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Original Research Article

Myricetin exerts potent anticancer effects on human skin tumor cells

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Abstract

Purpose: *To evaluate the anticancer activity of myricetin against skin cancer A431 cell lines.*
Methods: *Cell viability was determined by MTT and colony formation assays. Apoptosis was determined by DAPI and annexin V/PI staining. Cell cycle, ROS and MMP analysis were performed by flow cytometry. Cell migration and invasion were assessed by Boyden Chamber assay, while protein expression was determined using western blotting.*
Results: *Myricetin showed considerable anticancer activity against skin A431 cancer cell lines. However, lower cytotoxic effects were observed in normal FR2 cells. The anticancer activity of myricetin was due to ROS-prompted alterations in mitochondrial membrane potential and initiation of apoptotic cell death. The expressions of Bcl-2 and Bax were altered in response to myricetin treatment. Myricetin also induced cell cycle arrest and suppressed the migration and invasion of A431 cells.*
Conclusion: *These results suggest that myricetin may be an important lead molecule for the development of a suitable treatment of skin cancer.*

Keywords: *Skin carcinoma, ROS, Apoptosis, Myricetin, Cell migration*

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INTRODUCTION

Cancer is one of the most deadly diseases responsible for significant mortality and morbidity [1]. Skin cancer being one of the prevalent cancers causes of mortality across the world [1]. In the USA, it is estimated that one in every five Americans is a victim of skin cancer [2]. With the increasing frequency of skin cancer, new treatment approaches need to be developed. Although skin cancer is treated by chemotherapy or surgical interventions, the tumors show frequent relapse [3]. Besides, the adverse effects of synthetic drugs significantly affect the health of the patients.

Flavonoids constitute one the common types of metabolites in the plant kingdom. They form a vital part of almost all edible plant parts which include, but are not limited to fruits and vegetables. It has been shown that humans consume flavonoids in milligrams every day [4]. Flavonoids present in a number of medicinally important plants and herb base medicine have been well-utilized in conventional systems of medicine [5]. With recent developments in the

field of medicinal plant research, flavonoids are being screened for a wide array of bioactivities. Some of their pharmacological properties include anti-inflammatory, antibacteral, antifungal, anticancer, and antitumor activities; and inhibition of enzymes [4,6].

Several epidemiological investigations on high intake of dietary flavonoids have shown that they are associated with a reduced risk of cancer development [7]. Since flavonoids are prevalent in eatable plants and beverages, they are expected to exhibit negligible toxicity [8].

In this investigation, the anticancer effects of myricetin were evaluated on A431 skin cancer cell line.

EXPERIMENTAL

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich Co. The antibodies were procured from Santa Cruz Biotechnology Inc. The fluorescent probes 4′-6-diamidino-2-phenylindole (DAPI), propidiumiodide (PI), fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics were obtained from Invitrogen Life Technologies.

Cell line and culture conditions

The cell lines skin carcinoma (A431) and normal fibroblasts (FR-2) were procured from Cancer Research Institute of Beijing, China, and were cultured continuously in RPMI-1640 supplemented with 10 % FBS containing antibiotics streptomycin (100 µg/ml))and penicillin G (100 U/ml), and incubated at 37 ˚C in an atmosphere containing 5 % CO_2.

Anti-proliferative assay

The impact of myricetin on the proliferation rate of A431 cancer cells was investigated using MTT assay. The cells were cultured to $1x10^6$ cells per well in 96well plates for 12 h and then administrated with 0 - 100 µM myricetin for 24 h. Thereafter, addition of MTT solution (20 µl) was done. Before the addition of 500µl of DMSO for solubilizing the formazan crystals, dimethylsulfoxide (DMSO, 500 µL) was added. Absorbance was thereafter read in an ELISA plate reader.

Clonogenic assay

To assess the impact of myricetin on the colony development capacity of A431cell lines, the cells were collected at exponential phase of growth and counted using a hemocytometer. The platting of the cells was carried out at 200 cells in each well. The plates were then kept for 48 h at 37 ˚C to allow adhesion. This was followed by the addition of various concentrations (0, 10, 120 and 40 µM) of myricetin. The plates were again subjected to incubation for 6 days, after which they were subjected to washing with FBS and fixed with methanol. The A431 cells were then stained with crystal violet, followed by microscopy, and counted under light microscope.

Apoptosis assay

A431 cells were grown in 6-well platesat1 × 10^6cells per well and then treated with 0, 10, 20 and 40 µM myricetin for a period of 24 h. This was immediately followed by DAPI staining. The cells were examined and the photomicrographs taken with the help of a fluorescence microscope. Thereafter, the cells were harvested, washed with FBS, and then treated with Annexin V/FITC and Propidium iodide for 20 min. The percentage of apoptotic cells was checked by flow cytometry.

Cell cycle analysis

Approximately 1 x 10^5 cells in each well in 6-well plates were kept at 37 °C for 24 h to allow the cells to adhere and subsequently treated with various doses of myricetin. The cells were then subjected to incubation at 37 ˚C. The distribution of the A431 cells in various cell cycle phases was determined by flow cytometry.

Determination of ROS and MMP

A431cells were grown in six-well plates with a density of 2 × 10^5 cells per well followed by incubation at 37 ˚C overnight. The cells were then subjected to treatment with 0, 10, 20 and 40 µM myricetin for 24 h at 37 °C. The cells were then harvested and subjected to FBS washing. They were then re-suspended in 500 µl of DCFH-DA (10 µM) for determination of ROS, and $DiOC_6$ (1 µmol/L) for assessment of MMP levels at 37 °C in the dark, for 30 min. The samples were then analyzed using a flow cytometer.

Cell migration and invasion assay

The A431 cells were suspended in 2 % fetal bovine serum (FBS) and placed in an upper chamber with transwells of 8 µm pore size. The medium was subsequently supplemented with 10 % FBS, and the cells were subjected to 24 h incubation at 37 ˚C. The un-migrated A431 cells were removed from the upper side of the

membrane, while migrated cells on the lower surface were subjected to fixation with 100 % methanol followed by Giemsa staining. Cell migration was determined by estimating the number of the migrated cells, using microscopy. The cell invasion assay was performed alongside the migration assay as described above, except that the pores were coated with martigel.

Western blotting analysis

A431 cells were lysed and the concentration of the proteins in each cell lysate was determined by BCA assay. The expressions of the proteins of interest were determined by western blotting as described previously [6].

Statistical analysis

Data are presented as mean ± SD and were statistically analyzed using Students Newman Keul's test or *t*-test. The levels of significance were determined at $p < 0.01$, $p < 0.001$ or $p < 0.0001$.

RESULTS

Anti-proliferative effects of myricetin on A431 cell line

Myricetin displayed significant anti-cancer effects against A431 cell lines with an IC_{50} of 20µM. Nonetheless, its showed lower growth inhibitory activity on normal human FR2 cells (IC_{50} of 85 µM). The effect of myricetin on the growth of A431 cells followed a concentration-dependent pattern (Figure 1A). It was also observed that myricetin treatment caused significant reduction in A431 colonies dose (Figure 1B).

Apoptosis in A431 cells

Myricetin caused apoptotic cell death in A431 cells dose-dependently (Figure 2). The analysis of apoptotic cell population was performed with the help of flow cytometry (Figure3). The apoptotic A431 cells augmented from 1.25 % in control to 46.3 % at 40 µM of myricetin. To assess if the apoptosis was due to the activation of mitochondrial apoptotic pathway, the protein expressions of Bax and Bcl-2 were studied. The results showed that while the expression of Bax was increased in a dose-dependent pattern, Bcl-2 expression decreased (Figure 4).

Activation of ROS and reduction of MMP

It was revealed that the ROS levels of myricetin-administrated cells were increased up to 210 % at 100 µM myricetin (Figure 5A). These observations showed that myricetin may trigger apoptosis through accretion of ROS in A431 cells. Reactive oxygen species generation is related to mitochondrial dysfunction. They disrupt the outer mitochondrial potential leading to the discharge of the apoptosis-triggering proteins [9]. Myricetin-treated A431 cells exhibited a dose-dependent and significant reduction in MMP. The MMP declined up to 85 % at 100 µM of myricetin (Figure 5B).

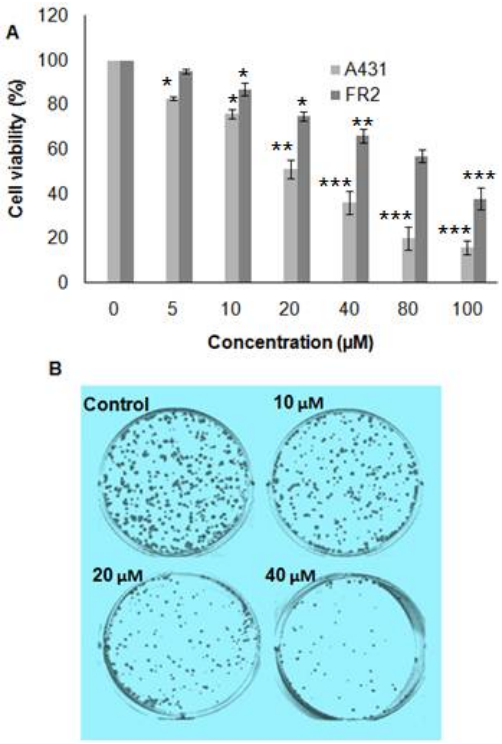

Figure 1: Effect of myricetin on (A) viability of cells, and (B) colony development capacity. Experiments were repeated thrice. (*$p < 0.001$, **$p < 0.001$ or ***$p < 0.0001$)

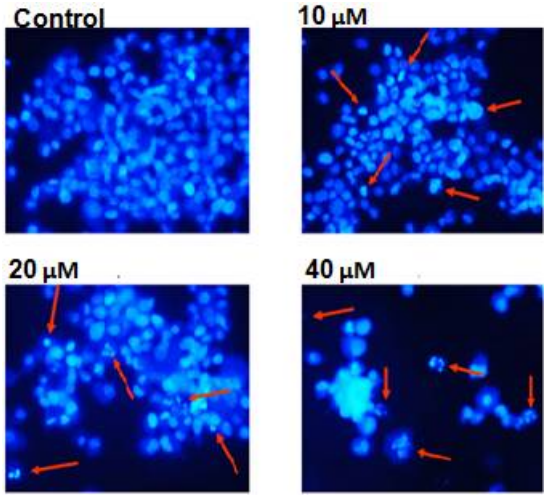

Figure 2: Induction of apoptosis bymyricetin as depicted by DAPI staining

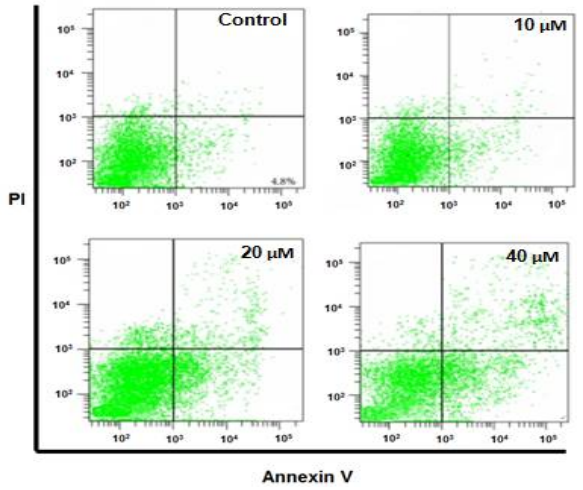

Figure 3: Effect of myricetin on apoptotic cell populations as shown by annexin V/PI staining and flow cytometry

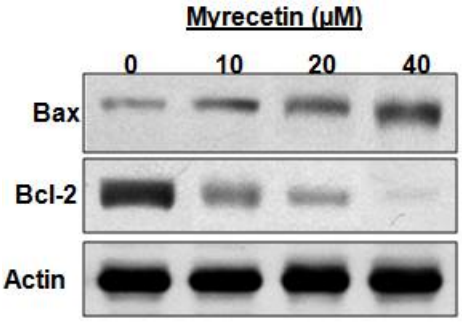

Figure 4: Impact of myricetin on the protein expressions of Bax and Bcl-2 as seen through immuno blotting

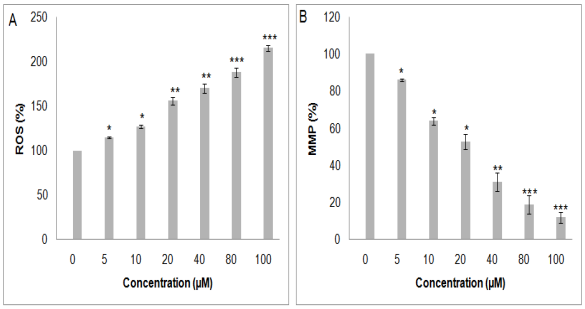

Figure 5: Effect of myricetin on (A) ROS levels (B) MMP levels. Data is shown as mean of three replicates ± SD (*p < 0.001, **p < 0.001 or ***p < 0.0001)

Myricetin triggered cell cycle arrest

It was found that the number of the A431 cells at sub-G1 phase increased in a dose-dependent manner causing cell cycle arrest (Figure 6). At 40 µ M myricetin, there was a marked increase in sub-G1 phase cells.

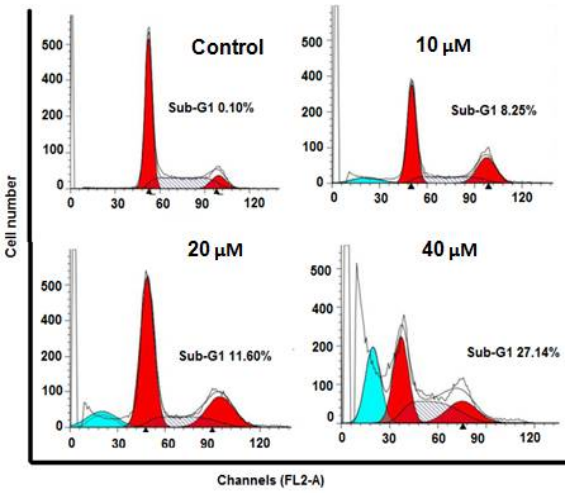

Figure 6: Effect of myricetin on cell cycle arrest at the G2/M phase

Myricetin inhibited cell migration and invasion

Myricetin reduced the motility and migration of the A431 cells in a dose-dependently. It inhibited A431 cells invasion up to 66 % (Figure 8).

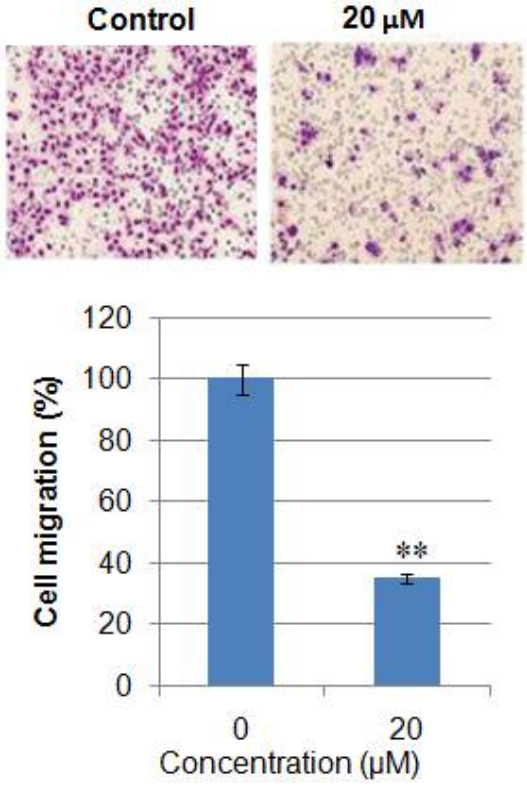

Figure 7: Effect of myricetin on the migration of A431 cells. (**p < 0.001)

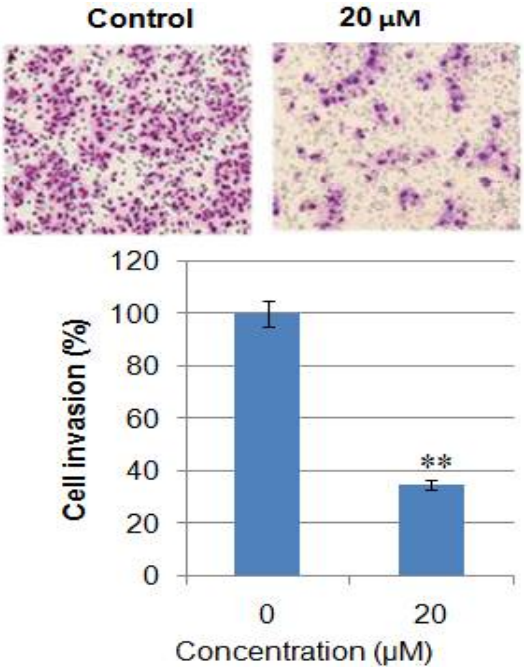

Figure 8: Effect of myricetin on the invasion of A431 cells. (**p< 0.001)

DISCUSSION

Skin cancer is a lethal disease and responsible for considerable deaths in humans, especially among the Caucasians. The mounting prevalence of skin malignancies demands establishment of multiple and novel treatment approaches [1]. So far, a number of secondary metabolites from plants are used as anticancer drugs [9]. Myricetin is a flavonoid frequently reported to have a wide range of medicinal importance [10]. The effects of myricetin on bladder and thyroid tumors have been reported [10].

In this study, the anticancer activity of myricetin against A431 human skin cancer cell lines and normal human fibroblasts FR2 cells were evaluated. The results showed that myricetin exerted potent anti-proliferative effects on A431 cells and decreased their colony-forming capacity as was evident from MTT and clonogenic assays, respectively. Many chemotherapeutic agents like cisplatin, taxol and 5-fluorouracil have been observed to induce explicit apoptotic signaling pathways [10,11]. However, the chemo-resistance of cancer cells to drugs is in part because of the capability of the cells to escape apoptosis [12,13].

Results from this study showed that myricetin induces apoptosis in a dose-dependently. Myricetin-treated cells showed ROS-prompted MMP loss. These results are in agreement with previous reports [14,15], and suggest that myricetin may induce apoptotic damage via increased production of ROS and decline in MMP.

Several anti-cancer drugs exert growth inhibitory effects on cancer cells by generation of ROS [16]. For instance, capsaicin disrupts MMP and causes oxidative stress causing apoptotic cell death in cancerous pancreatic cells [17].

The results of the present study are interesting and show great promise in the treatment of skin cancer. For instance, myricetin significantly inhibited the migration and invasion of skin cancer cells, an indication that it may prove beneficial in halting the metastasis of cancer cells *in vivo.*

CONCLUSION

The results of this study show that myricetin, by inducing apoptosis and cell cycle arrest, may be a potent lead molecule for the treatment of skin cancer. As a flavonoid, myricetin may exhibit low toxicity, and so deserves further investigations *in vivo*.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named earlier and all liabilities pertaining to claims and content of this article will be borne by them. Wei Sun and Youming Tao contribute to this work equally. Daojiang Yu collected materials. Tianlan Zhao gave a help in statistical analysis. Wei Sun, Daojiang Yu, Tianlan Zhao, Lijun Wu, Wenyuan Yu, Wenya Han did this experience under the supervision of Youming Tao.

REFERENCES

1. *de Gruijl FR. Skin cancer and solar UV radiation: Eur J Cancer 1999; 35: 2003-2009.*
2. *Donaldson MR, Coldiron BM. No end in sight: the skin cancer epidemic continues. In Seminars in cutaneous*

medicine and surgery. Frontline Medic Commun 2011; 30: 3-5.

3. *Aitken JF, Elwood M, Baade PD. Clinical whole-body skin examination reduces the incidence of thick melanomas. Int J Cancer 2010; 126: 450-458.*
4. *Hollman PC, Katan MB. Dietary flavonoids: Intake, health effects and bioavailability. Food Chem Toxicol 1999; 37:937–942.*
5. *Nagaoka T, Banskota AH, Tezuka Y, Saiki I, Kadota S. Selective antiproliferative activity of caffeic acid phenethyl ester analogues on highly liver metastatic murine colon 26-L5 carcinoma cell line. Bioorg Med Chem 2002; 10: 3351-3359.*
6. *Takagaki N, Sowa Y, Oki T, Nakanishi R, Yogosawa S, Sakai T. Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. Inter J Oncol 2005; 26:185-190,*
7. *Le Marchand L. Cancer preventive effects of flavonoids—A review. Biomed Pharmacother 2002; 56:296–301.*
8. *Alonso DF, Farias EF, Urtreger A, Ladeda V, Vidal MD CC, Bal De Kier Joffe E. Characterization of F3II, a sarcomatoid mammary carcinoma cell line originated from a clonal subpopulation of a mouse adenocarcinoma. J Sr Oncol 1996; 62: 288-297,*
9. *Azuma M, Tamatani T, Ashida Y, Takashima R, Harada K, Sato M. Cisplatin induces apoptosis in oral squamous carcinoma cells by the mitochondria-mediated but not the NF-kappaB-suppressed pathway. Oral Oncol 2003; 39: 282–289.*
10. *Ha TK, Jung I, Kim ME, Bae SK, Lee JS. Anti-cancer activity of myricetin against human papillary thyroid cancer cells involves mitochondrial dysfunction–mediated apoptosis. Biomed Pharmacother 2017; 91:378-84.*
11. *Ferreira CG, Epping M, Kruyt FA, Giaccone G. Apoptosis. Clin Cancer Res 2002; 8: 2024-34.*
12. *Lopez J, Tait SW. Mitochondrial apoptosis: killing cancer using the enemy within. British journal of cancer. 2015; 112(6):957.*
13. *Ding H, Han C, Guo D, Chin YW, Ding Y, Kinghorn AD, D'Ambrosio SM. Selective induction of apoptosis of human oral cancer cell lines by avocado extracts via a ROS mediated mechanism. Nutr Cancer 2009; 61: 348-356.*
14. *Munagala R, Kausar H, Munjal C, Gupta RC. Withaferin A induces p53-dependent apoptosis by repression of HPV oncogenes and upregulation of tumor suppressor proteins in human cervical cancer cells. Carcinogenesis 2011; 32: 1697-1705.*
15. *Pramanik KC, Boreddy SR, Srivastava SK. Role of mitochondrial electron transport chain complexes in capsaicin mediated oxidative stress leading to apoptosis in pancreatic cancer cells. PloS one 2011; 6: e20151.*