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ORIGINAL ARTICLE

Tormentic acid induces anticancer effects in cisplatin-resistant human cervical cancer cells mediated via cell cycle arrest, ROS production, and targeting mTOR/PI3K/AKT signalling pathway

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Summary

Purpose: *Tormentic acid has been shown to exert remarkable anti-cancer potential against different cancer cell types. In this study, the anti-cancer potential of tormentic acid was examined in cisplatin-resistant cervical cancer cells (HeLa cells). Further, the effects of tormentic acid on cell cycle, reactive oxygen species (ROS) production, and mTOR/PI3K/AKT signalling pathway were evaluated as well.*

Methods: *Cell viability was evaluated by MTT assay and its impact on mTOR/PI3K/AKT signalling pathway was estimated via western blot assay. Colony formation was analysed through clonogenic assay and phase-contrast microscopy was used for the determination of apoptotic cell morphology along with DAPI staining. Fluorescence-activated cell sorting was performed for cell cycle analysis and ROS production was monitored by fluorescence microscopy.*

Results: *The results indicated that tormentic acid significantly suppresses the proliferation of HeLa cells. These antiproliferative effects of tormentic acid were dose-dependent. Clonogenic assay revealed anti-colony formation potential of tormentic acid. Tormentic acid also induced remarkable morphological changes in HeLa cells, indicative of apoptosis. Further, DAPI staining assay showed formation of apoptotic bodies along with dead cells bearing apoptotic nuclei. Western blotting showed impressive increase in the expressions of pro-apoptotic proteins and decreased expression of anti-apoptotic proteins. Fluorescence-activated cell sorting (FACS) analysis revealed that tormentic acid induced G2/M phase cell cycle arrest and its effectiveness increased with increased doses. Fluorescence intensity indicated amplified ROS production after tormentic acid exposure. The expression of tormentic acid on mTOR/PI3K/AKT pathway revealed blocking of this pathway with a concentration-dependent manner.*

Conclusions: *The outcomes of the present investigation suggest that tormentic acid-induced apoptotic effects in cisplatin-resistant HeLa cells were mediated via cell cycle arrest, ROS production and targeting of mTOR/PI3K/AKT signalling pathway. Thus, tormentic acid may be considered as a lead molecule in cancer therapeutics.*

Key words: *cervical cancer, apoptosis, cell cycle arrest, reactive oxygen species*

Introduction

Tormentic acid (TA) is a triterpene extracted from the plant *Vochysia divergens* (stem bark). TA is a potent pharmacological agent demonstrating different actions including anti-inflammatory, antioxidant, hypoglycemic and anticancer [1-4]. This molecule has been shown to suppress hyperlipidemia and diabetes (high-fat diet) through adenosine monophosphate (AMP) and glucose transporter 4- activated protein kinase phosphorylation [5]. TA was previously reported to show remarkable

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tendency of suppressing vascular smooth muscle cells (A7r5 cell line) (VSMC) survival rate and proliferation rate [6]. In addition, in RAW264.7 cell line TA has shown significant inhibition of lipopolysaccharide-iduced cyclooxygenase-2, nitric oxide synthase (NOS), and tumor necrosis factor-α (TNF-α) [7]. Therefore, the current study was designed to investigate the anticancer potential of TA against cisplatin-resistant cervical cancer (CC) cells along with its effects on cell cycle, ROS production, and mTOR/PI3K/AKT signalling pathway.

Cervical cancer is a deadly gynecological malignancy and has been ranked as the fourth most frequent cause of cancer-related deaths in women. With over 528,000 new cases and more than 266,000 deaths in 2012 alone, CC is the fourth most common cancer in women worldwide and second in ages 15 to 44 years [8]. Persistent human papilloma virus (HPV) infections are among the risk factors of developing CC. Among different HPVs, HPV-18 and -16 are the major risk factors for CC [9]. There is strong evidence of the vital role of PI3K/AKT signalling pathway in cervical carcinogenesis [10-12]. A recent investigation in CC has shown activation of PI3K/AKT and downregulation of PTEN play a pivotal role in cervical carcinogenesis [13]. It has also been reported that PI3K/AKT signalling pathway regulated by STAT3 is involved in CC poor prognosis [14,15]. Moreover, various factors play a pivotal role in CC development like miRNAs (micro RNAs), oncogenic signalling molecules, tumor suppressive genes and oncogenic expression modulators like small noncoding RNAs [16]. Currently, chemotherapy is the most frequently used treatment for advanced CC. However, chemotherapy involves serious challenges like toxic side effects and development of chemoresistance. Thus, there is an urgent need for the development of novel therapeutic agents with lower toxicity and better results against CC.

Thus, the main aim of this study was to examine the anticancer effects of tormentic acid against cisplatin-resistant CC cells (HeLa cells) and also to evaluate the impact of this molecule on cell cycle, reactive oxygen species (ROS) production and mTOR/PI3K/AKT signalling pathway.

Methods

Estimation of cell viability

The estimation of cytotoxic effects of TA on HeLa CC cells were performed via 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. HeLa cells were cultured at a concentration of 7×10^3 cells in each well of 96-well plate. Culturing was performed a day before TA treatment at varying doses (0, 5, 20, 50, 100 µM). TA-treated cells were then incubated in a CO_2 incubator for 24-72 h. Afterwards, a working concentration of MTT solution, with concentration of 5mg/ml, was added and again left for 3 h for incubation. Thereafter, the purple formazan crystals evolved were dissolved with DMSO. Finally, the absorbance was recorded at 650 nm and 570 nm with a microplate reader (Thermo Fisher Scientific Inc., Waltham, United States).

Clonogenic survival determination

HeLa cells were treated with TA at varying doses (0, 20, 50 and 100 µM) for 3 h. Cell colonies were thereafter diluted to 0.3% of agar solution and redisposed into Dulbecco's Modified Eagle's medium (DMEM) in 6-well culture plates. in 35-mm culture plates. Each plate contained 3000 cells that were cultured for 2 weeks. Finally, using 2-mm grid culture dish (Corning) cell colonies were calculated.

Phase contrast microscopy for cell morphology determination

Identification of significant morphological changes was performed via phase contrast microscopy. In brief, 4×10^4 HeLa cells were first incubated for 48 h in 6-well plates. Afterwards, cells were exposed at varying doses of TA (0, 20, 50 and 100 µM). Then, the cells were washed with phosphate buffered saline (PBS) after discarding DMEM. Apoptotic cells were finally observed under phase contrast inverted microscope (Leica DMI 3000B, Germany) for cell morphology determination.

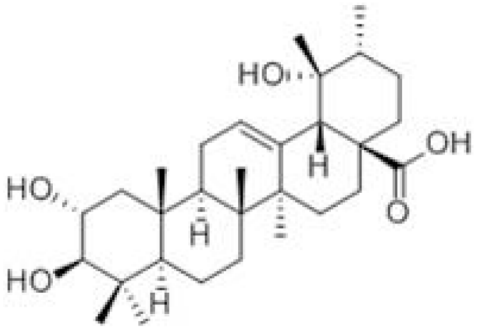

Figure 1. Chemical structure of tormentic acid

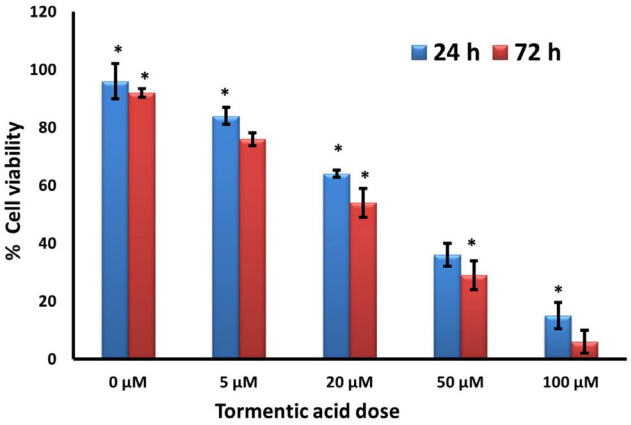

Figure 2. MTT assay representing cell viability of HeLa cervical cancer cells. Data are shown as mean±SD of experiments performed in triplicate. *p<0.05

DAPI staining assay for apoptosis determination

HeLa cells were seeded in 96-well plates for apoptosis estimation at 2×10^5 cells per well. Seeded HeLa cells were exposed to TA (0, 20, 50 and 100 µM) for 24-h incubation. Further, DAPI solution was added to the treated cells following washing with PBS. Thereafter, DAPI-stained cells were fixed with 10% formaldehyde. Apoptosis analysis was finally carried out under fluorescence microscope.

Cell cycle analysis

For the determination of distribution of cell cycle phases, HeLa cells were evaluated through fluorescence-activated cell sorting (FACS). Briefly, HeLa cells were treated with different TA doses (0, 20, 50 and 100 µM), followed by trypsinization. Treated cells were then washed with PBS and were re-suspended in 95% ethanol (900ml). Fixation of cells was performed overnight at 4°C, followed by re-suspension in staining solution bearing RNase, acridine orange/propidium iodide (PI) and PBS. Finally, cells were observed with FACsort (fluorescence-activated sorter) recording up to 40,000 events. Each experiment was performed at least three times.

Measurement of ROS production via fluorescence microscopy

Prewarmed HeLa cells were suspended in PBS bearing 5-(and-6)-carboxy-2',7'-dichlorodihydrofluoresceindiacetate (H2DCFDA) (5 µmol/L) for about 20 min. Afterwards, cells were washed with DMEM for removal of the dye, then returned to growth DMEM medium. Thereafter, cells were further incubated for 30 min and were supplemented with 10 mmol/L of N-acetyl-L cysteine. Cells were then treated with different TA concentrations (0, 20, 50 and 100 µM). Finally, using Wallace VICTOR2 1420 multilabel counter (EG&G Wallace) the fluorescence intensity was analyzed at 485 nm and 535 nm wavelengths.

Western blotting assay

Cells were treated with varying doses of TA (0, 20, 50 and 100 µM) in 6-well plates. After TA treatment, cells were placed on ice followed by lysis buffer (100 µL) (1 mmol/L EDTA, 1 mmol/L Na3VO4, 120 mmol/L NaCL, 50 mmol/L Tris-HCL (pH 7.6), 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 µg/mL leupeptin and aprotinin). A total of 10 µg of protein was loaded onto 8%

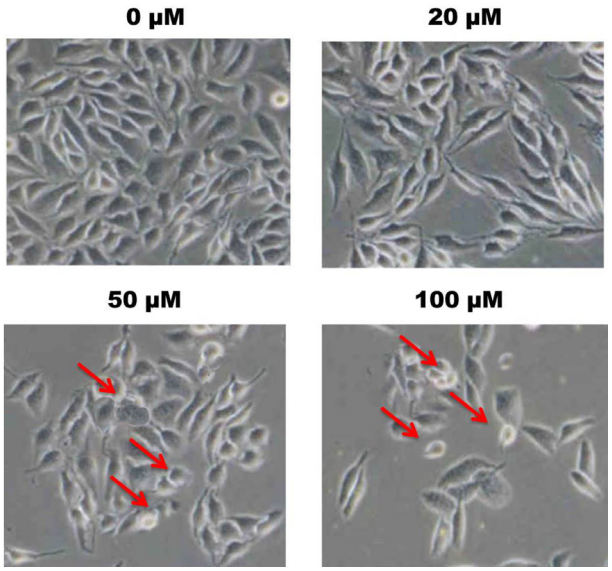

Figure 4. Phase contrast microscopy revealing morphological modifications (arrows) in tormentic acid treated HeLa cells at indicated doses. The experiments were repeated three times.

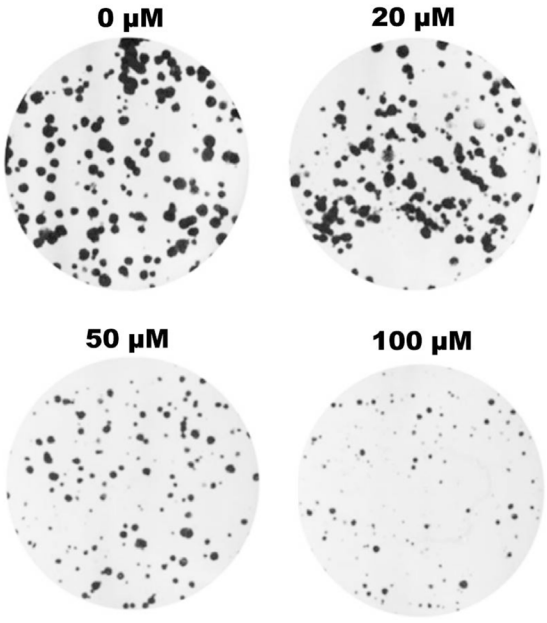

Figure 3. Clonogenic assay presenting HeLa cell colonies after tormentic acid treatment. The experiments were repeated thrice.

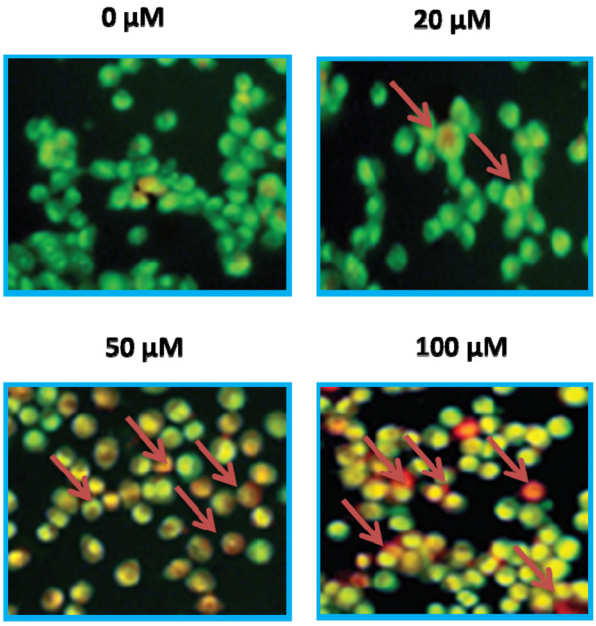

Figure 5. DAPI staining assay showing apoptotic cells after tormentic acid treatment (arrows). The experiments were repeated thrice.

SDS-PAGE gel. Electrophoresis was performed for protein estimation and thereafter protein sample was transferred to polyvinylidene difluoride membranes (PVDF). Next, membranes were blocked at room temperature with fat-free milk for 1 h. Afterwards, PVDF membranes were exposed to primary antibodies followed by secondary antibodies overnight at 4°C. Finally, using Actin as loading control, signal was recorded with Odyssey Infrared Imaging System (LI-COR, USA).

Statistics

The results are presented as mean±standard deviation from 3 independent experiments. Student's *t*-test was used for comparison of differences of controls and treated groups. SPSS 17.0 was used for statistical analyses and $p<0.05$ was considered to indicate statistical significance.

Results

Cytotoxicity induced by tormentic acid

To check the impact of TA on the viability of HeLa CC cells, MTT cytotoxicity assay was performed (Figure 1). The viability of HeLa cells was significantly inhibited with increasing doses of TA. The cell viability was nearly 85%, 65%, 40% and 20%, and nearly 80%, 60 %, 30% and 5% at 5, 20, 50 and 100 µM concentrations after 24-72 h of drug exposure, respectively (Figure 2), showing dose and time-dependent growth inhibition of HeLa cells.

Effects of tormentic acid on colony formation of HeLa cells

Colony formation was performed to evaluate the impact of TA on colony potential of HeLa cells. The results indicated that TA reduced the trend of HeLa cells of colony formation. The effect was dose-dependent (Figure 3).

Morphology determination of apoptotic HeLa cells after tormentic acid treatment

Cellular morphology was observed through phase contrast microscopy. The results demonstrated that after treatment with TA significant morphological changes occurred in HeLa cells. Morphological changes included cell shrinkage, membrane blebbing, condensation of nuclei and bubbling (Figure 4). The effects were increased after increasing the drug doses.

Induction of apoptosis in HeLa cervical cancer cells by tormentic acid

Further, investigation was carried out for checking whether the cytotoxicity of TA was apo-

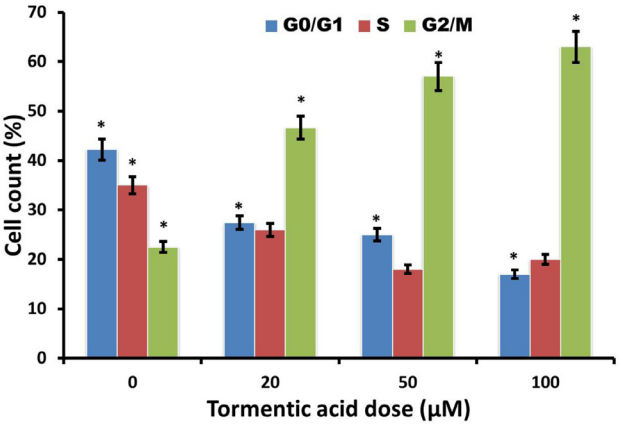

Figure 7. FACS analysis representing different cell cycle phases. Data are shown as mean±SD of experiments performed three times. *$p<0.05$.

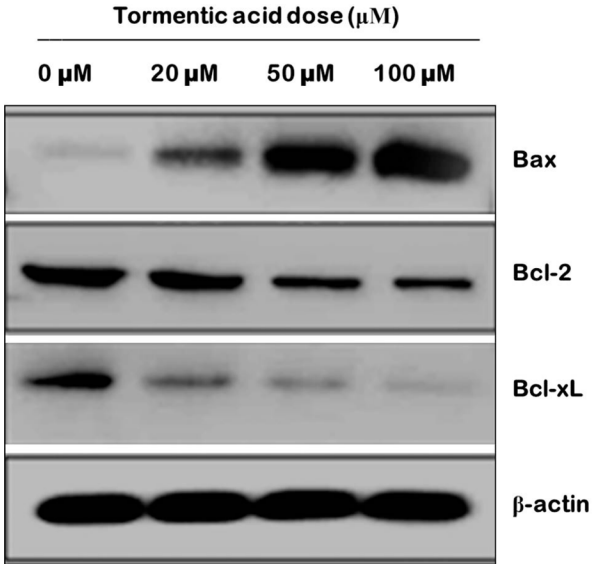

Figure 6. Western blotting analysis showing the expressions of BAX, BCL-2 and BCL-XL. Data are shown as mean±SD of experiments performed three times. *$p<0.05$.

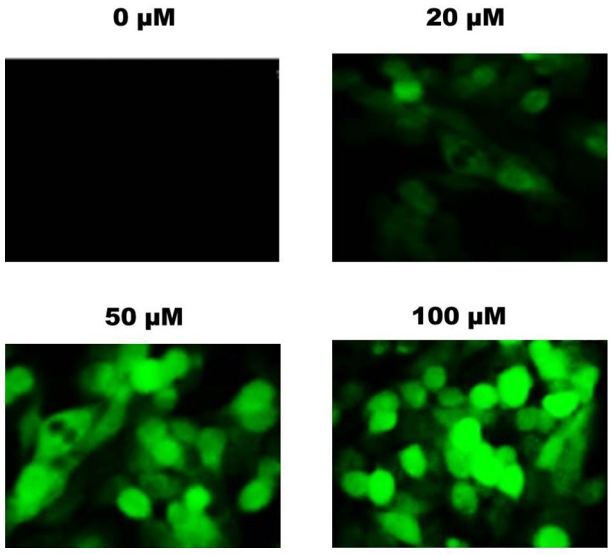

Figure 8. Presentation of ROS production after tormentic acid treatment. The experiments were performed in triplicate.

ptosis-related. Apoptosis estimation was performed through DAPI staining assay. The results revealed remarkable dose-dependent induction of apoptosis by drug exposure (Figure 5). In control untreated cells there were no signs of apoptosis, but at increasing doses of TA the cells emitted yellow and orange fluorescence indicating onset of apoptosis. The percentage of these cells was concentration-dependent. Further, the effect of TA on pro- and anti-apoptotic proteins was assessed via western blotting which showed downregulation of BCL-2 and BCL-XL and upregulation of BAX with increasing TA concentration (Figure 6).

Impact of tormentic acid on different cell cycle phases

To assess different cell cycle phases FACS was performed. The results indicated significant increase in G2/M phase cells, evidencing cell cycle arrest. The percentage of G2/M phase cells increased from about 20% in controls to 40% at 20 μM of drug concentration and up to 70% at 100 μM (Figure 7).

Effect on reactive oxygen species (ROS) of HeLa cells after tormentic acid treatment

Further, the effect on ROS production of HeLa cells was assessed through fluorescence microscopy. The results revealed that the ROS production increased significantly with increasing doses of TA (Figure 8).

Tormentic acid targeting mTOR/PI3K/AKT signalling pathway in HeLa cells

The effect on mTOR/PI3K/AKT signalling pathway by TA was analyzed through western blotting analysis. The results showed remarkable inhibitory effects on mTOR/PI3K/AKT signalling pathway. It was observed dose-dependent decrease in p-PI3K, p-mTOR and p-AKT and dose-dependent increase in mTOR and AKT protein expressions after drug exposure. PI3K expression remained unchanged at lower as well as higher drug doses.

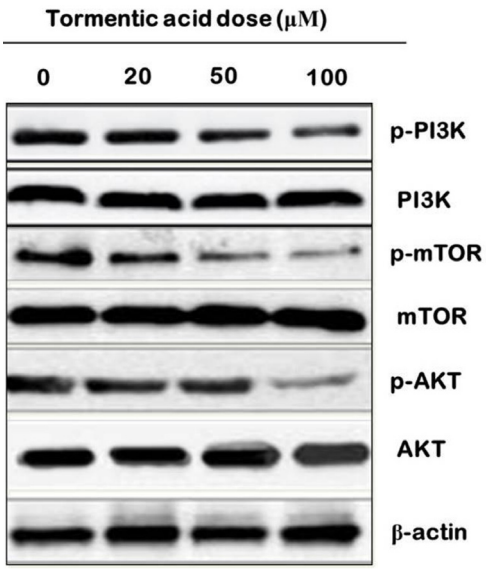

Figure 9. Western blotting analysis presenting the expressions of PI3K/AKT signalling pathway related proteins. Data are shown as mean±SD of experiments performed three times. *P<0.05.

Discussion

As a result of aerobic metabolism in human body, there is the production of different free radicals including O_2^-, nitric oxide, H_2O_2, hypochloric acid, and peroxynitrile and many more ROS [17,18]. Universally, all the mammalian cells produce ROS for signal generation and the production of oxidative species are also associated to long-term exposure to physiological stress which induces protein, RNA and DNA damage. Regulation of ROS is essential for normal functioning of the cell, regulating differentiation, proliferation and apoptosis. Apoptosis is the main form of programmed cell death (PCD) and is categorized as type I PCD. It is one of the essential mechanisms that operate homeostasis and development of multicellular organisms. Apoptosis is characterized by identification of certain specific morphological and biochemical modifications including fragmentation, nuclear condensation, cell shrinkage, and apoptotic bodies. The current investigation was designed to determine the anticancer potential of TA against cisplatin-resistant HeLa cells mediated via apoptosis, cell cycle arrest, ROS production, and targeting mTOR/PI3K/AKT signalling pathway. The effect of TA on cell viability of HeLa cells was assessed by MTT assay. The results revealed dose as well as time-dependent inhibition in cell viability. Colony formation assay indicated significant dose-dependent suppression in colony formation ability of HeLa cells. Next, phase contrast microscopy was performed to assess the TA impact on cellular morphology of HeLa cells which revealed different morphological changes such as cell shrinkage, membrane blebbing, condensation of nuclei and bubbling, which were dose-dependent and indicative of apoptosis. This was further supported by western blotting assay, which revealed enhancement in the expressions of pro-apoptotic proteins rather than in anti-apoptotic proteins. Cell cycle analysis was performed via FACS technique, and the results showed remarkable increase in the number of G2/M phase cells, indicating cell cycle arrest. Thereafter, TA was evaluated for ROS production via fluorescence microscopy which showed ROS production increased strongly with increased TA

doses. Finally, the effect of this molecule on PI3K/AKT signalling pathway was shown as a potential downregulator of p-PI3K, p-mTOR and p-AKT and potential upregulator of mTOR and AKT, with increasing drug doses, indicating dose-dependent blockade of PI3K/AKT signalling pathway.

Conclusion

In conclusion, the current investigation of TA treatment in HeLa cells revealed its potential as cell viability inhibitor. TA also induced cell cycle arrest, enhanced ROS production, and targeted PI3K/AKT signalling pathway. In our opinion, TA deserves further *in vitro* and *in vivo* investigation as potential anticancer agent against human cervical cancer.

Conflict of interests

The authors declare no conflict of interests.

References

1. Park GH, Lee JY, Kim DH, Cho YJ, An BJ. Anti-oxidant and antiinflammatory effects of Rosa multiflora root. J Life Sci 2011;21:1120-6.
2. Banno N, Akihisa T, Tokuda H et al. Triterpene acids from the leaves of Perilla frutescens and their anti-inflammatory and antitumor-promoting effects. Biosci Biotechnol Biochem 2004;68:85-90.
3. Ivorra M, Paya M, Villar A. Hypoglycemic and insulin release effects of tormentic acid: A new hypoglycemic natural product. Planta Medica 1988;54:282-5.
4. Bortalanza LB, Ferreira J, Hess SC, Delle Monache F, Yunes RA, Calixto JB. Anti-allodynic action of the tormentic acid, a triterpene isolated from plant, against neuropathic and inflammatory persistent pain in mice. Eur J Pharmacol 2002;453:203-8.
5. Wu JB, Kuo YH, Lin CH, Ho HY, Shih CC. Tormentic acid, a major component of suspension cells of Eriobotrya japonica, suppresses high-fat diet-induced diabetes and hyperlipidemia by glucose transporter 4 and AMP-activated protein kinase phosphorylation. J Agric Food Chem 2014;62:10717-26.
6. Fogo AS, Antonioli E, Calixto JB, Campos AH. Tormentic acid reduces vascular smooth muscle cell proliferation and survival. Eur J Pharmacol 2009;615:50-4.
7. An HJ, Kim IT, Park HJ, Kim HM, Choi JH, Lee KT. Tormentic acid, a triterpenoid saponin, isolated from Rosa rugosa, inhibited LPS-induced iNOS, COX-2, and TNF-α expression through inactivation of the nuclear factor-κb pathway in RAW 264.7 macrophages. Int Immunopharmacol 2011;11:504-10.
8. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7-30.
9. Moore EE, Wark JD, Hopper JL, Erbas B, Garland SM. The roles of genetic and environmental factors on risk of cervical cancer: a review of classical twin studies. Twin Res Hum Genet 2012;15:79-86.
10. Page C, Huang M, Jin X et al. Elevated phosphorylation of AKT and Stat3 in prostate, breast, and cervical cancer cells. Int J Oncol 2000;17:23-8.
11. Wei LH, Kuo ML, Chen CA et al. The anti-apoptotic role of interleukin-6 in human cervical cancer is mediated by up-regulation of Mcl-1 through a PI 3-K/Akt pathway. Oncogene 2001;20:5799-809.
12. Lee MS, Jeong MH, Lee HW et al. PI3K/AKT activation induces PTEN ubiquitination and destabilization accelerating tumourigenesis. Nat Communications 2015;6:7769.
13. Chen CL, Hsiek FC, Liebein JC et al. Stat3 activation in human endometrial and cervical cancers. Br J Cancer 2007;96:591-9.
14. Prasad SB, Yadav SS, Das M et al. PI3K/AKT pathway-mediated regulation of p27(Kip1) is associated with cell cycle arrest and apoptosis in cervical cancer. Cell Oncol 2015;38:215-25.
15. Hou MM, Liu XC, Wheler J et al. Targeted PI3K/AKT/mTOR therapy for metastatic carcinomas of the cervix: A phase I clinical experience. Oncotarget 2014;5:11168-79.
16. Wang X, Tang S, Le SY et al. Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. PLoS One 3, 2008;e2557.
17. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994;344:721-4.
18. Poulsen HE, Prieme H, Loft S. Role of oxidative DNA damage in cancer initiation and promotion. Eur J Cancer Prev 1998;7:9-16.