferine, liensinine, and isoliensinine share a bisbenzylisoquinoline chemical structure; however, no study discussed their potency and efficiency against prostate cancer based on structure activity relationships (SAR). Furthermore, few studies reported the pharmacological mechanism of action of neferine, liensinine, and isoliensinine in prostate cancer (Erdogan and Turkekul 2020; Nazim et al. 2020). Herein, we are reporting the potential anticancer activities of these compounds in androgen-dependent and castration-resistant prostate cancer cells. In the present study, the inhibitory effects of neferine, liensinine, and isoliensinine on the PI3K/AKT signaling pathway and its relevance to the induction of apoptosis and autophagy were examined. Moreover, the effects of these compounds on the protein expression of 5-α-reductase, cell migration, androgen receptor and PSA by these compounds were examined.

2. Investigations and results

2.1. Effects of neferine, isoliensinine, and liensinine treatments on prostate cancer cells

The chemical structure of neferine, liensinine, and isoliensinine is shown in Fig. 1. We investigated their cytotoxicity on the growth

of human prostate cancer cell lines. LNCaP, DU-145 and PC3 cells were treated with 1, 10, and 100 µM of neferine, isoliensinine, and liensinine for 24 h and 48 h. The compounds showed cytotoxicity in a dose- and time-dependent manner on prostate cancer cells (Fig. 2). Neferine was the most potent alkaloid, but liensinine had the least anti-growth activity in prostate cancer cells. At 48 h, 100 µM of neferine, isoliensinine, and liensinine reduced DU-145 cell growth to 61.33 ± 5.7%, 62.27 ± 3.28%, and 83.75 ± 11.49%, LNCaP cell growth to 47.67 ± 1.22%, 39.33 ± 5.3%, and 64 ± 7.83%, and PC-3 cell growth to 35.75 ± 6.24%, 56.75 ± 14.58%, and 80.25 ± 7.18%, respectively, compared to the non-treated control.

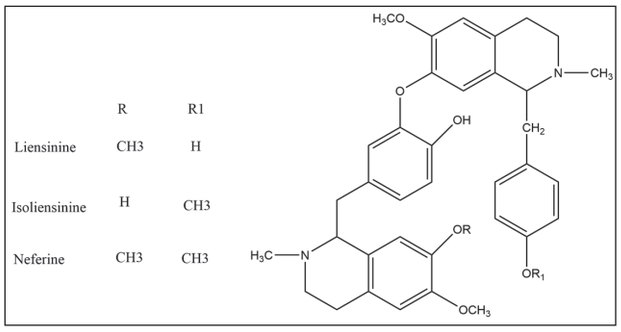

Fig. 1: Chemical structures of nerferine, isoliensinine, and liensinine.

2.2. Neferine, isoliensinine, and liensinine inhibit cell motility of PC3 cells

LNCaP is an androgen-dependent prostate cancer cell, while DU145 and PC3 are castration-resistant prostate cancer cells and have more potential metastatic activity than LNCaP cells. To determine the *in vitro* effect of neferine, isoliensinine, and liensinine on the migration of DU145 and PC3, migration assays were performed, and the results indicated that DU145 had higher migration activity than PC3 cells at the 24 h time point. Neferine, isoliensinine, and liensinine (100 µM) significantly reduced cell migration in PC3 cells. However, the compounds did not significantly inhibit cell migration in DU145 cells. Taken together, these results show that neferine had the most anti-migratory activity in PC3 cells (Fig. 3).

2.3. Neferine, isoliensinine, and liensinine trigger apoptosis and autophagy in LNCaP cells

The results of the MTT assay indicated that neferine, isoliensinine, and liensinine had significant cytotoxicity in LNCaP cells. Therefore, we chose LNCaP cells for further studies. To evaluate whether neferine, isoliensinine, and liensinine modulate apoptosis signaling molecules in LNCaP cells, we examined the protein expression of intrinsic apoptotic markers, such as Bcl-2, Bax, PARP, and cleaved-PARP by Western Blot. The results showed that neferine, isoliensinine, and liensinine (100 µM) increased the expression of Bax, cleaved-caspase-9, and cleaved-PAPR, whereas the expression of Bcl-2 and PARP were inhibited (Fig. 4). We then investigated whether neferine, isoliensinine, and liensinine mediate autophagy in LNCaP cells by investigating the level of LC3B-II accumulation that is associated with the autophagosome formation. The results indicated that neferine, isoliensinine, and liensinine treatments increase LC3B-II protein expression in LNCaP cells after 24h of treatments (Fig. 4). These results demonstrated that autophagy mediated by these compounds could promote cell death.

2.4. Neferine, isoliensinine, and liensinine induced apoptosis through the PI3K/AKT signaling pathway

LNCaP cells were treated with 100 µM of neferine, isoliensinine, and liensinine for 24 h. The presence of apoptosis was determined by M30 CytoDeath ELISA kit. The treatments were combined with caspase inhibitor (Z-VAD-FMK), Akt inhibitor (MK2206) and PI3K inhibitor (LY294002). The treated cells were then subjected to MTT and apoptosis assays to determine cytotoxicity. Using the ELISA assay, the results indicated that neferine, isoliensinine, and liensinine induce apoptosis. To determine if the caspase is involved in neferine-, isoliensinine-, and liensinine-induced apoptosis and

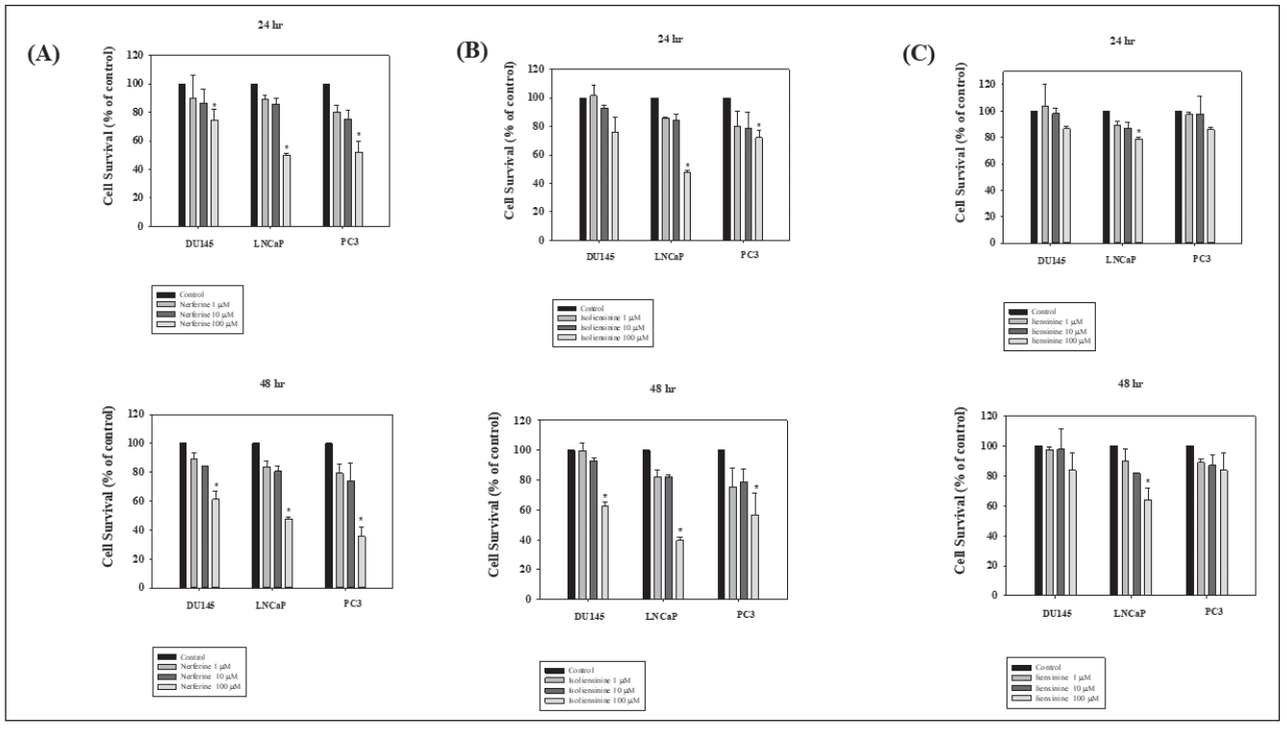

Fig. 2: Nerferine, isoliensinine, and liensinine inhibited the growth of prostate cancer cells. DU145, LNCaP, and PC3 cells were treated with various concentrations (1, 10, 100 µM) of nerferine (A), isoliensinine (B), and liensinine (C) for 24 and 48 h by MTT assay. Data are presented as mean ± SEM from three independent experiments. * $p < 0.05$ vs. control group.

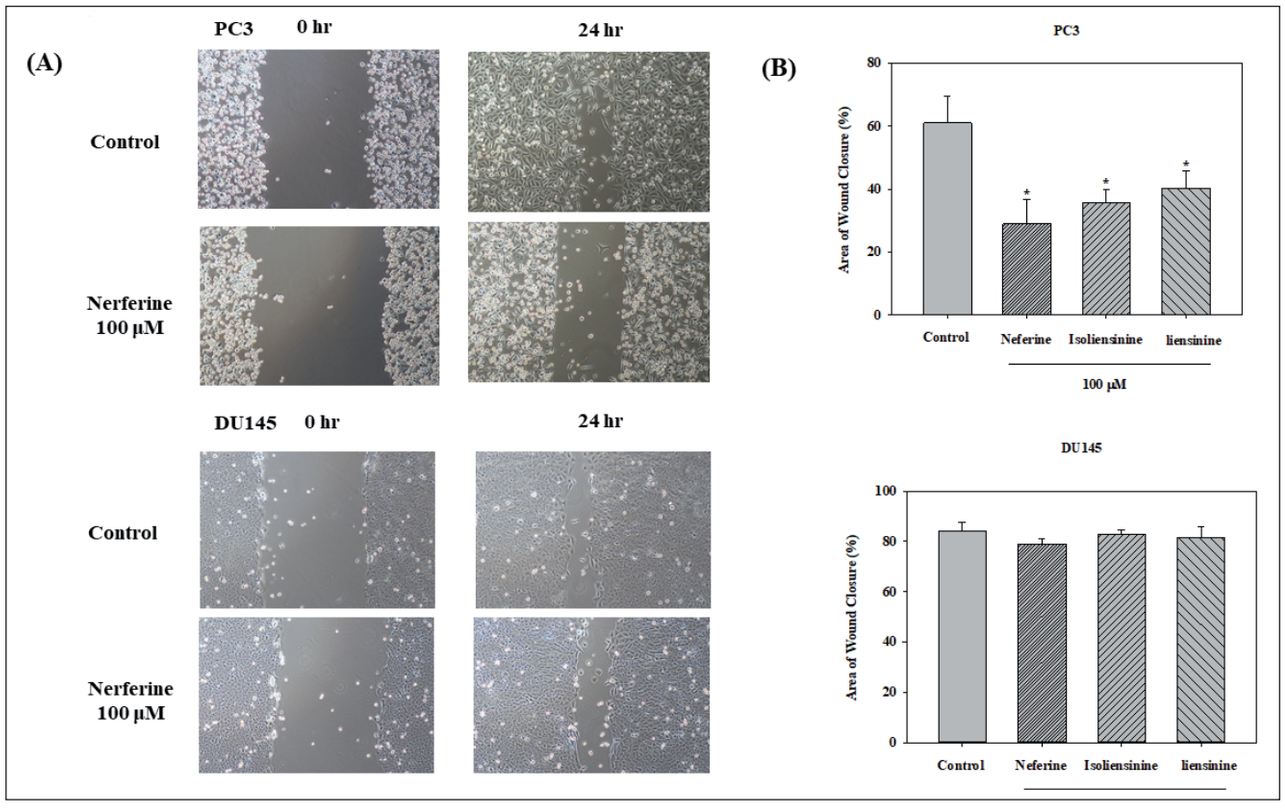

Fig. 3: Nerferine, isoliensinine, and liensinine suppresses wound closure in PC3 and DU145 cells at 24 h. For wound healing assay, PC3 and DU145 cells were treated with nerferine, isoliensinine, and liensinine for 24 h (A). Figures are representative of three independent experiments performed (B). Data are presented as mean ± SEM from three independent experiments. p* < 0.05 compared with control.

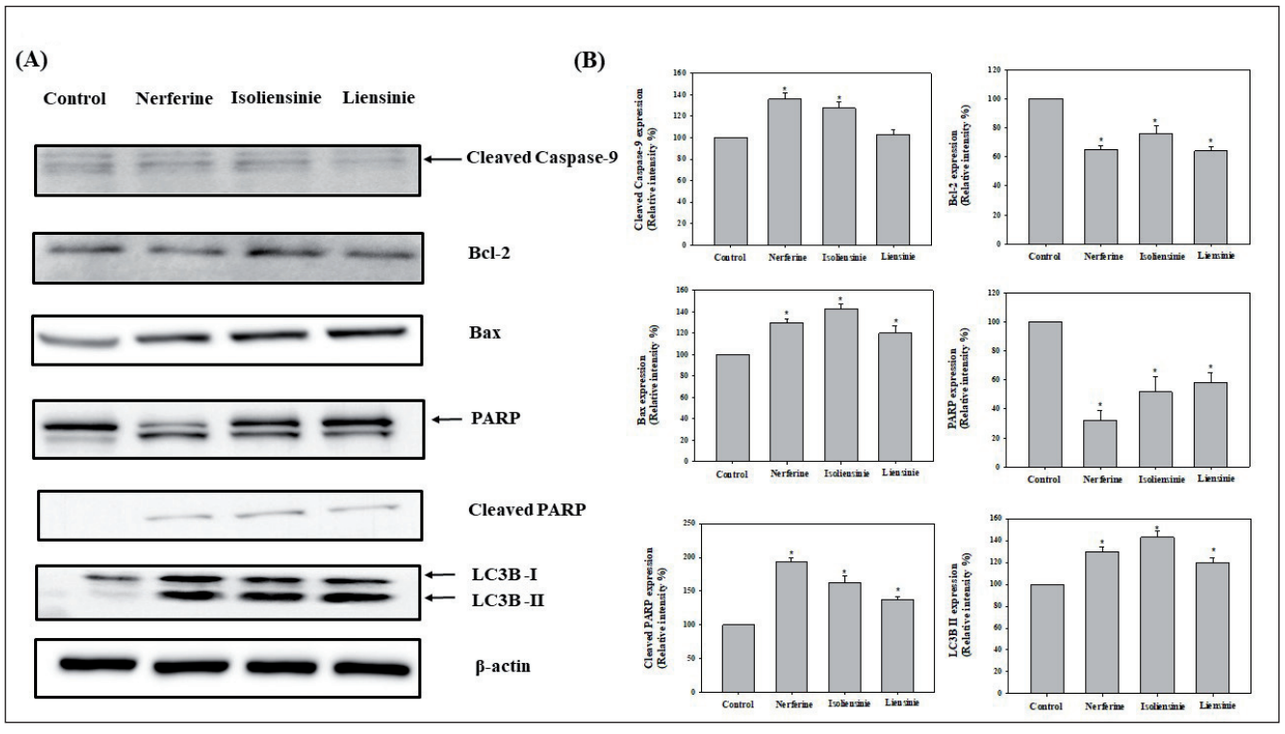

Fig. 4: Effects of nerferine, isoliensinine, and liensinine on protein expression in LNCaP cells. The LNCaP cells were treated with nerferine, isoliensinine, and liensinine (100 µM) for 24 h. The expression levels of cleaved Caspase-9, Bcl-2, Bax, PARP, cleaved PARP, LC3B-I and LC3B-II were assessed by Western Blotting (A). Beta-actin was used as an internal control. Figures are representative of at three experiments performed. Each value represents the mean ± SEM of independent experiments. p* < 0.05 compared with control (B).

cell death, LNCaP cells were co-treated with a broad-spectrum caspase inhibitor. After the treatments, the apoptotic induction and cell death were reduced (Figs. 5, 6). To confirm that the PI3K/AKT signaling pathway is involved in neferine-, isoliensinine-, and liensinine-induced apoptosis, Akt and PI3K inhibitors were used in the study. The data showed that these inhibitors enhance the apoptotic

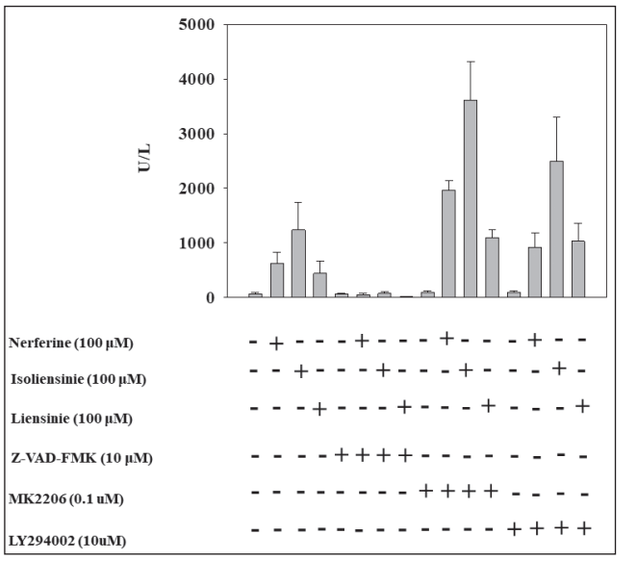

Fig. 5: The LNCaP cells were treated with nerferine, isoliensinine, and liensinine in the presence or absence of the Z-VAD-FMK caspase inhibitor (10 μM), AKT inhibitor (MK2206, 0.1 μM), PI3K inhibitor (LY294002, 10 μM) for 24 h. The apoptosis effects were determined by ELISA assay. Each value represents the mean ± SEM of three independent experiments.

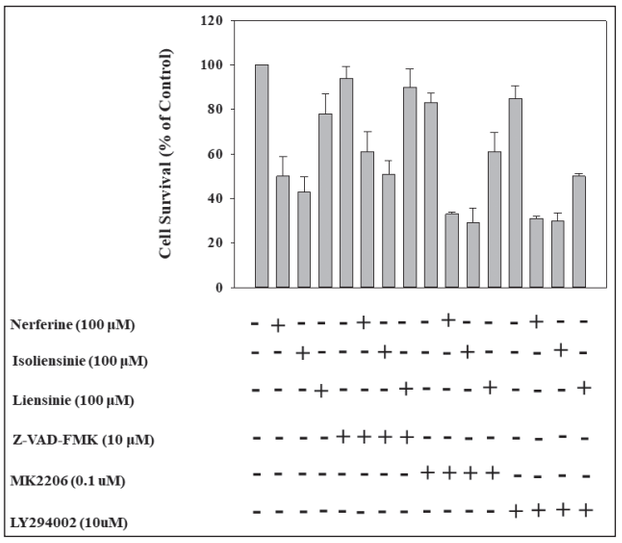

Fig. 6: The LNCaP cells were treated with nerferine, isoliensinine, and liensinine in the presence or absence of the Z-VAD-FMK caspase inhibitor (10 μM), AKT inhibitor (MK2206, 0.1 μM), PI3K inhibitor (LY294002, 10 μM) for 24 h. The cell survival rates were determined by MTT assays. Each value represents the mean ± SEM of three independent experiments.

activity and cytotoxicity of neferine, isoliensinine, and liensinine in LNCaP cells (Figs. 5, 6). It also suggested that the PI3K/AKT signaling pathway plays a critical role in neferine-, isoliensinine-, and liensinine-induced apoptosis.

2.5. Neferine, isoliensinine, and liensinine disturb the androgen receptor signaling axis

To determine whether the compounds affect the androgen receptor signaling pathway, we examined the protein expression of androgen receptor, 5-α-reductase and PSA in LNCaP cells. The cells were exposed to these compounds at the concentration of 100 μM for 24 h. The results showed that neferine, isoliensinine, and liensinine significantly decrease androgen receptor, type II 5-α-reductase and PSA protein expression (Fig. 7). The results also explained why neferine, isoliensinine, and liensinine had better anti-cancer activities in androgen-dependent prostate cancer compared to castration-resistant prostate cancer.

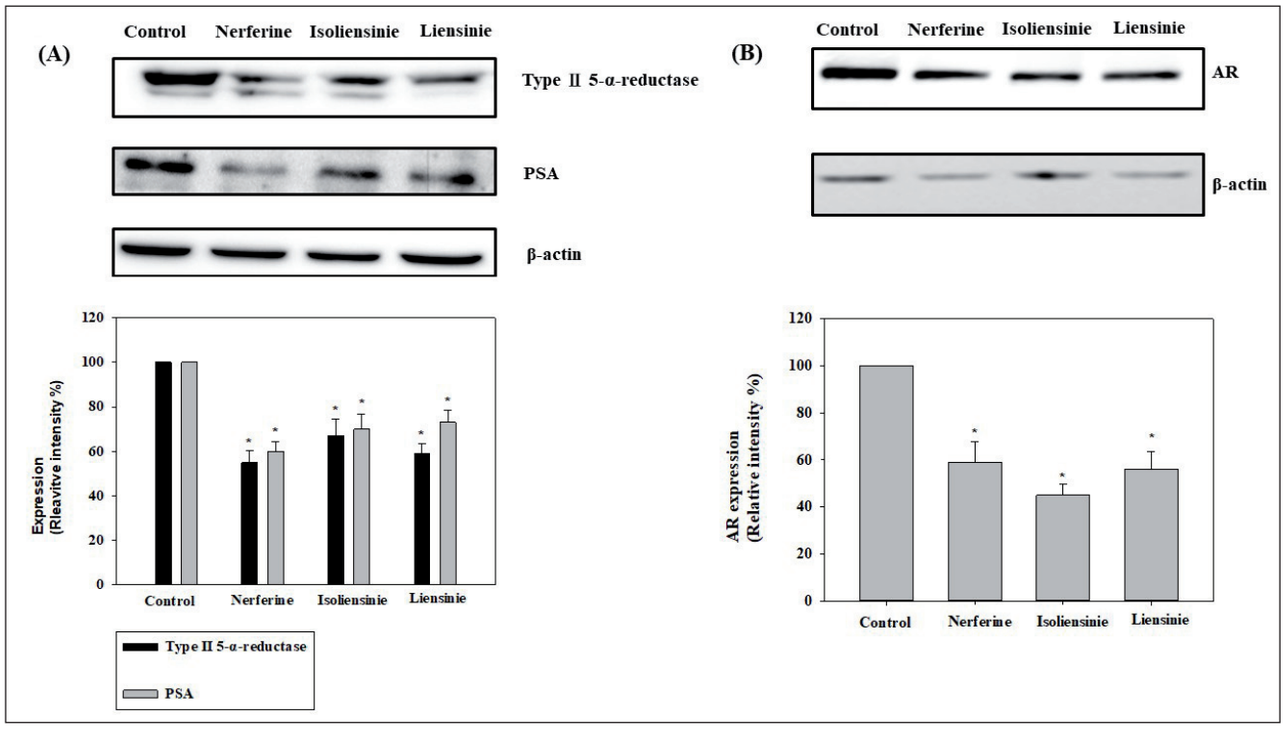

Fig. 7: The LNCaP cells were treated with nerferine, isoliensinine, and liensinine (100 μM) for 24 h. The protein expression levels of Type II 5-α-reducatse, PSA and androgen receptor were assessed by Western Blotting. Beta-actin was used as an internal control. Figures are representative of at three experiments performed. Each value represents the mean ± SEM of independent experiments. p* < 0.05 compared with control.

3. Discussion

Neferine, isoliensinine, and liensinine share a bisbenzylisoquinoline chemical structure. As shown in Fig. 1, the chemical structure of neferine, has a methyl group on R and R1 function group compared with liensinine and isoliensinine. The chemical structures of isoliensinine and liensinine are very similar. The R1 and R function groups are replaced with each other in liensinine, and isoliensinine. However, the anticancer activities are different.

In this study, we evaluated the potency and efficacy of neferine, isoliensinine, and liensinine against androgen-dependent (LNCaP) and castration-resistant (PC3 and DU145) prostate cancer cells. The compounds showed different cell growth inhibition effects on prostate cancer cells. Our results showed that they inhibited prostate cancer growth in dose-dependent and time-dependent manners in LNCaP, PC3 and DU145 cells. LNCaP and PC3 cells are more sensitive to the treatments than DU145 cells. Overall, the results indicated that neferine has the most potent anticancer activity against prostate cancer cells.

At the early stage of prostate cancer, cancer cells migrate and invade blood vessels and spread in the body. Cancer metastases involve migration, and patients with advanced prostate cancer often suffer from bone metastases. A previous study indicated that 22 genes are associated with bone metastasis in LNCaP, DU145 and PC3 cells (Jin et al. 2013). PC3 and DU145 have the higher migratory ability compared to LNCaP, and this ability has been shown to be associated with metastasis. In this study, we performed a wound-healing assay in DU145 and PC3 cells. At 24 h time point, 80% of the gap was closed in DU145 cells and 60% of gap was closed in PC3 cells; however, the neferine, isoliensinine, and liensinine inhibited the motility of PC3 cells. Taken together, these results demonstrated that neferine has the best anti-migratory activity.

Apoptosis is called programed cell death that can be evaded by cancer cells. Furthermore, apoptosis is an imbalance between cell division and cell death. Apoptosis plays a pivotal role in cancer treatment and a lot of chemotherapeutic agents can trigger apoptosis in cancer cells. Caspases are protease enzymes and play an important role in apoptosis (Van Opdenbosch and Lamkanfi 2019). Intrinsic (mitochondrial) and extrinsic (death receptor) pathways can be activated by caspases. When a cell is stimulated, it will release cytochrome c into the cytoplasm. The mitochondrial pathway is also regulated by several pro-apoptotic proteins (Bax) or anti-apoptotic proteins (Bcl-2). LNCaP cells are sensitive to neferine, isoliensinine, and liensinine treatment. In this study, we examined the expression of apoptotic molecules and the possible mechanism of action of neferine, isoliensinine, and liensinine in LNCaP cells. These compounds increased Bax expression, but decreased Bcl-2 expression in LNCaP cells. The alteration of the Bax/Bcl-2 ratio indicates the susceptibility of cells to apoptosis. Furthermore, the treatments activated the expression of cleaved PARP and cleaved caspase-9. PARP is cleaved by caspases and it is an early indicator of apoptosis. Additionally, the combination treatments of these compounds with caspase inhibitor, Z-VAD-FMK, significantly reduced cell death and apoptosis. In the present study, neferine, isoliensinine, and liensinine regulated different levels of Bax, Bcl-2, cleaved PARP, cleaved caspase-9 and PARP. These results demonstrated that these compounds induce apoptosis *via* the mitochondrial (intrinsic) pathway. The PI3K/AKT signaling pathway is involved in cell death and survival and this pathway is aberrantly activated in prostate cancer cells (Chen et al. 2016). The inhibition of the PI3K/AKT signaling pathway is associated with apoptosis and an anti-proliferative effect. Based on our results, the compounds enhanced apoptosis and cell death through the inhibition of PI3K and AKT using specific inhibitors in LNCaP cells. These results indicated that the PI3K/AKT signaling pathway is involved in apoptosis induced by neferine, isoliensinine, and liensinine.

Autophagy is a self-destructive process activated by cellular stress and plays a dual role in tumor proliferation and suppression (Sakanashi et al. 2019). Microtubule-associated protein light chain 3 (LC3) is a full-length cytosolic protein converted to LC3-I by Atg4 and conjugates to phosphatidylethanolamine to become LC3-II. LC3 is a marker of autophagosome. In the current study, neferine, isoliensinine, and liensinine treatments increased LC3B-II expression in LNCaP cells. Taken these results together, apoptosis, and autophagy led to cell death after neferine, isoliensinine, and liensinine treatments.

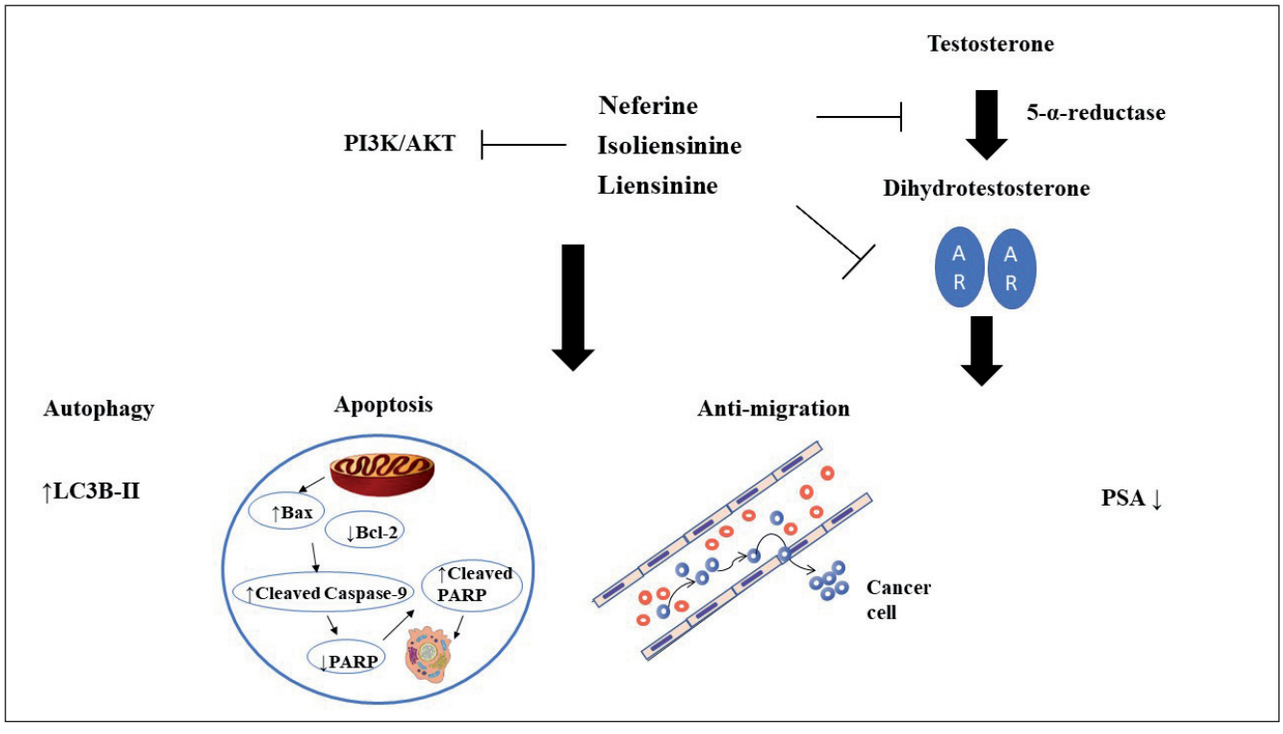

Fig. 8: The mechanism of action of nerferine, isoliensinine, and liensinine mediated anticancer effects in prostate cancer cells.

Testosterone binds to androgen receptors and the activated androgen receptor regulates prostate cell proliferation and survival. Androgen receptor (AR) antagonists, including flutamide impairs androgen binding to the AR and inhibits prostate cancer cell growth (Ornstein et al. 1996; Kai and Levenson 2011). The 5-α-reductase inhibitors, including finasteride and dutasteride, can prevent the conversion of testosterone into dihydrotestosterone and decrease the serum PSA concentrations (Choi et al. 2010). Although it has a low sensitivity, PSA is a marker for the detection of prostate cancer and benign prostatic hyperplasia. In our current study, neferine, isoliensinine, and liensinine reduced 5-α-reductase and AR protein expression in LNCaP cells, suggesting that bisbenzylisoquinoline alkaloids may possess 5-α-reductase and AR inhibition functions. Several studies indicated that Pao Pereira extracts are rich in alkaloids and the extracts have 5-α-reductase and AR inhibition activities (Liu et al. 2019). Neferine, isoliensinine, and liensinine are alkaloids and these compounds also possess 5-α-reductase and AR inhibitory effects in LNCaP cells, and exert anti-cancer and anti-migration activities (Fig. 8). This study demonstrated that the three bisbenzylisoquinoline alkaloids isolated from *Nelumbo nucifera* induce apoptosis and autophagy in LNCaP cells. These compounds induce apoptosis and autophagy by inhibiting 5-α-reductase and AR expression through the PI3K/AKT signaling pathway. We think that neferine, isoliensinine, and liensinine might serve as promising therapeutics agents for prostate cancer treatment.

4. Experimental

4.1. Cell lines and reagents

Neferine, liensinine, and isoliensinine were purchased from Chengdu Biopurify Phytochemicals Ltd. (China). The caspase inhibitor (Z-VAD-FMK), Akt inhibitor (MK2206) and PI3K inhibitor (LY294002) were purchased from Beyotime Biotechnology (China). The human prostate cancer lines, LNCaP, PC3, and DU145 were purchased from Shanghai Fuheng Biotechnology Co., Ltd. (China). The cells were maintained in a 37 °C humidified atmosphere with 5% CO_2. LNCaP cells were grown in RPMI-1640 medium, and DU145 and PC3 were grown in DMEM/Ham's F12K medium. The media were supplemented with 10 % fetal bovine serum (FBS), and 100 U/ml penicillin+100 µg/ml streptomycin. FBS and the media were obtained from LONSERA, ShangHai ShuangRu Biotech Co., Ltd (China).

4.2. Cell viability assay

Cells were seeded in 96-well plates and cultured in 100 µL medium with 10 % FBS. When the cells reached 80% confluence, they were incubated with media containing various concentrations of neferine, liensinine, and isoliensinine. After 24 h and 48 h treatments, the cell viability was determined by the MTT assay (Beyotime Biotechnology, China). The absorbance was measured by a microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad, USA).

4.3. Wound healing assay

PC3 and DU145 cells (10^6 cells/well) were maintained in 6 well plates and when they reached 100% confluence, a scratch wound was created using a 200 µL pipette tip. After washing the PC3 and DU145 cells with PBS, the cells were treated with different concentrations of neferine, liensinine, and isoliensinine.

4.4. Western blotting

The total protein was extracted by M-PER® (Thermo Scientific, USA). 20 µg of protein was separated by SDS-PAGE, and transferred into a PVDF membrane. The membrane was incubated with the following primary antibodies: cleaved PARP (Cell signaling technology, USA), PARP (Cell signaling technology, USA), Bax (Abways Technology, China), Bcl-2 (Abways Technology, China), androgen receptor (Abways Technology, China), Type II 5-α-reductase (Abways Technology, China), LC3B (Abways Technology, China), prostate-specific antigen (Abcam China, China), β-actin (Sigma-Aldrich, USA). The second antibodies were anti-rabbit IgG, HRP-linked Antibody (Cell signaling technology, USA), and anti-mouse IgG, HRP-linked Antibody (Cell signaling technology, USA). The blotted membrane was exposed to enhanced chemiluminescent kit (Epizyme Biotech, China).

4.5. M30 CytoDeathhs ELISA apoptosis assay

The M30 CytoDeathhs (Peviva AB, Bromma, Sweden) was used for the detection of apoptosis and is suitable for measuring cell death by apoptosis in cell culture. In the present study, LNCaP cells were seeded at 10^4 cells/well in a 96 well plate. After reaching 80% confluence, the cells were treated with the neferine, liensinine, and isoliensinine compounds. At the end of the treatment, 0.5% NP40 was added to the culture medium and the level of apoptosis was measured following the manufacturer's protocol.

4.6. Statistical analysis

The statistical difference between two variables was compared by unpaired, two-tailed Student's *t*-test. One-way analysis of variance (ANOVA) was used for comparing control group with more than one treatment group. All data are presented as mean ± standard error of mean (SEM). A p-value of <0.05 was regarded as statistically significant.

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Conflicts of interest: The Authors declare no conflicts of interest regarding this study.

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