JBUON 2019; 24(3): 975-981
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Kaempferol exerts anti-proliferative effects on human ovarian cancer cells by inducing apoptosis, G0/G1 cell cycle arrest and modulation of MEK/ERK and STAT3 pathways

Shuli Yang[1], Lihui Si[1], Yan Jia[1], Wenwen Jian[1], Qing Yu[1], Min Wang[1], Ruixin Lin[2]

[1]Department of Obstetrics and Gynecology, the Second Hospital of Jilin University, Changchun, Jilin 130000, China; [2]Department of General Surgery, the Second Hospital of Jilin University, Changchun, Jilin 130000, China.

Summary

Purpose: *Ovarian cancer causes significant mortality in women and is one of the most prevalent types of gynaecological cancer world over. Ovarian cancer is often diagnosed at advanced stages and the currently used anticancer drugs produce several adverse effects. Herein, we examined the anticancer effects of a natural flavonoid Kaempferol against a panel of ovarian cancer cells.*

Methods: *WST-1 and colony formations assays were used to examine the anti-proliferative effects of Kaempferol. AO/EB, DAPI and annexin V/PI staining assays were used to check apoptosis. Cell cycle analysis was performed by flow cytometry and western blotting was used to check the expression of the proteins.*

Results: *The results showed that Kaempferol could inhibit the growth of ovarian cancer cells with IC_{50} ranging between 25 to 50 μM. However, the cytotoxic effects of Kaempferol were comparatively negligible against the normal SV40 cells with an IC_{50} of >120 μM. Exploration of the mechanism of action revealed that Kaempferol exerts growth inhibitory effects on the OVACAR-3 ovarian cancer cells by apoptotic cell death. This was also accompanied with upregulation of apoptotic proteins such as caspase 3, 8 and 9 and Bax. Kaempferol also induced arrest of the OVACAR-3 cells at the G2/M check point of the cell cycle. In addition, Kaempferol could also inhibit the MEK/ERK and STAT3 signal transduction pathways.*

Conclusion: *Taken together, these results suggest that Kaempferol exerts potent anticancer effects on ovarian cancer cells and may prove useful in the management of ovarian cancer.*

Key words: *Kaempferol, ovarian cancer, autophagy, apoptosis*

Introduction

Ovarian cancer is the leading cause of gynaecological cancer-related mortality [1]. The majority of ovarian cancers are sporadic and only up to 10% of ovarian cases are familial [2]. The 5-year overall survival rate for localised ovarian cancer is 93% but the survival rate is as low as 3% for metastatic ovarian cancers [3]. Owing to its diagnosis at advanced stages, this cancer is difficult to manage. Besides, adverse affects connected with the currently available drugs negatively affect the patient quality of life [4]. Nature has bestowed mankind with an inexhaustible array of chemical scaffololds. These amazing chemical entities stand as an exceptional source of drugs for the management of human diseases [5]. Although usage of herbal extracts dates back to centuries, the use of pure isolated compounds started only in the 19th century [6]. Since then, a wide array of molecules have been isolated, evaluated and used for the treatment of several diseases and disorders [7].

Corresponding author: Ruixin Lin, MD. Department of General Surgery, the Second Hospital of Jilin University, no.218 Ziqiang street, Changchun, Jilin 130000, China.
Tel & Fax: +86 0431 81136555, Email: RLakeshiattebe@yahoo.com
Received: 12/09/2018; Accepted: 03/11/2018

Among plant metabolites, the ubiquitous flavonoids have shown promising potential for drug development. Flavonoids are common components of human diet and it is believed that diet rich in flavonoids lowers the risk of cancer development [8]. Kaempferol is an important flavone that is believed to possess immense pharmacological potential [9]. It is commonly isolated from plants and has been reported to halt the growth of several types of cancer cells [10]. For example, Kaempferol has been shown to inhibit the growth of glioblastoma cells [11]. However, the anticancer effects of Kaempferol on ovarian cancer are yet largely unknown. Therefore, this study was designed to investigate the anticancer effect of Kaempferol against a panel of ovarian cancer cell lines and one normal cell line and an attempt was made to explore the underlying mechanisms.

Methods

WST-1 cell viability and colony formation assay

The effect of Kaempferol on the viability of the ovarian cancer cells was assessed on by WST-1 assay. In brief, the ovarian cancer cells were cultured at a density of 2.5×10^5 cells/well in 96-well plates and subjected to treatment with varied concentrations of Kaempferol. This was followed by incubation of the ovarian cancer cells with WST-1 for 3 h at 37°C and the proliferation rate was determined by taking absorbance at 450 nm. Cell morphology of the Kaempferol-treated ovarian cancer cells was also examined by phase-contrast microscopy. The effect of Kaempferol on the formation of OVACAR-3 colonies was investigated as described earlier [12].

Apoptosis assay

For acridine orange/ethidium bromide (AO/EB) staining, the ovarian cancer OVACAR-3 cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, the OVACAR-3 cells were subjected to Kaempferol treatment for 24 h at 37°C. As the cells sloughed off, 25 µl cell cultures were put onto glass slides and subjected to staining with a solution (1 µl) of AO and EB. The slides were covered with cover slips and examined with a fluorescent microscope. DAPI and annexin V/propidium iodide (PI) staining were performed as described previously [14].

Table 1. Anticancer effects of Kaempferol on different ovarian cancer cell lines as depicted by WST-1 assay and expressed as IC_{50}

S. No.	*Cell Line*	*IC_{50} (µM)*
1	Caov-3	30
2	TOV-112D	30
3	SKOV-3	50
4	OVACAR-3	25
5	SV40 (normal cells)	10

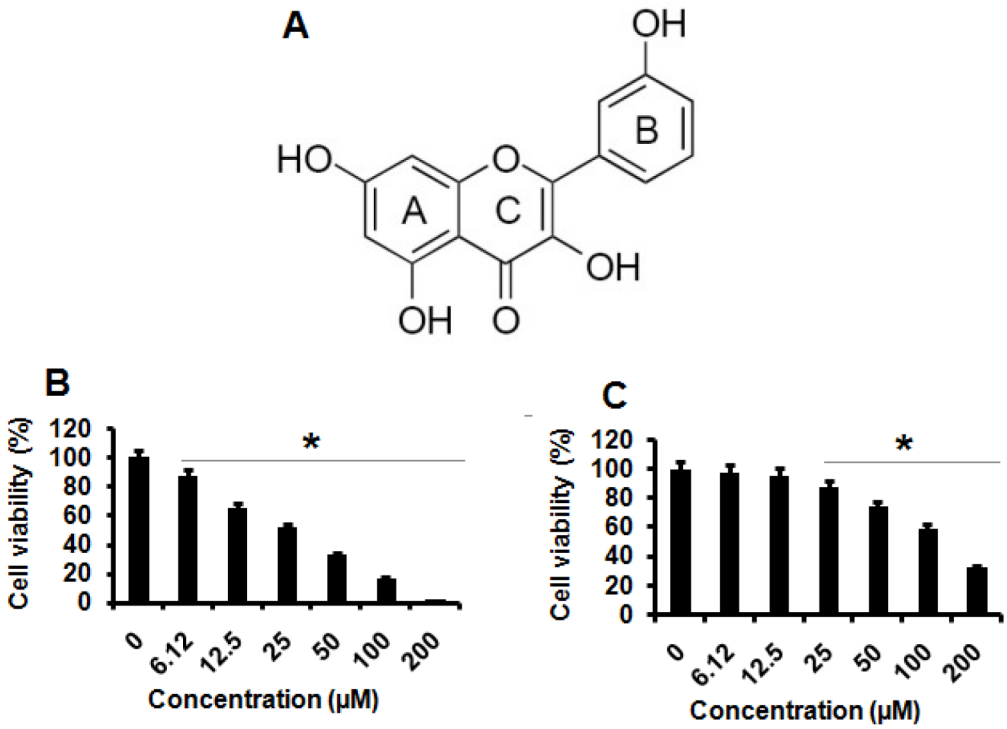

Figure 1. A: Chemical structure of Kaempferol. **B:** Effect of Kaempferol on the viability of the OVACAR-3 ovarian cancer cells, and **C:** normal SV40 ovarian cells. The values represent mean ± SD of three independent experiments (*p< 0.05).

Cell cycle analysis

After incubating the ovarian OVACAR-3 cells with varied concentrations of Kaempferol (0, 12.5, 25 and 50 μM) for 24 h the cells were subjected to washing with phosphate buffered saline (PBS). Afterwards, the OVACAR-3 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Western blotting

The OVACAR-3 cells were firstly subjected to washing with ice-cold PBS and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, 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 subjected to treatment with tris-buffered saline (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 assay.

Statistics

Data is represented as mean ± SD of 3 independent experiments and was statistically analyzed using Student's Newman Keul's test or t-test using GraphPad prism 7 software. $P<0.05$ was taken as significant difference.

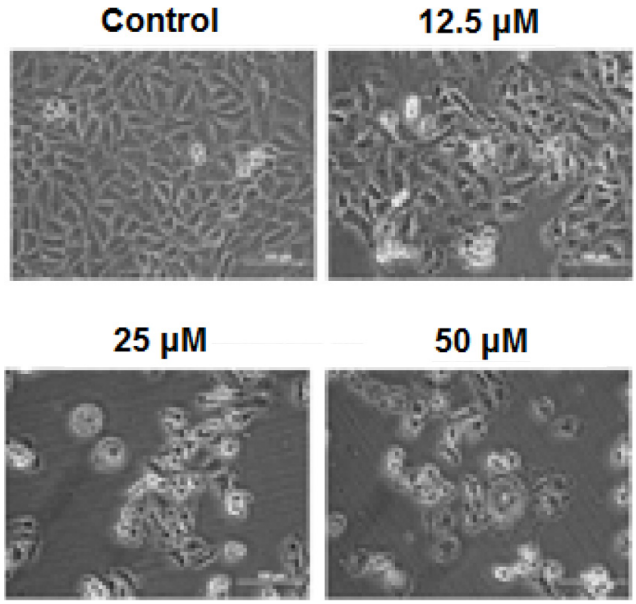

Figure 2. Effect of Kaempferol on the morphology of the OVACAR-3 ovarian cancer cells. The Figure depicts that Kaempferol causes remarkable changes in the morphology of OVACAR-3 cells concentration-dependently. The experiments were performed in triplicate.

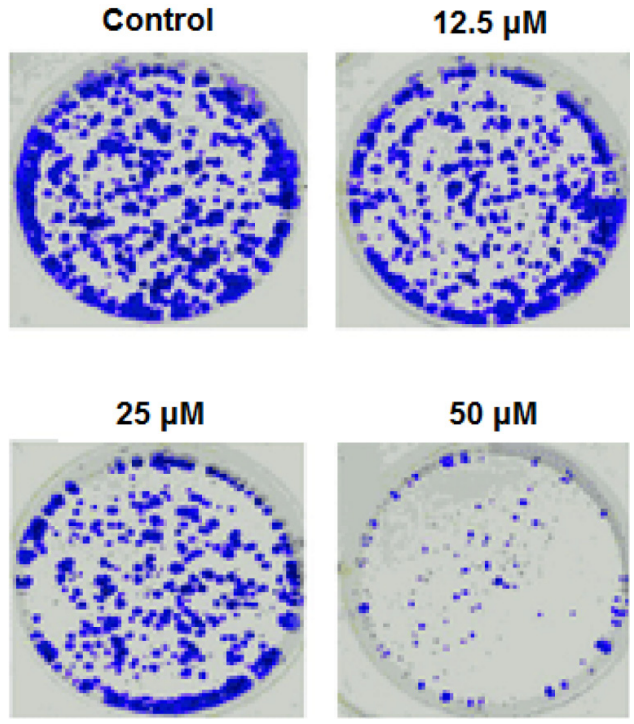

Figure 3. Effect of Kaempferol on the colony formation of the OVACAR-3 ovarian cancer cells. The Figure shows that Kaempferol inhibits the colony formation of the cells in a concentration-dependent manner. The experiments were performed in triplicate.

Results

Kaempferol inhibits the growth of ovarian cancer cells

The anti-proliferative effects of Kaempferol (Figure 1A) were assessed on a panel of ovarian cancer and normal cell lines by WST-1 assay. It was found that that Kaempferol triggers anti-pro-

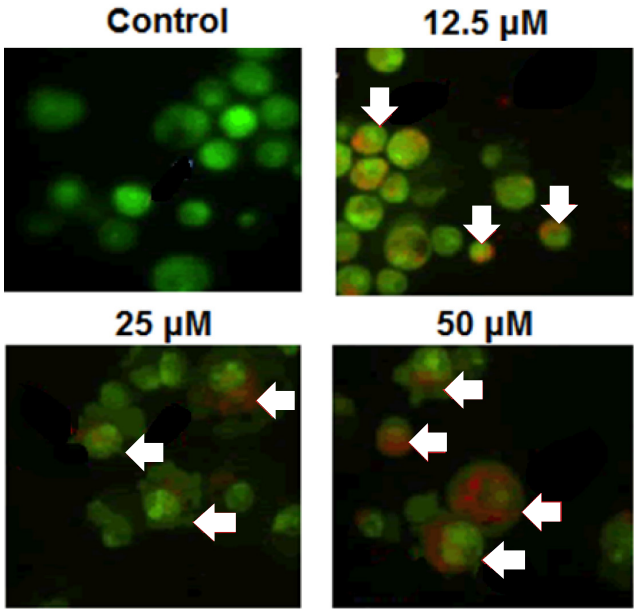

Figure 4. Kaempferol triggers apoptosis in OVACAR-3 ovarian cancer cells as depicted by AO/EB staining. The arrows depict the apoptotic cells. The experiments were performed in triplicate.

liferative effects on all the ovarian cancer cell lines (Table 1). The maximum anti-proliferative effects were observed against the OVACAR-3 cells with an IC_{50} of 25 µM (Figure 1B). Nonetheless, the IC_{50} of Kaempferol was found to be comparatively higher against the normal SV40 ovarian cells (IC_{50}; 120 µM) (Figure 1B). Additionally, it was found that the anticancer effects of Kaempferol on the ovarian cancer cells were concentration-dependent and Kaempferol also produced morphological changes in the OVACAR-3 cells (Figure 2). In addition, Kaempferol exerted inhibitory effects on the colony development of OVACAR-3 cells concentration-dependently (Figure 3).

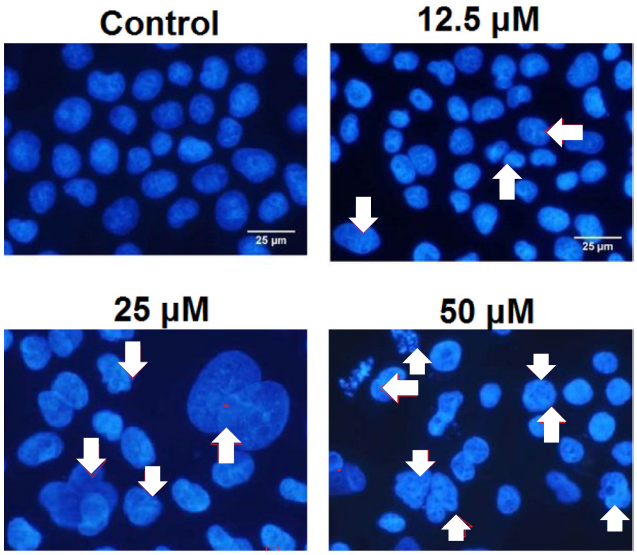

Figure 5. Kaempferol triggers apoptosis in OVACAR-3 ovarian cancer cells as depicted by DAPI staining. Arrows depict apoptotic cells. The experiments were performed in triplicate.

Kaempferol induces apoptosis in ovarian cancer cells

To ascertain whether Kaempferol prompts apoptosis in the OVACAR-3 cells, AO/EB staining was performed which showed remarkable changes in the nuclear morphology and membrane blebbing of the OVACAR-3 cells (Figure 4). Furthermore, DAPI staining also showed increased number of white color nuclei indicative of apoptosis (Figure 5). The percentage of the apoptotic OVACAR-3 cells was determined by Annexin V/PI staining which showed that the apoptotic cell percentage increased significantly from 3.46% in control to 34.16% in cancer cells at 50 µM Kaempferol (Figure 6). The apoptosis was further confirmed by the increased expression of Caspase 3, 8, 9 and Bax and decreased expression of the Bcl-2 in OVACAR-3 cells (Figure 7).

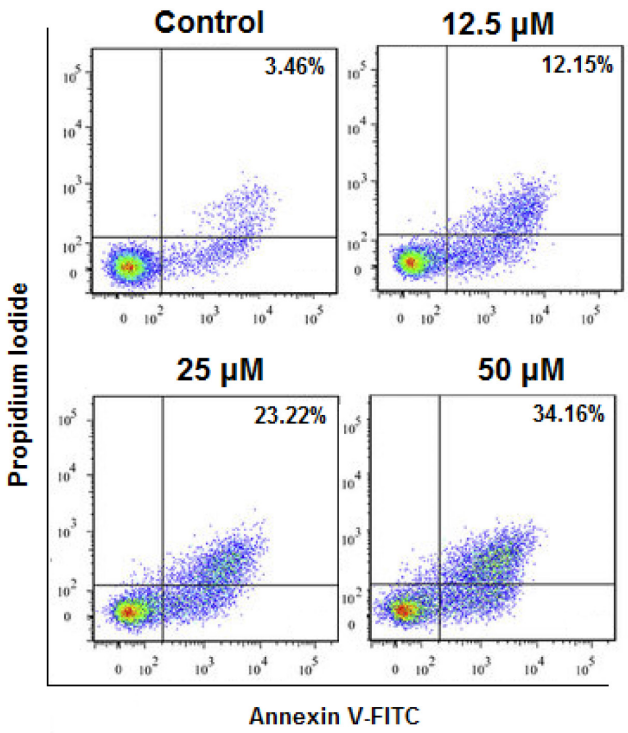

Figure 6. Estimation of apoptotic cell populations of Kaempferol-treated OVACAR-3 cancer cells as depicted by Annexin V/PI staining. The Figure shows that the apoptotic cells increase with the increase in the concentration of Kaempferol. The experiments were performed in triplicate.

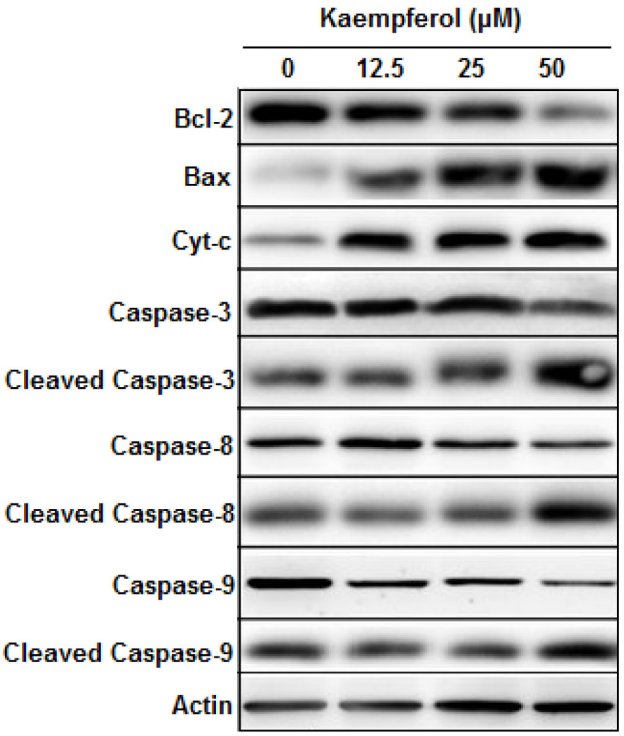

Figure 7. Effect of Kaempferol on the apoptosis-related protein expressions as depicted by western blotting. The Figure depicts that Kaempferol alters the expression of apoptosis-related proteins in a concentration-dependent manner. The experiments were performed in triplicate.

Kaempferol causes the G0/G1 arrest of ovarian cancer cells

The effects of Kaempferol on the distribution of OVACAR-3 cells in various cell cycle phases was assessed by flow cytometry. It was found that Kaempferol caused remarkable increase in the percentage of the OVACAR-3 cells in the G0/G1 phase of the cell cycle. The percentage of OVACAR-3 cells in the Go/G1 phase increased from 67.11% to 78.16% upon treatment with Kaempferol (Figure 8A). These results clearly indicate that Kaempferol induces G0/G1 cell cycle arrest of ovarian cancer cells. The cell cycle arrest of OVACAR-3 cells was also associated with downregulation of Cyclin B1 and Cdc2 expression (Figure 8B).

Kaempferol inhibits the PI3K/AKT and STAT3 signalling pathway

Next, we tried to know the effects of Kaempferol on the MEK/ERK and STAT3 signalling pathway of OVACAR-3 ovarian cancer cells. What it was revealed was that Kaempferol caused concentration-dependent decline in the phosphorylation of p-MEK and p-ERK while no obvious effect was observed on the expression of total MEK and ERK (Figure 9A). Similarly, the phosphorylation of p-

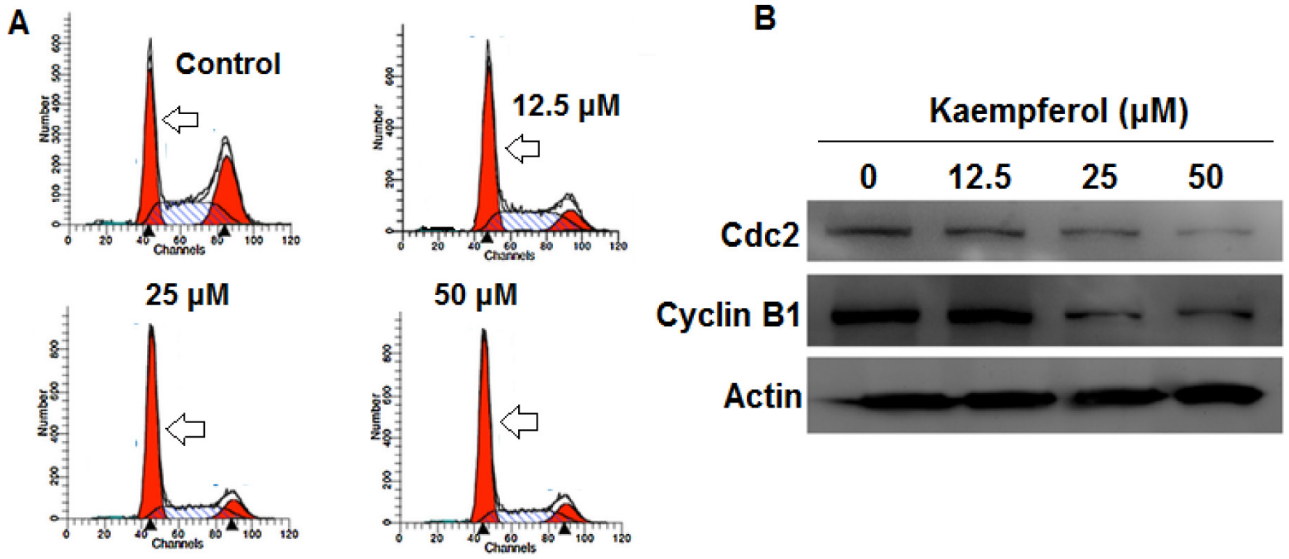

Figure 8. A: Kaempferol triggers G0/G1 cell cycle arrest in the OVACAR-3 cells as depicted by flow cytometry. The Figure shows that Kaempferol triggers G2/M cell cycle arrest in a concentration-dependent manner. **B:** Kaempferol affects the cell cycle related protein expression in OVACAR-3 cells as depicted by western blotting. The Figure shows that Kaempferol suppresses the expression of Cdc2 and Cyclin B1. The experiments were performed in triplicate.

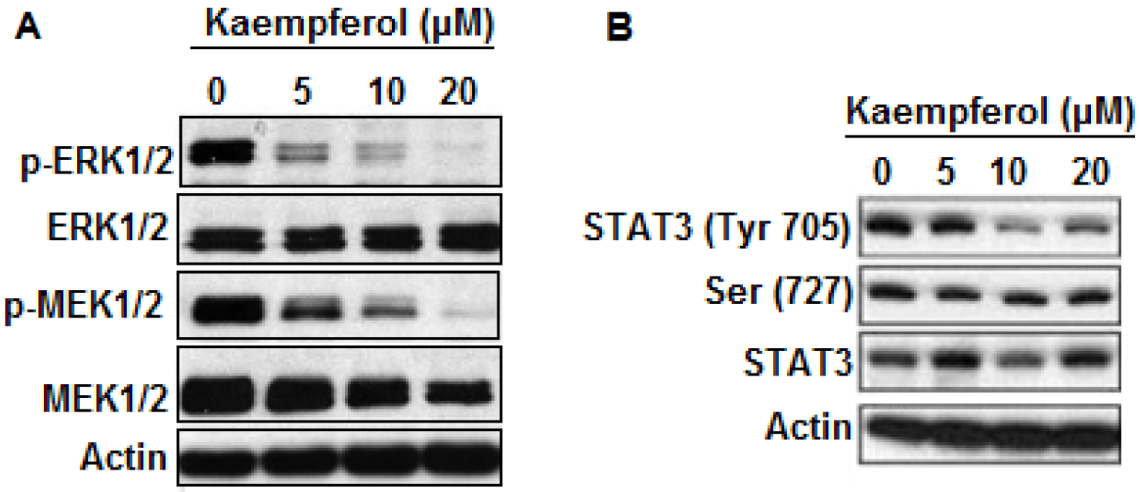

Figure 9. Kaempferol inhibits **A:** MEK/ERK and **B:** STAT3 signalling pathways as depicted by western blot analysis. The Figures show that Kaempferol suppresses the MEK and ERK signalling pathways concentration-dependently. The experiments were repeated thrice.

STAT3 (Tyr 705) and p-STAT3 (Ser 727) was significantly decreased while total STAT3 remained unaltered (Figure 9B).

Discussion

Ovarian cancer is a lethal type of cancer in women accounting for approximately 0.3 million new cases and 0.152 million deaths annually throughout the globe [14]. The clinical outcome is unsatisfactory due its diagnosis at advanced stages and treatment strategies exhibit a number of flaws. Besides, emergence of chemoresistance in cancer cells further makes it difficult to treat ovarian cancer [3]. Herein, we report that the natural flavonoid Kaempferol exerts growth inhibitory effects on the human ovarian cancer cells. The anticancer effects were dose-dependent and were also complemented by the clonogenic assay. Previous studies have also reported the anticancer potential of Kaempferol. Kaempferol has been reported to inhibit the growth of breast and pancreatic cancer to name a few [15,16]. Furthermore, it was observed that Kaempferol exhibits limited cytotoxicity on the normal SV40 cells, indicating that Kaempferol specifically targets the cancer cells. To decipher the mechanism of action, we investigated if Kaempferol triggers apoptotic cell death in the OVACAR-3 cells. The results of AO/EB staining showed that Kaempferol induced membrane blebbing and apoptosis. DAPI staining also showed similar results and annexin V/PI staining showed that the percentage of the apoptotic cell populations increased with increasing concentrations of Kaempferol. The apoptosis in OVACAR-3 cells was further confirmed by examining the expression of the apoptosis-related proteins. Kaempferol increased the expression of Caspase 3, 8 and 9 and Bax, and decreased the expression of Bcl-2. Apoptosis removes the defective or cancer cells and maintains the tissues homeostasis. Besides, apoptosis also prevents the development of drug resistance in cancer cells [17]. Previous studies have also shown that Kaempferol induces apoptotic cell death in glioblastoma cells, breast cancer, non-small lung cancer and colon cancer cells [18-20]. Cell cycle arrest also helps to halt the growth of cancer cells [21]. Kaempferol has also been reported to trigger cycle arrest in colon and renal cancer cells [22,23]. Herein, we found that Kaempferol treatment blocks the cells at the G0/G1 checkpoint which was also accompanied with suppression of Cyclin B1 and Cdc2 expression. Finally, the effects of Kaempferol were also investigated on the MEK/EK and STAT3 signal transduction pathways. These pathways have been shown to be aberrantly activated in cancer cells [23] and Kaempferol could inhibit both these pathways in the OVACAR-3 ovarian cancer cells, suggestive of its potential anticancer effects.

Conclusion

Taken together, Kaempferol exerts considerable anticancer effects on the human ovarian cancer cells by induction of autophagy and apoptosis. Besides, Kaempferol also triggers G0/G1 cell cycle arrest in the ovarian cancer cells. We believe that Kaempferol may prove a potential therapeutic lead molecule and warrants further investigations.

Conflict of interests

The authors declare no conflict of interests.

References

1. La Vecchia C. Ovarian cancer: epidemiology and risk factors. Eur J Cancer Prev 2017;26:55-62.
2. Collaborative Group on Epidemiological Studies of Ovarian Cancer. Menopausal hormone use and ovarian cancer risk: individual participant meta-analysis of 52 epidemiological studies. The Lancet 2015;385:1835-42.
3. Reid BM, Permuth JB, Sellers TA. Epidemiology of ovarian cancer: a review. Cancer Biol Med 2017;14:9.
4. Whittmore AS, Harris R, Itnyre J; Collaborative Ovarian Cancer Group. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies: II. Invasive epithelial ovarian cancers in white women. Am J Epidemiol 1992;136:1184-203.
5. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Products 2016;79:629-61.
6. Patridge E, Gareiss P, Kinch MS, Hoyer D. An analysis of FDA-approved drugs: natural products and their derivatives. Drug Discov Today 2016;21:204-7.
7. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 2015;14:111.
8. Felice MR, Giuffre L, El LA et al. Looking for new antifungal drugs from flavonoids: impact of the genetic diversity of Candida albicans on the in-vitro response. Curr Medicinal Chem 2017;4:2-9.
9. Khan NM, Haseeb A, Ansari MY, Devarapalli P, Haynie S, Haqqi TM. Kaempferol, a plant derived small

molecule, exerts potent anti-inflammatory and chondroprotective effects through the activation of ROS/ERK/Nrf2 signaling pathways in human Osteoarthritis chondrocytes. Free Radical Biol Med 2017;106:288-301.

10. Kashyap D, Sharma A, Tuli HS, Sak K, Punia S, Mukherjee TK. Kaempferol–A dietary anticancer molecule with multiple mechanisms of action: Recent trends and advancements. J Funct Foods 2017;30:203-19.
11. Nakatsuma A, Fukami T, Suzuki T, Furuishi T, Tomono K, Hidaka S. Effects of kaempferol on the mechanisms of drug resistance in the human glioblastoma cell line T98G. Die Pharmazie 2010;65:379-83.
12. Nakamura N, Kusunoki Y, Akiyama M. Radiosensitivity of CD4 or CD8 positive human T-lymphocytes by an in vitro colony formation assay. Radiat Res 1990;123:224-7.
13. Hua F, Li CH, Chen XG, Liu XP. Daidzein exerts anticancer activity towards SKOV3 human ovarian cancer cells by inducing apoptosis and cell cycle arrest, and inhibiting the Raf/MEK/ERK cascade. Int J Molec Med 2018;41:3485-92.
14. Lukanova A, Kaaks R. Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Prev Biomarkers 2005;14:98-107.
15. Choi EJ, Ahn WS. Kaempferol induced apoptosis via cell cycle arrest in human breast cancer MDA-MB-453 cells. Nutrition Res Pract 2008;2:322-5.
16. Zhang Y, Chen AY, Li M, Chen C, Yao Q. Ginkgo biloba extract kaempferol inhibits cell proliferation and induces apoptosis in pancreatic cancer cells. J Surg Res 2008;148:17-23.
17. Koff JL, Ramachandiran S, Bernal-Mizrachi L. A time to kill: targeting apoptosis in cancer. Int J Molec Sci 2015;16:2942-55.
18. Sharma V, Joseph C, Ghosh S, Agarwal A, Mishra MK, Sen E. Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. Molec Cancer Ther 2007;6:2544-53.
19. Leung HW, Lin CJ, Hour MJ, Yang WH, Wang MY, Lee HZ. Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes. Food Chem Toxicol 2007;45:2005-13.
20. Li W, Du B, Wang T, Wang S, Zhang J. Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia Mutated-p53 pathway with the involvement of p53 Upregulated Modulator of Apoptosis. Chemico-Biol Interactions 2009;177:121-7.
21. Saleem M, Asif J, Asif M, Saleem U. Amygdalin, from Apricot Kernels, Induces Apoptosis and Causes Cell Cycle Arrest in Cancer Cells: An Updated Review. Anti-Cancer Agents Medic Chem 2018;18:1650-5.
22. Cho HJ, Park JH. Kaempferol induces cell cycle arrest in HT-29 human colon cancer cells. J Cancer Prev 2013;18:257.
23. Steelman LS, Chappell WH, Abrams SL et al. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. Aging (Albany NY). 2011;3:192.