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ORIGINAL ARTICLE

Icariin inhibits the growth of human cervical cancer cells by inducing apoptosis and autophagy by targeting mTOR/PI3K/AKT signalling pathway

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Summary

Purpose: *Cervical cancer causes significant morbidity and mortality among women worldwide. The currently available treatment options are not efficacious and also create severe adverse effects. It is apparent that new therapeutic approaches are needed for this cancer. In this study, we examined the anticancer effects of a natural flavonoid, Icariin, against human cervical cancer cells.*

Methods: *The anti-proliferative effects of Icariin were evaluated on cervical cancer HeLa cell line and normal HCvEpC cells by cell counting assay.*
The effect of Icariin on colony formation was assessed by colony formation assay. Apoptotic effects were determined by acridine orange/ethidium bromide (AO/EB) and DAPI staining. Autophagy was investigated by electron microscopy. Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) levels were estimated by flow cytometry. Protein expression was evaluated by western blotting.

Results: *Icariin inhibited the growth of HeLa cervical cancer cells dose-dependently. IC_{50} of Icariin was 20 μM against the HeLa cells with comparatively negligible toxic effects on normal HCvEpC cells. The anticancer effects of Icariin were due to induction of apoptosis which was accompanied with cleavage of caspase 3 and 9, upregulation of Bax and downregulation of Bcl-2. Icariin also prompted autophagy in HeLa cells and enhanced the LC3 II expression concentration-dependently. Icariin also induced the generation of ROS and diminished the MMP levels in HeLa cells and blocked the mTOR/PI3K/AKT signalling cascade, suggestive of its potent anticancer activity.*

Conclusions: *Taken together Icariin may prove potent and efficacious lead molecule for the development of therapy for cervical cancer.*

Key words: *apoptosis, autophagy, cervical cancer, icariin*

Introduction

Natural products have drawn much attention as anticancer drugs owing to their potent activities and comparatively lower side effects [1]. Many of the plant derived natural products such as taxanes, podoplylotoxins, vinca alkaloids, etc are being used for the therapy of cancer [2]. Flavonoids constite a large assortment of molecules and are prevalently found across plant kingdom [3]. Owing to their presence in edible plant parts, they have been considered safe for human consumption [4]. Accumulating evidence suggests that consumption of plants products rich in flavonoids lower the risk of cancer development [5]. Icariin is a flavonoid glucoside prevalently biosynthesized by the Epimedium species both in roots and aerial parts. Icariin has broad pharmacological activities such as antican-

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cer and anti-inflammatory to name a few [6,7] and it has been shown to halt the growth of breast and gastric cancer cells [8,9]. In a recent study, Icariin was found to suppress the growth of esophageal cancer via induction of apoptosis [10]. However, the anticancer effect of Icariin has not been examined in cervical cancer. Cervical cancer, being one of the common types of cancers in women, is ranked as second most prevalent cancer world over [11]. Although, cervical cancer is more frequent in underdeveloped countries, it still accounts for 10% of all the cancers in women [12]. Approximately 0.37 million new cervical cancer cases are detected annually worldwide [13]. The treatment for cervical cancer involves surgery, chemotherapy and/or radiotherapy [14]. However, the currently available chemotherapeutic agents can create adverse effects, negatively impacting the patient quality of life [15]. Hence, the identification of novel, effective and safer anticancer agents is required to improve the therapeutic results of cervical cancer. In the present investigation, the anticancer effects of Icariin were examined against the human cervical HeLa cancer cells and its underlying mechanisms were deciphered.

Methods

Cell culture conditions

The HeLa cervical cancer cell line and the normal HCvEpC cell line were purchased from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Massachusetts, USA),100 µg/ml streptomycin and 100 U/ml penicillin G (Himedia, Pennsylvania, USA) in an incubator at 37°C with 5% CO_2.

Cell viability and colony formation assays

In brief, 5×10^4 HeLa cells/well were seeded in 12-well plates and incubated for 24 hrs with different concentrations of Icariin. The aliquots of cells were then removed and counted in triplicate following trypan blue staining. The effect of Icariin on the cell morphology was also performed by phase contrast microscopy and its effect on the formation of HeLa colonies was investigated as described earlier [17]. In brief, HeLa cells were subjected to treatment with 0, 10, 20 and 40 µM Icariin concentrations and cultured for 24 hrs at 37°C. The cells were collected at the exponential growth phase and counted with a hemocytometer. Seeding of the cells was done at 200 cells per well which were incubated for 6 days and subjected to washing with phosphate buffered saline (PBS). Afterwards, methanol was used to fix the colonies and stained with crystal violet for about 30 min before being counted under light microscope.

AO/EB and annexin V/PI staining for apoptosis

The detection of apoptosis in HeLa cervical cancer cells was performed by AO/EB staining. In brief, HeLa cells (0.6×10^6) were grown in 6-well plates. Following incubation of around 12 hrs, the HeLa cells were subjected to Icariin treatment for 24 hrs at 37°C. 25 µl of cell culture were put onto a glass slide and stained with 1 µl of AO and EB. The slides were covered with cover slip and examined under fluorescence microscope. Annexin V/propidium iodide (PI) staining of the Icariin-treated HeLa cells was performed as described previously [18]. In brief, the estimation of the apoptotic cells was further carried out by double staining of the cultures with PI and annexin V-FITC. The Icariin-treated HeLa cells were washed and incubated at room temperature for 15 min in the dark in 100 µl of 1X binding buffer containing 5 µl of Annexin V-FITC and 5 µl of PI. Afterward, apoptosis was analyzed by FACScan laser flow cytometer (FACS Calibur, Becton Dickinson, USA).

Transmission electron microscopy (TEM) for autophagy detection

Detection of autophagy was used to assess the induction of autophagy in HeLa cells. The cells were fixed in a solution of 4% glutaraldehyde 0.05 M sodium cacodylate, postfixed in 1.5% OsO4, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and then observed using Zeiss CEM 902 electron microscope.

Determination of ROS and MMP

The ROS and MMP levels were estimated by culturing HeLa cells for 24 hrs at 37°C and subsequently treated with varied doses of Icariin for 24 hrs. Next, Dulbecco's modified Eagle's medium was decanted and the cells were treated 5 µM DCH-DA for estimation of ROS or rhodamine 123 (Rh123) for estimation of MMP by examining the cells by fluorescence microscopy.

Western blotting

Briefly, the HeLa cells were washed with ice-cold PBS and suspended in a lysis buffer at 4°C which was then shifted to 95°C. Afterwards, the protein content of each cell extract was checked by Bradford assay. About, 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then treated with Tert-butyldimethylsilyl chloride (TBS) and then exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

Statistical analyses were done using one way analysis of variance (ANOVA) followed by Tukey's *post hoc* test, using SPSS software package v.9.05 (SPSS Inc, Chicago, Ill, USA). Data is presented as mean±standard deviation and $p<0.05$ was considered to indicate statistically significant difference.

Results

Icariin suppresses the growth of cervical cancer cells

The antiproliferative effects of Icariin (Figure 1A) were assessed on the HeLa cervical cancer cell line and HCvEpC non-cancer cell line. Icariin treatment caused dose-dependent inhibition of the growth of HeLa cells. The IC_{50} of Icariin against the HeLa cells was 20 μM (Figure 1B). Nonetheless, the cytotoxicity of Icariin on the HCvEpC non-cancer cells was comparatively negligible (IC_{50} 95 μM). Moreover, Icariin also induced some morphological changes such as shrinkage and membrane blebbing of the HeLa cells (Figure 1C). The effects of Icariin treatment were also assessed on the colony development potential of HeLa cells and it was revealed that this molecule exerted dose-dependent inhibition on the colony formation of HeLa cells (Figure 1D).

Icariin activates apoptotic cell death of cervical cancer cells

To investigate the mechanism for the anticancer effects of Icariin, AO/EB staining was performed and remarkable changes were observed in the nuclear morphology and membrane blebbing of HeLa cells (Figure 2A). Furthermore, annexin V/PI staining showed that Icariin increased the apoptotic cells from 3.8% in the control to around 41% at 40 μM (Figure 2B). Western blot analysis showed that this molecule also caused considerable increase in the expression of caspases 3, 9 and Bax, and decreased the expression of Bcl-2 in HeLa cells (Figure 2C).

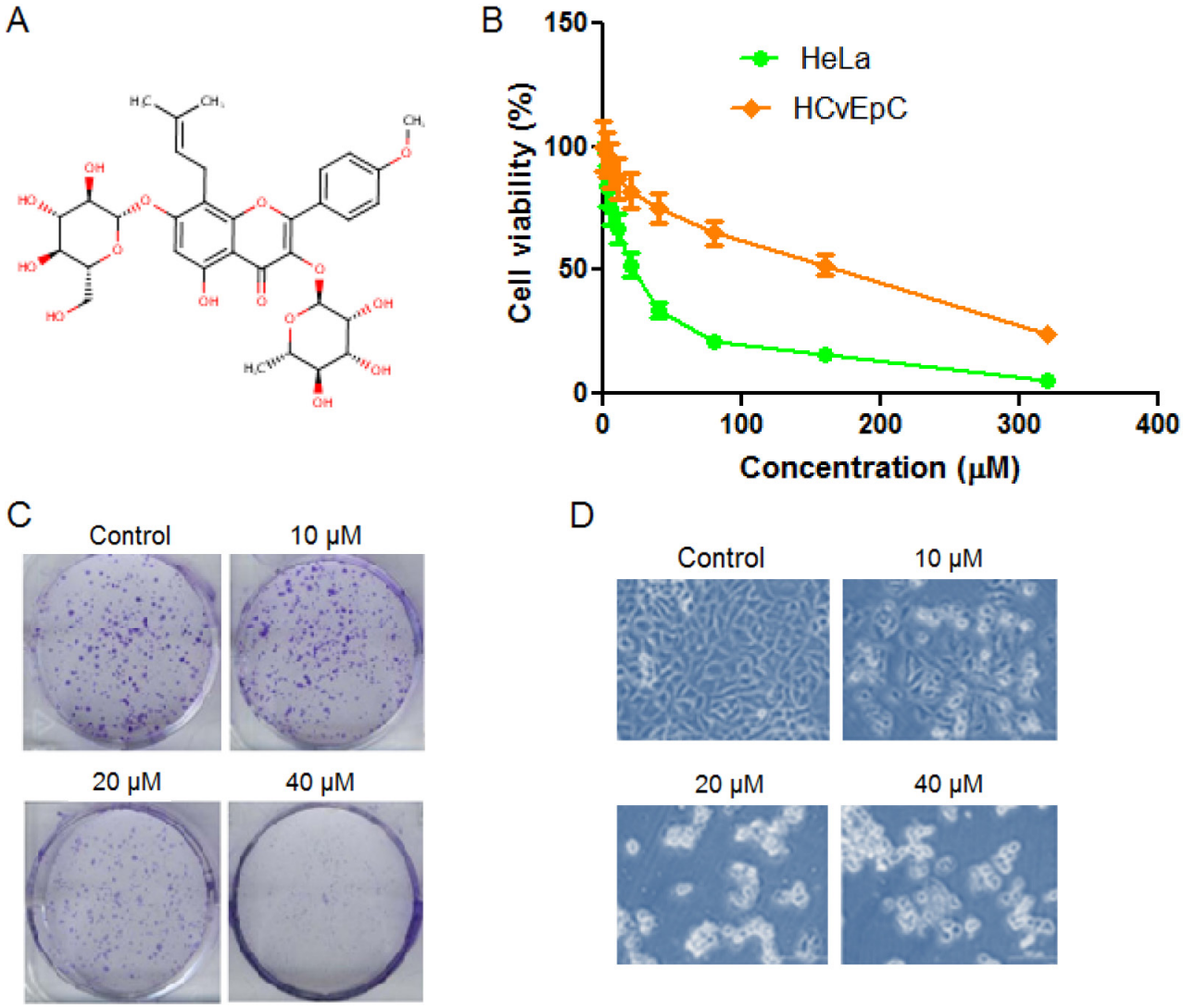

Figure 1. (A): Chemical structure of Icariin. **(B):** MTT assay showing that Icariin inhibits the viability of the cervical cancer HeLa with minimal effects on the non-cancerous HCvEpC cells ($p<0.05$). **(C):** Colony formation assay showing inhibitory effects of Icariin on the colony formation of the HeLa cells. **(D):** Effect of Icariin on the morphology of the HeLa cells. The experiments were performed in triplicate and shown as mean ± SD.

Icariin induces autophagy in the cervical cancer cells

Electron microscopic analysis showed that Icariin caused development of autophagic vesicles in HeLa cells in a concentration-dependent manner (Figure 3A). Western blot analysis further confirmed the induction of autophagy in the HeLa cells wherein LC3 II expression was found to increase and that of p62 decreased considerably. Nonetheless, the expression of LC3 I showed no remarkable change (Figure 3B).

Icariin causes increase in ROS and decrease in MMP levels

The effects of Icariin were examined on the ROS and MMP levels of the HeLa cells at 0, 10, 20 and 40 μM concentrations. The results showed that Icariin caused considerable increase in the ROS levels (Figure 4A) which was also associated with decrease in the MMP levels (Figure 4B). These effects of Icariin were found to be concentration-dependent.

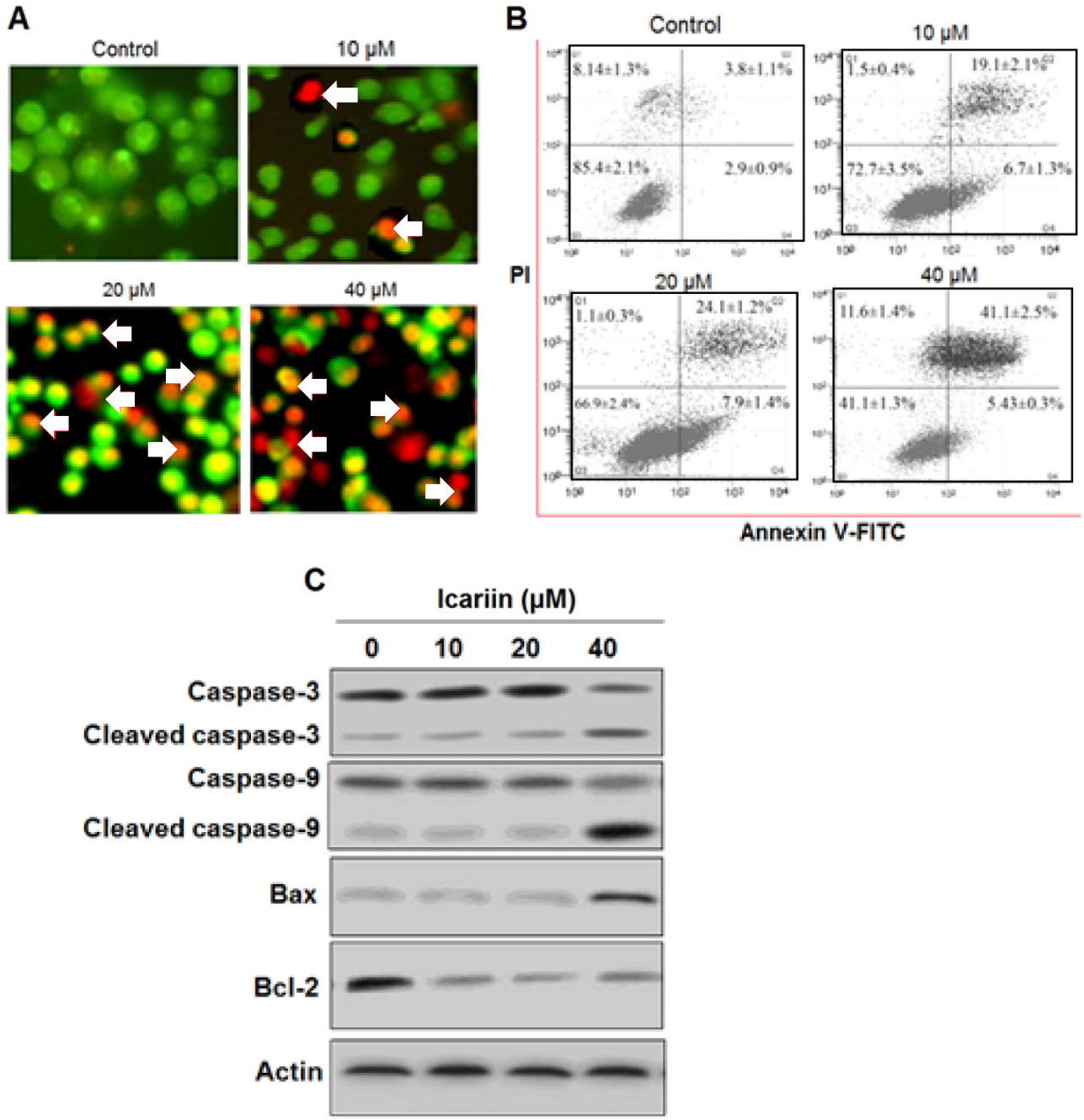

Figure 2. (A): AO/EB staining of the Icariin-treated HeLa cells showing that Icariin induces apoptosis in HeLa cells concentration-dependently (arrows depict apoptotic cells). **(B):** Annexin V/PI staining showing that the percentage of apoptotic cells increases with increase in the concentration of Icariin. **(C):** Effect of Icariin on the expression of apoptosis-related proteins showing that Icariin activates the cleavage of caspase-3 and 9, decreases the expression of Bcl-2 and increases the expression of Bax. The experiments were performed in triplicate.

Icariin inhibits the PI3K/AKT signalling pathway

Next, we sought to know the effects of Icariin on the PI3K/AKT signalling pathway of HeLa cells. What was revealed was that Icariin caused concentration-dependent decline in the phosphorylation of m-TOR, PI3K and AKT, while no apparent effect was observed on the expression of total PI3K and AKT (Figure 5).

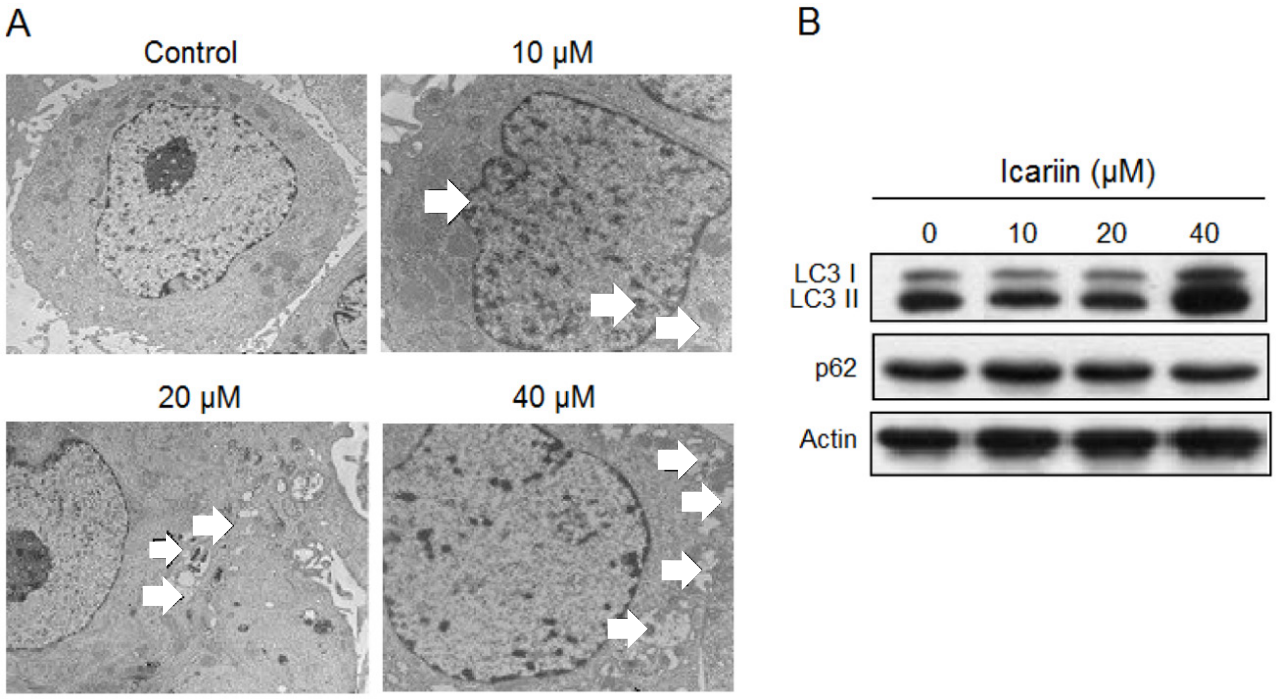

Figure 3. (A): Electron microscopic analysis of Icariin-treated HeLa cells showing induction of autophagy (arrows depict autophagic vesicles). **(B):** Western blot showing the effect of Icariin on the expression of the autophagy-related proteins at indicated concentrations, proving that Icariin increases LC3 II and p62 expression concentration-dependently. The experiments were performed in triplicate.

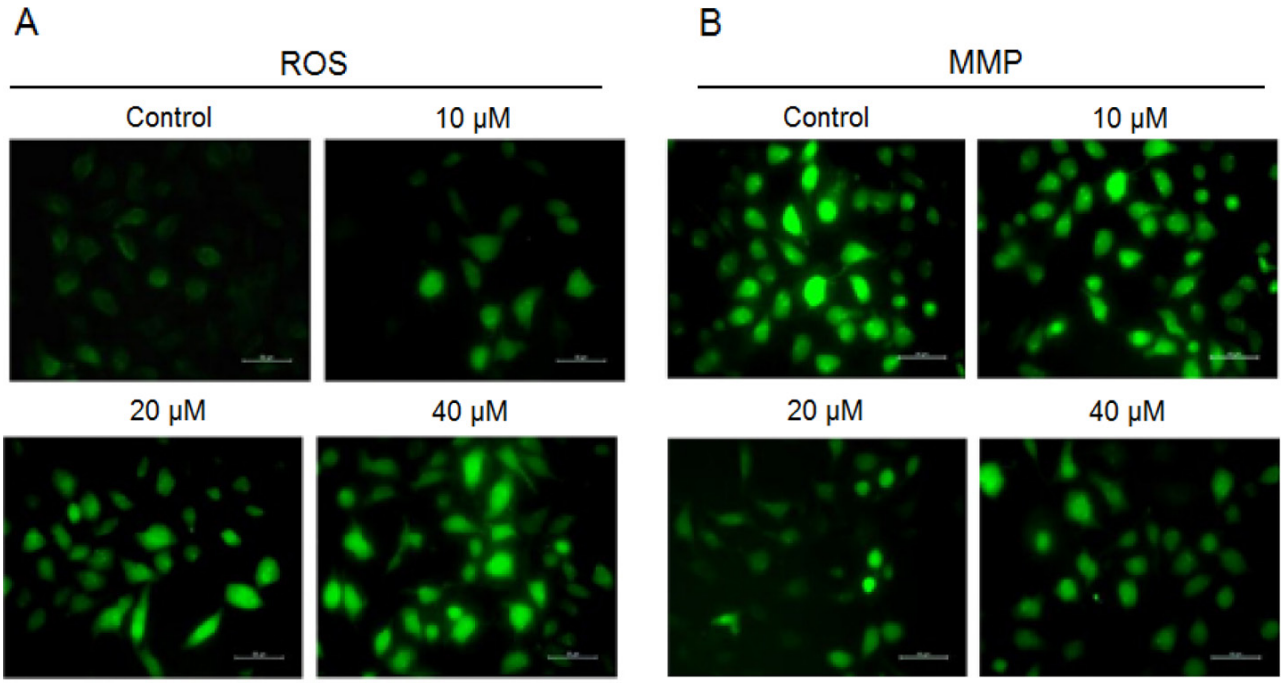

Figure 4. Effect of Icariin at indicated concentrations on **(A)** ROS and **(B)** MMP levels of HeLa cells as determined by fluorescence microscopy. The figure depicts that ROS levels increase and the MMP levels decrease in HeLa cells upon treatment with Icariin. The experiments were performed in triplicate.

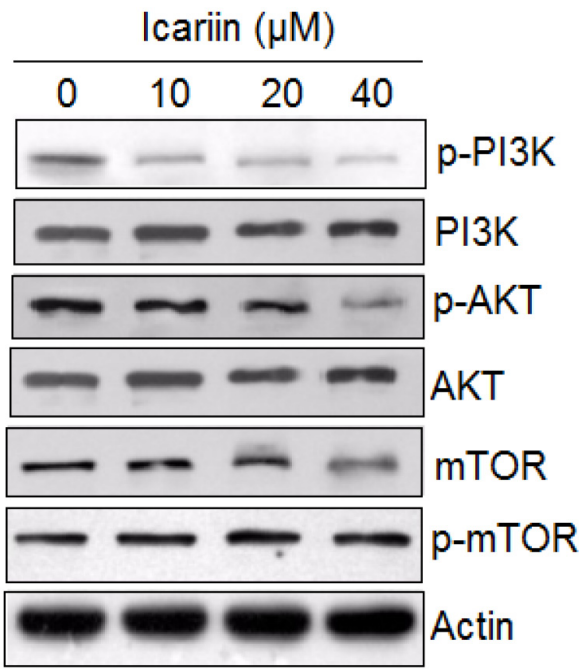

Figure 5. Western blot showing the effect of Icariin on the PI3K/AKT/mTOR signalling pathway at indicated concentrations. The western blot shows that Icariin blocks the phosphorylation of PI3K, AKT and mTOR in a concentration-dependent manner. The experiments were performed in triplicate.

Discussion

Cervical cancer is considered as the second most common type of cancer in women across the world [19]. Since the clinical outcomes are far from satisfactory and the existing drugs exhibit a number of side effects, the identification of novel anticancer molecules and subsequent development of efficient and safer treatment regimens for clinical cancer therapy are required [15]. Herein, we examined the anticancer potential of Icariin. It was found that this molecule exerted growth inhibitory effects on HeLa cervical cancer cell line. Nonetheless, the cytotoxic effects of Icariin were comparatively negligible on the normal HCvEpC non-cancer cells, indicative of the cancer cell specific activity of Icariin. The antiproliferative effects of Icariin were also confirmed by colony formation assay. Previous studies have also shown that Icariin inhibits the growth of breast and gastric cancer cells [8,9]. In order to understand the mechanism for the anticancer activity of Icariin, we performed the AO/EB staining which clearly showed membrane blebbing and induction of apoptotic cell death. The annexin V/PI staining showed that Icariin increased the apoptotic HeLa cells concentration-dependently. The apoptotic cell death of HeLa cells was confirmed by examining the expression of marker proteins of apoptosis. It was found that Icariin treatment prompted increase in the expression of Bax and cleavage of caspases 3 and 9. Moreover, the expression of Bcl-2 was considerably downregulated. These results are also supported by previous investigations wherein Icariin has been shown to induce apoptosis in human ovarian and esophageal cancer cells [10,20]. Apoptosis causes elimination of defective, harmful or cancer cells from the body and helps in the maintenance of tissue homeostasis. It also prevents the development of chemoresistance in cancer cells [21]. Icariin was found to induce autophagy in the HeLa cells which was accompanied with upregulation of LC3 II and downregulation of p62. Several of the flavonoids have been shown to induce autophagy in cancer cells, for example Silibinin, a natural flavonoid, induces autophagy in breast cancer cells via generation of substantial amounts of ROS [22]. We also examined the ROS and MMP levels in the HeLa cancer cells and found that Icariin increases ROS production which is often accompanied by reduction in the MMP levels. mTOR/PI3K/AKT signal pathway is considered an important pathway that regulates the proliferation and tumorigenesis of several types of cancers [16]. In this study we found that Icariin blocks this pathway by inhibiting the phosphorylation of mTOR, PI3K and AKT proteins.

Conclusions

Taken together, it is concluded that Icariin is an important molecule with anticancer potential. Icariin inhibits the growth of cervical cancer cells by inducing apoptosis and autophagy. Hence, it may prove beneficial in the treatment of cervical cancer and therefore warrants *in vivo* evaluation.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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