

ORIGINAL ARTICLE

Anticancer effects of kaempferol in A375 human malignant melanoma cells are mediated via induction of apoptosis, cell cycle arrest, inhibition of cell migration and downregulation of m-TOR/PI3K/AKT pathway

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Summary

Purpose: Melanoma is an aggressive form of human cancer with limited treatment options currently available. The present study was aimed to evaluate the anticancer activity of kaempferol (KAM) against the human malignant melanoma A375 cell line along with evaluation of its effects on apoptosis, cell cycle, cell migration and m-TOR/PI3K/AKT pathway.

Methods: Effects on cell viability were assessed by MTT assay while clonogenic assay measured the effects of KAM on colony formation. Annexin V assay evaluated the apoptotic effects of KAM in these cells using flow cytometry. Effects on cell cycle were determined by using flow cytometry with propidium iodide (PI) as probe. The effects of KAM on m-TOR/PI3K/AKT signalling pathway were evaluated by western blot assay.

Results: MTT assay indicated that KAM exhibits a significant anticancer activity against A375 cells with an IC₅₀

of 20 µM. These antiproliferative effects of KAM were also supported by the colony formation assay wherein KAM reduced the colony formation in a dose-dependent manner. The anticancer effect of KAM was found to be due to the initiation of apoptosis in human malignant melanoma A375 cells. Additionally, KAM also exhibited the capacity to trigger G2/M cell cycle arrest and to inhibit the cell migratory potential of A375 cells. KAM caused significant downregulation of m-TOR, phosphorylated (p) m-TOR, PI3K, p-PI3K and Akt protein levels in A375 malignant melanoma cells.

Conclusions: KAM exerts potent anticancer effects via induction of apoptosis, G2/M cell cycle arrest, cell migration inhibition and downregulation of m-TOR, pm-TOR, PI3K, p-PI3K and Akt protein levels.

Key words: apoptosis, cell cycle arrest, kaempferol, malignant melanoma, m-TOR

Introduction

Malignant melanoma is an aggressive form of human cancer originating from the malignant conversion of the pigmented cells of skin [1]. Despite the advances in modern biological sciences, the chemotherapeutic treatment options for melanoma remain a failure owing to the dismal insensitivity of melanoma cells to existing anticancer drugs [1]. However, it is believed that molecules

of natural origin may prove handy in the management of malignant melanoma. Among the natural products, flavonoids form a large group of compounds ubiquitously found across that plant kingdom [2]. These molecules have been reported to possess tremendous pharmacological properties which include antimicrobial, antioxidant and anticancer activities [3]. The bioactivities of flavonoids

are attributed to their capacity to interact with a diversity of cellular enzymes. Moreover, flavonoids act as scavengers of reactive oxygen species (ROS), and also avert their formation by chelating metals [4]. The use of flavonoids as prospective chemopreventive agents has gained interest in the recent past. One such molecule is the flavonol KAM (Figure 1) which has been shown to exhibit considerable anticancer activity against human cancers [5]. Additionally, KAM is present in several edible plants such as piper [6] and other plant sources and is therefore likely to exhibit minimal toxicity in humans. Against this background, this study was designed to examine the anticancer activity of KAM against human A375 cancer cells and to explore the possible underlying mechanism. Our results indicated that KAM exhibits significant anticancer activity against malignant melanoma A375 cells via initiation of apoptosis, G2/M cell cycle arrest, suppression of cell migration and modulation of mTOR/PI3K/AKT signaling pathway. We therefore strongly believe that KAM may prove beneficial in the treatment and management of malignant melanoma.

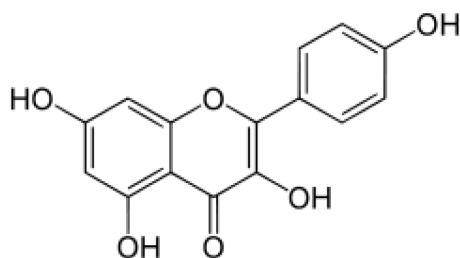


Figure 1. Chemical structure of Kaempferol.

Methods

Chemicals, reagents and cell culture

KAM, PI, RNase A triton X-100 dimethyl and sulfoxide (DMSO), were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Human malignant melanoma A375 cancer cell line was procured from Cancer Research Institute of Beijing, China, and it was maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in an incubator at 37°C (5% CO₂ and 95% air).

Antiproliferative activity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] assay [7] was used to determine the antiproliferative activity of KAM against the A375 human malignant melanoma cell line. The A375 cells

in 100 µL culture medium were seed in a 96-well plate at a density of 3×10³ cells/mL and kept at 37°C in 5% CO₂ for 24 hrs. After 24 hrs, an extra 100 µL of complete medium with different concentrations (10-80 µM) of KAM was added. Thereafter, the cells were kept for 72 hrs. This was followed by the addition of 20 µL of MTT solution (5mg/mL) and incubation for 4 hrs. Afterwards, the medium was discarded and 150 µL DMSO were added. The absorbance was determined at 490 nm using a Tunable Mi-185 croplate Reader (EL-x800, BioTek Instruments, USA).

Colony formation assay

For colony formation assay A375 cells were seeded in a 12-well culture plate with approximately 500 cells per well and permitted to adhere for 12 hrs. Then, they were administered 0, 10, 20 and 40 µM of KAM. This was followed by an incubation period of one week. The cells were then washed with PBS and finally fixed with methanol for 30 min. Thereafter, the cells were stained with crystal violet (0.5%) solution.

Analysis of apoptotic cells by flow cytometry

After exposing the A375 cells to 0, 10, 20, 40 and 80 µM of KAM for 24 hrs, the apoptotic cell death was estimated by using an FITC-Annexin V/PI Apoptosis Detection Assay according to the manufacturer's instructions (Beijing Biosea Biotechnology, China).

Cell cycle analysis

For cell cycle analysis the A375 cells were administered 0, 10, 20, 40 and 80 µM of KAM and the percentage of cells in each phase of the cell cycle was estimated by using Muse™ Cell Analyzer and Muse™ Cell Cycle Kit according to the manufacturer's protocol (Merck Millipore).

Wound healing assay

A375 cells were seeded in 96-well plates at a 5×10⁴ cell density and administered with IC₅₀ of KAM and then kept overnight to attach. As the cells reached 98% confluence, a wound was scratched across each well by wound Maker device. Afterwards, the cells were treated with PBS to remove the detached cells. The cells were monitored after 20 hrs and photographed.

Western blotting

The protein lysates of each cell sample (untreated and KAM-treated) were prepared as described previously [8]. The protein concentrations of the lysates were quantified by bicinchoninic acid assay using specific antibodies. β-actin was used as control. From each sample similar amounts of protein were loaded and resolved by electrophoresis on a 12% denaturing sodium dodecyl sulfate (SDS) gel. Afterwards, the proteins were electroblotted on polyvinylidene difluoride membranes (0.45 µm pore size).

Statistics

All experiments were carried out in triplicate and the data was presented as mean±SD. The data was

analysed by Tukey's test and one-way ANOVA using GraphPad prism 7 software. A p value <0.05 was considered as statistically significant.

Results

Antiproliferative effect of KAM on A375 cells

In the present study KAM was evaluated for its antiproliferative activity against A375 melanoma cells at 0, 10, 20, 40 and 80 μM concentrations. The results indicated that KAM exerted its antiproliferative activity in a dose-dependent manner and the antiproliferative activity increased with increasing concentration of KAM (Figure 2). The IC_{50} value was found to be 20 μM at the incubation period of 48 hrs.

KAM inhibited the cell migration of A375 cells

Further, we examined if KAM could inhibit the migration of human A375 cells at the IC_{50} concentration after 48 hrs of incubation by wound healing assay. The results of wound healing assay showed that KAM reduced the migratory capability of A375 cells as evidenced from the wound healing assay cells. While in the control the cells showed fairly good capacity to migrate, in the treatment group the cells showed reduced potential to migration as depicted in Figure 3.

KAM reduced the colony formation potential of malignant melanoma A375 cells

We also determined the impact of KAM on the colony formation potential of A375 cancer cells at the concentrations of 0, 10, 20 and 40 μM . The results revealed that KAM decreased the colony formation potential of human malignant melanoma

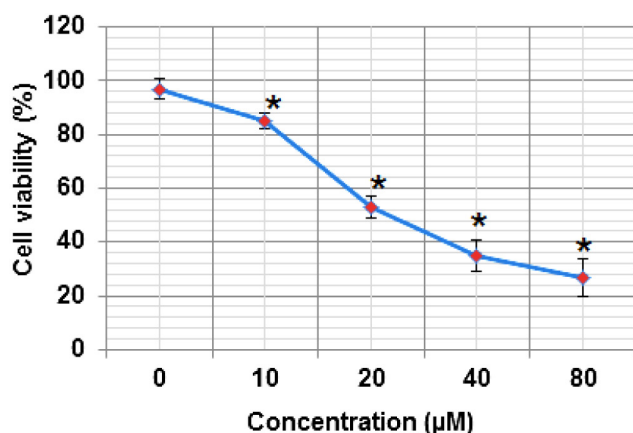


Figure 2. Assessment of cell viability at indicated doses of KAM. With increasing KAM dose the cell viability decreased significantly indicating KAM caused cancer cell death. The results are the mean \pm SD of three biological experiments and values were considered significant at * p <0.05.

A375 cells in a concentration-dependent manner (Figure 4) further confirming the antiproliferative activity of KAM.

KAM induced apoptosis in human A375 cancer cells

To investigate whether KAM exerts its antiproliferative activity on malignant melanoma A375 cells via induction of apoptosis, the cells were administered varied doses of KAM and subjected to FITC-Annexin V/PI staining and examined by a flow cytometer (Figure 5). The results revealed that KAM could induce apoptosis in

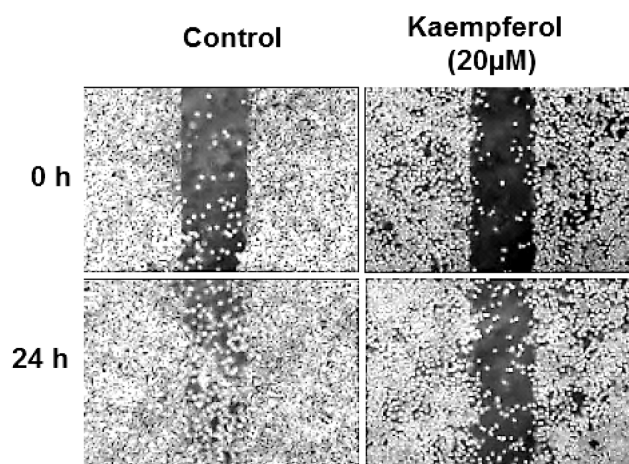


Figure 3. Effect of KAM at IC_{50} concentration of cell migration of A375 cells as determined by cell migration assay. The images are representatives of three biological experiments. KAM caused cancer cell inhibition of migration at 20 μM , indicating its potential as an anticancer agent.

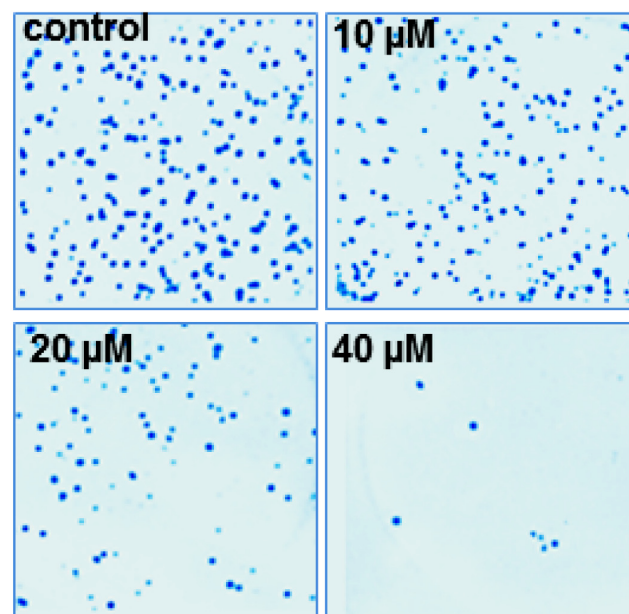


Figure 4. Clonogenic potential of A375 cells at indicated doses of KAM. The results are representatives of three biological experiments. Increasing KAM doses caused significant decrease in cancer cell colonies with only few of them detectable at 40 μM dose.

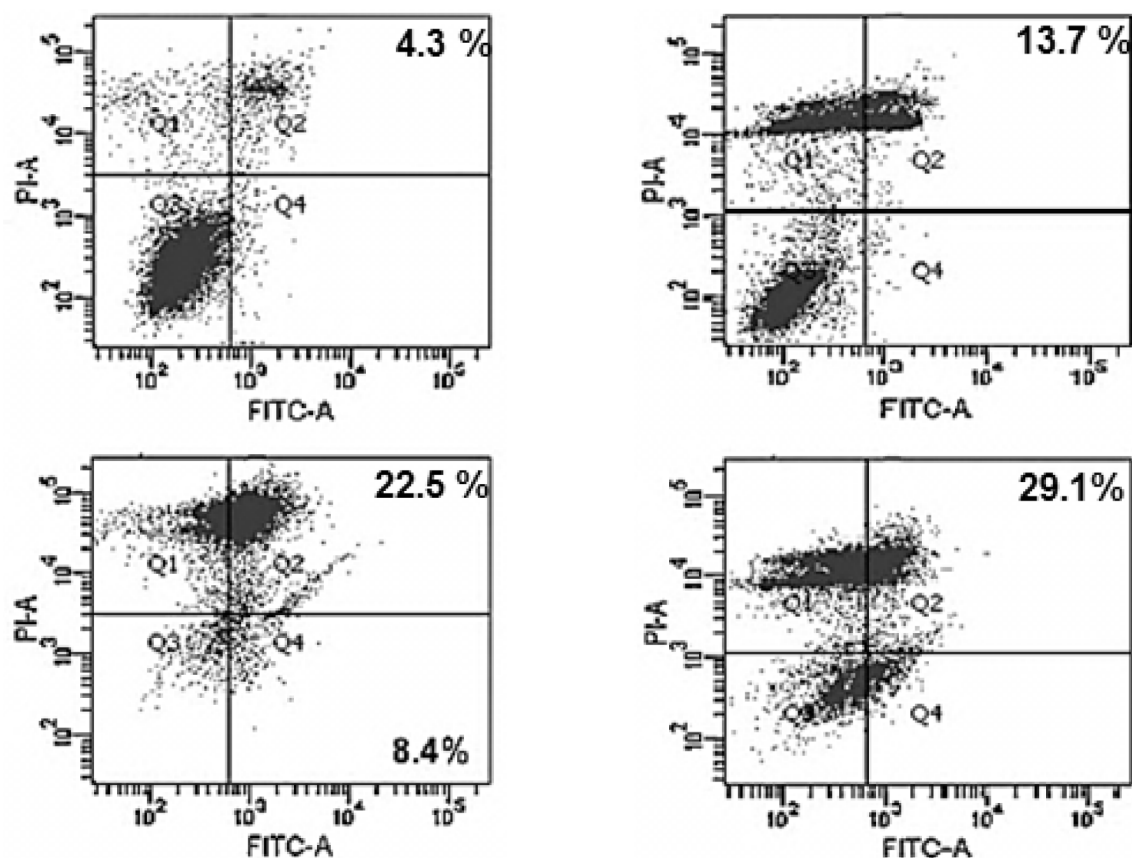


Figure 5. Estimation of apoptotic A375 populations at indicated doses by annexin V/PI using flow cytometric analysis. The images are representatives of three biological experiments. The Figure indicates that the fraction of apoptotic cells (both early and late apoptosis) increased considerably with the increasing concentrations of KAM.

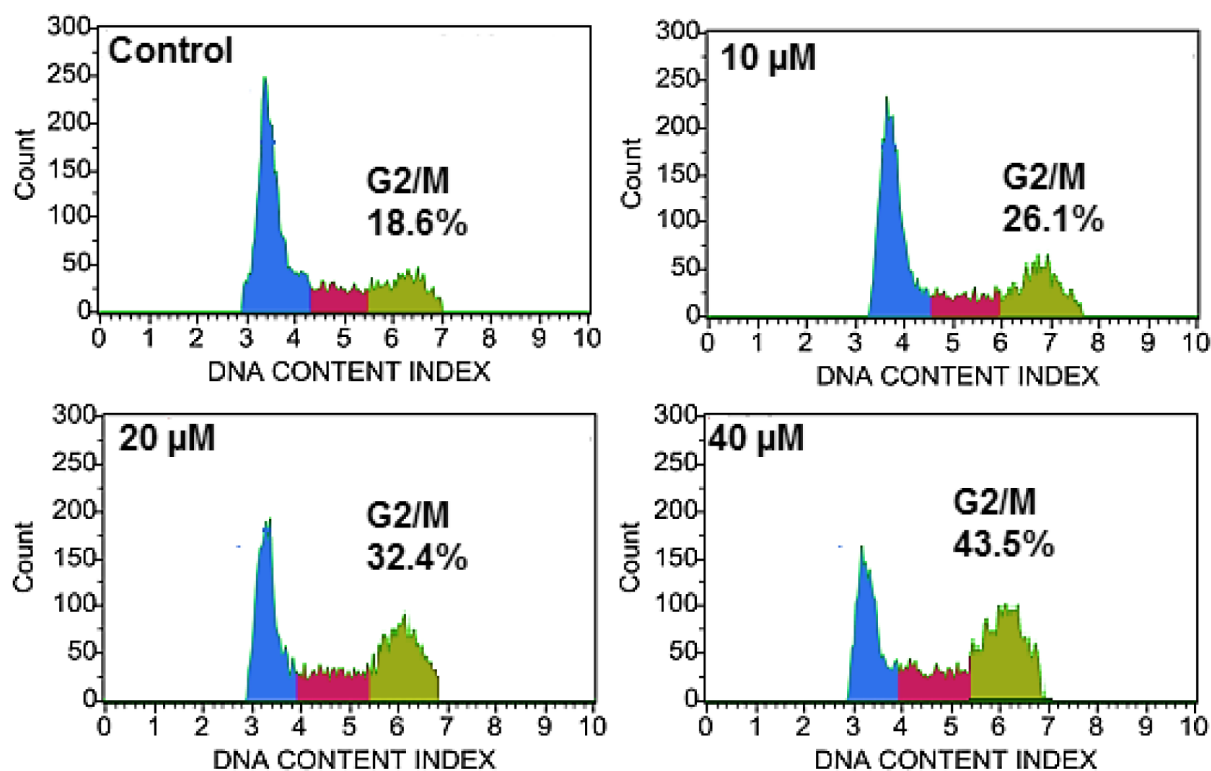


Figure 6. Cycle distribution of A375 cells at indicated doses of KAM. The results are representatives of three biological experiments. The Figure clearly indicates that with increasing KAM concentrations the fraction of cells with G2/M cell cycle arrest also increased, indicating that KAM leads to G2/M cell cycle arrest in A375 cells.

A375 cells dose-dependently. The percentage of apoptotic populations were 13.7, 22.5 and 29.1% in A375 cells as compared to the untreated control with just 4.3% of apoptotic cells.

KAM caused G2/M cell cycle arrest in A375 cancer cells

Apart from apoptosis, cell cycle arrest is another mechanism by which anticancer agents cause cell death. To assess whether KAM causes cell cycle arrest, A375 cells were administered varied doses of KAM. We observed that KAM caused increase in the G2 cell populations and also caused G2/M cell cycle arrest in a concentration-dependent manner (Figure 6). The apoptotic cell populations increased to 26.1, 32.4 and 43.5% at concentrations of 10, 20 and 40 μ M as compared to 18.6% in the untreated control.

KAM inhibited mTOR/PI3/AKT signalling pathway

The mTOR/PI3/AKT signalling cascade is an important pathway that is activated and is respon-

sible for the transformation of normal cells to cancer cells. Therefore, this pathway is considered as an important target for cancer drug development. In the current study we evaluated the effect of KAM at different concentrations on the protein expression of several key proteins of mTOR/PI3/AKT signalling pathway by western blotting. The results showed that in comparison with the untreated cells, KAM-treated cells showed a concentration-dependent downregulation of m-TOR and pm-TOR proteins (Figure 7). KAM also showed downregulation of PI3K/Akt protein expressions. Thus it may be concluded that KAM induced anticancer activity via m-TOR/PI3K/Akt signalling pathway.

Discussion

Melanoma is a ferocious form of human cancer with limited treatment options available currently [1]. The present study was therefore directed to evaluate the anticancer effect of KAM against the human malignant melanoma A375 cell line. KAM showed potential antiproliferative activity against A375 cells as evidenced from the MTT assay. The antiproliferative effects were found to be dose-dependent and the IC_{50} of KAM against A375 cells was found to be 20 μ M. The antiproliferative activity of KAM was further supported by the results of colony formation assay wherein KAM reduced the colony formation potential of A375 cells dose-dependently.

It has been reported that many anticancer drugs, such as 5-fluorouracil, exert their anticancer effects by inducing apoptosis in the cancer cells [9]. Our results also revealed that KAM prompted apoptosis in A375 cells through induction of apoptosis as clearly evidenced from the flow cytometric analysis.

Apart from apoptosis, cell cycle arrest is considered as another important mechanism by which several anticancer agents cause growth inhibition of cancer cells [10]. Flow cytometry using propidium iodide as a probe was used to study the effects of KAM on cell cycle progression and it was observed that KAM induced G2/M cell cycle arrest and led to a significant rise in G2 phase of A375 cells. These findings are promising since it is well recognized that malignant melanoma is one of the lethal cancers and KAM exhibits the capacity to halt its growth.

Moreover, anticancer drugs that cause cell cycle arrest have been reported to be clinically more effective and efficient [10]. In the current investigation we also determined the effect of KAM on the migratory potential of A375 malignant melanoma cells. Interestingly, KAM considerably reduced the

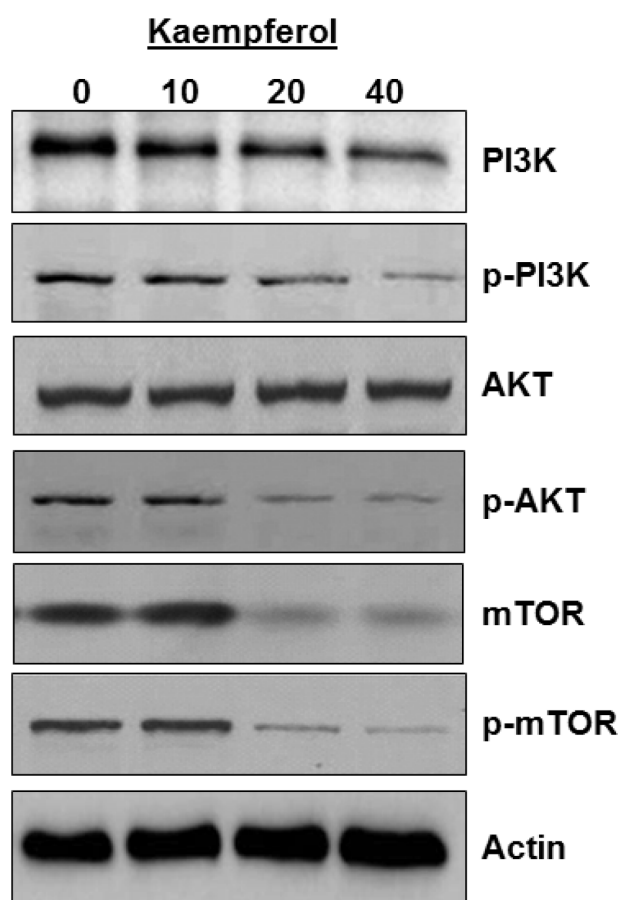


Figure 7. Effect of indicated doses of KAM on the protein expression of key proteins of mTOR/PI3/AKT signalling pathway. The images are representatives of three biological experiments. KAM treatment led to concentration-dependent downregulation of mTOR and PI3K/AKT protein expressions.

migratory potential of A375 cells at IC_{50} concentration. It is well established that cell migration is the vital characteristic of cancer progression and metastasis and inhibition of cell migration may prove handy in the suppression of metastasis *in vivo* [11]. Lastly, we attempted to determine the impact of KAM on the protein expression levels of some key proteins of mTOR/PI3K/AKT pathway. The results revealed that KAM causes significant downregulation of m-TOR, pm-TOR, PI3K, p-PI3K and Akt protein levels in A375 human malignant melanoma cells. Earlier studies have reported that mTOR/PI3K/AKT pathway is an important pathway that is activated in different cancer cells and contributes to tumorigenesis and cancer progression and therefore is considered as an important target for anticancer drug development [12-15].

In conclusion, we propose that KAM exhibits significant anticancer effects on A375 cells via induction of apoptosis, inhibition of cell migration, cell cycle arrest and more importantly by targeting mTOR/PI3K/AKT/ pathway. Therefore, KAM may prove a key molecule in the treatment and management of malignant melanoma.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests

References

- Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* 2006;203:1651-6.
- Marder M, Viola H, Bacigaluppo JA et al. Detection of benzodiazepine receptor ligands in small libraries of flavone derivatives synthesized by solution phase combinatorial chemistry. *Biochem Biophys Res Comm* 1998;249:481-5.
- 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;10:3351-9.
- 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. *Int J Oncol* 2005;26:185-90.
- Kim SH, Choi KC. Anti-cancer effect and underlying mechanism (s) of kaempferol, a phytoestrogen, on the regulation of apoptosis in diverse cancer cell models. *Toxicol Res* 2013;29:229-34.
- Patanasethanont D, Nagai J, Yumoto R, Murakami, T, Sutthanut K. Effects of Kaempferiaparviflora extracts and their flavone constituents on P-glycoprotein function. *J Pharm Sci* 2007;96:223-33.
- Ma L, Diao A. Marizomib, a potent second generation proteasome inhibitor from natural origin. *Anticancer Agents Med Chem* 2015;15:298-306.
- Hernandez-Vargas H, Ballestar E, Carmona-Saez P et al. Transcriptional profiling of MCF7 breast cancer cells in response to 5-Fluorouracil: relationship with cell cycle changes and apoptosis, and identification of novel targets of p53. *Int J Cancer* 2006;119:1164-75.
- Yoneda K, Yamamoto T, Osaki T. p53- and p21-independent apoptosis of squamous cell carcinoma cells induced by 5-fluorouracil and radiation. *Oral Oncol* 1998;34:529-37.
- Yin XM. Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. *Cell Res* 2000;10:161-7.
- Sun SY, Hail N Jr, Lotan R. Apoptosis as a novel target for cancer chemoprevention. *J Natl Cancer Inst* 2004;96:662-72.
- Huang J, Zhang L, Greshock J et al. Frequent genetic abnormalities of the PI3K/AKT pathway in primary ovarian cancer predict patient outcome. *Genes Chromosomes Cancer* 2011;50:606-18.
- Tetiana A, Krupodorova IA, Shmarakov VY et al. Anti-cancer potential of *Trametes versicolor* (L.) Lloyd and *Auriporiaaurea* (Peck) Ryvarden mycelia in rat *Guerins* carcinoma. *Adv Biomed Pharma* 2016;3: 1-8.
- Aadil K, Manzoor AR, Rafiya R. Plant-based natural compounds and herbal extracts as promising apoptotic agents: their implications for cancer prevention and treatment. *Adv Biomed Pharma* 2016;3:245-69.
- Wani BA, Ramamoorthy D, Manzoor AR et al. Induction of apoptosis in human pancreatic MiaPaCa-2 cells through the loss of mitochondrial membrane potential ($\Delta\Psi_m$) by *Gentiana kurroo* root extract and LC-ESI-MS analysis of its principal constituents. *Phytomedicine* 2013;20:723-33.