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Regular paper

Betulinic acid targets drug-resistant human gastric cancer cells by inducing autophagic cell death, suppresses cell migration and invasion, and modulates the ERK/MEK signaling pathway

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The main purpose of this study was to examine the anticancer effects of betulinic acid – a plant triterpene, against gastric cancer, along with demonstrating its underlying mechanism. The MTT assay and clonogenic assays were executed to assess cellular viability in control and betulinic acid treated cells. Transmission electron microscopy and western blotting were implemented to study autophagy stimulation by betulinic acid. The ERK/MEK signaling pathway was monitored by western blotting. Migration and invasion of SGC-7901 cells was investigated *via* transwell chamber assay. Results of this investigation indicated that betulinic acid induced remarkable cytotoxicity against gastric cancer SGC-7901 cells, in contrast to normal gastric GES-1 cells. The cytotoxicity of betulinic acid was observed due to its autophagy stimulation tendency in target cells. Autophagic cell death was supported by the data attained from western blotting showing enhanced LC3-II, and lowered LC3-I and p62 expressions. Moreover, betulinic acid was observed to block the ERK/MEK signaling pathway in SGC-7901 cells, which was associated with declined levels of expressions of the phosphorylated ERK and MEK proteins. Finally, the transwell chamber assay revealed a potential lowering of migration and invasion by betulinic acid in the SGC-7901 cells. In conclusion, these results demonstrated that betulinic acid exhibited significant anti-gastric cancer effects mediated *via* autophagy induction, blocking of ERK/MEK signaling and suppression of migration and invasion. Therefore, betulinic acid may prove as a lead molecule in gastric cancer management and research.

Keywords: gastric cancer, triterpenes, betulinic acid, autophagy, cell migration

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Abbreviations: ECL, enhanced chemiluminescence

INTRODUCTION

Natural products (phytochemicals) possess a huge molecular, structural and behavioral diversity which leads to their extraordinary medicinal value and dominant role in drug discovery (Newman *et al.,* 2003). Phytochemicals occur as secondary metabolites in plants and typically assist their defensive mechanism against other plants and insects (Dewick, 2001). Terpenoids ($C_{30}H_{48}$) are a huge class of phytochemicals with several subclasses, including the mono-, di-, tri- and tetra-terpenoids. More than 200 different triterpenes have been identified and structure was obtained for the pentacyclic-triterpenes as dominant ones (Khursheed *et al.,* 2016). Triterpenes have been identified with remarkable biological and medicinal activities, including antioxidant, cardioprotective, anti-inflammatory, hepatoprotective, analgesic, anti-HIV, anti-nociceptive, anxiolytic and anticancer activities (Yadav *et al.,* 2010; Pearson *et al.,* 2003; Hikino *et al.,* 1984; Somova *et al.,* 2003; Fujioka *et al.,* 1994; Liu *et al.,* 2014; Pawar & Bhutani, 2005). Triterpenes have been recognized to induce anticancer effects against cancers of colon, breast, T-cell leukemia, and oral mucosa (Petronelli *et al.,* 2009; Ellington *et al.,* 2005; Konopleva *et al.,* 2002). Betulinic acid is a triterpene with a significant bioactivity and medicinal profile. It has been identified in the barks of a number of plant species, principally the *Betula pubescens* (white birch). Betulinic acid has been reported to display growth suppressive effects against liver cancer, lung cancer and malignant melanoma (Xu *et al.,* 2017; Kessler *et al.,* 2007). Betulinic acid is a renowned inhibitor of growth of human melanoma, migrant cancer cells and neuroectodermal cancer cells. Betulinic acid induces cytotoxic effects in several human cancer cells, including neuroblastoma, glioblastoma, medulloblastoma, and the Ewing sarcoma (Schmidt *et al.,* 1997; Fulda *et al.,* 1999). It has been shown to have an apoptosis stimulation potential, cell cycle inhibitory potential, free radical scavenging potential, migration and invasion inhibitory potential, and potential to inhibit several survival signaling pathways (Gao *et al.,* 2011). Betulinic acid results in modulation of mitochondrial functions (both, intrinsic and extrinsic) in cancer cells which leads to apoptosis stimulation and ultimately programed cell death (Fulda & Kroemer, 2009).

Gastric cancer is a highly rated lethal cancer occurring in human gut, associated with immense mortality and ranked as the second most common cancer across the globe (Crew & Neugut, 2006; Correa *et al.,* 1990). Global incidences of gastric cancer are rising by every year and about 0.99 M patients are yearly diagnosed with gastric cancer, out of which about 0.73 M patients suffer death (Machlowska *et al.,* 2020). Despite recent advances made in the gastric cancer targeted and combination therapy with improved clinical outcome, the drug-resistance in gastric cancer cells poses a biggest challenge for re-

searchers and scientists worldwide. Considering that betulinic acid is of natural origin, less toxic and possesses remarkable anticancer potency, this study was designed to investigate its anticancer effects against drug-resistant SGC-7902 gastric cancer cells. The effects of inducing autophagy, suppressing cell migration and invasion, and modulation of the ERK/MEK signaling pathway by betulinic acid were also studied.

EXPERIMENTAL

Chemicals, reagents, cell culture and conditions

Betulinic acid (>98% purity by HPLC) was obtained from Chengdu Biopurify Phytochemicals Ltd, (Chengdu, China). All of the chemicals and reagents, if otherwise not mentioned, were procured from Sigma Aldrich, MO, United States. The cancerous and normal human gastric cell lines SGC-7901 and GES-1 were procured from the Chinese Academy of Sciences Shanghai Cell Bank, Shanghai, China. All cell lines were cultured in DMEM (Thermo Fisher Scientific), containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). This mixture was placed in a humid environment, at 37°C, in a 5% CO_2 incubator. Cells were maintained under these conditions till further use.

Viability assay

Antiproliferative potency of betulinic acid against gastric SGC-7901 cancer and normal gastric GES-1 cells was studied with the MTT assay (Wang *et al.,* 2016). Briefly, 5000 of SGC-7901 and GES-1 cells were loaded onto 96-well plates containing DME (Dulbecco's modified Eagle's) medium and precultured for 24 h at 37°C. Precultured SGC-7901 cells were then subjected to different betulinic acid doses, viz 0, 5, 25, 50 and 150 μM for 48 h. Afterwards, betulinic acid treated SGC-7901 cells were washed using PBS (Sigma), followed by addition of the MTT stock solution (Sigma Aldrich, MO, United States) to each well of the 96-well plate. Cells were then incubated for 20 min until formazan crystals were evolved. These crystals were then dissolved within 200 μl of DMSO (dimethyl sulphoxide). Finally, optical density measurements were taken at 570 nm with a multimode reader (Infinite F200 pro, TECAN, Switzerland).

Colony formation assay

The proliferation of SGC-7901 colonies was analyzed using the clonogenic assay (Zhao *et al.,* 2015). A 6-well plate with top layer of 0.5 mL of agar and bottom layer of 1.5 mL of agar (Difco Laboratories, Detroit, United States) with a concentration of 5.1 mg/mL was used in culturing of the tested cells. Each well was filled with different concentrations of the betulinic acid drug, viz 0, 25, 50 and 150 μM, prior to incubation with 5% CO_2 at 37°C for 7 consecutive days. The medium was replaced after every two days. Finally, SGC-7901 cells were subjected to the Giemsa (Gibco® KaryoMAX®) staining, and cell colonies were counted with the help of a light microscope (Olympus, Japan).

Autophagy assay

Transmission electron microscopy was used to study induction of the autophagic effects exerted by betulinic acid in the SGC-7901 cells. Gastric cancer SGC-7901 cells were exposed to betulinic acid of variant concentrations, viz 0, 25, 50 and 150 μM, for 48 h. Afterwards, betulinic acid treated cells were pelleted, followed by 30 min of fixation with the cacodylate buffer (0.1 M) bearing glutaraldehyde (1.5%), pH of 7.4. These fixed pellets were left untouched overnight in the same buffer containing 2.5% of glutaraldehyde at 4°C. Then, the betulinic acid treated SGC-7901 cells were post-fixed for 60 min with cacodylate buffer (0.1 M) containing osmium tetroxide (1%), followed by 16 h of staining with uranyl acetate (1%), at 4°C. Propylene oxide and ethanol were used for cell dehydration, followed by embedding in the epoxy resin, and thin sectioning was done at 80 nm. These thin sections were placed on copper grids post stained with 3.5% sodium citrate/2.7% lead nitrate. Finally, images were captured using the HITACHI H7500 transmission electron microscope.

Western blotting assay

Western blotting was performed to analyze the activity levels of proteins associated with autophagy and the ERK/MEK signaling pathway. SGC-7901 cells were harvested at logarithmic growth phase and treated with betulinic acid at different concentrations, viz 0, 25, 50 and 150 μM, for 24 h. The betulinic acid treatment of the cells was followed by lysing with the RIPA lysis buffer and then BCA assay for estimation of protein content in the lysates. Equal amounts of proteins was electrophoresed using SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were then blocked in TBS maintaining 5% non-fat milk powder and 0.05% Tween-20 (TBST). These membranes were blotted with anti-LC3-I, anti-LC3-II, anti-p62, anti-MEK, anti-p-MEK, anti-p-ERK, anti-beclin-1, anti-GAPDH and anti-ERK primary antibodies (purchased from Cell Signalling Technology, MA, United States), at 4°C for 24 h with 1:1 000 dilutions of each individual antibody. Thereafter, the membranes were washed thrice in TBST, followed by treatment with HRP conjugated secondary antibody (Santa Cruz Biotechnology, cat. no. sc-2372), for 55 min at 25°C. Finally, a reagent for enhanced chemiluminescence (ECL) was used to visualize protein bands.

Migration and invasion assay

The ability of SGC-7901 cells to migrate and invade after betulinic acid (0, 25, 50 and 150 μM) exposure was analyzed using a transwell chamber assay (Chen *et al.,* 2016). In brief, 10 000 of SGC-7901 cells were seeded within upper transwell chambers fitted with polycarbonate filters of 8-μm pore size. Then, the cells from the upper chambers were relocated into 24-well plates and were incubated for 48 h at 37°C. In case of invasion, these inserts were coated with 50 μl of Matrigel (ECM, Sigma) prior to relocation. The un-migrated and un-invaded cells were detached from the upper surface using a cotton swab. However, the lower surfaces, bearing the migrated and invaded cells, were subjected to fixation with 70% methanol for 35 min. These cells were stained for 50 min using 0.5% crystal violet, followed by washing with PBS (phosphate buffered saline, Sigma). Finally, the migrated and invaded gastric cancer SGC-7901 cells were counted under a light microscope (OLYMPUS, Japan).

Statistical analysis

Each individual experiment was executed three times and the data collected was represented as mean ±S.E.

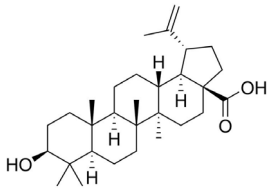

Figure 1. Chemical structure of the betulinic acid molecule.

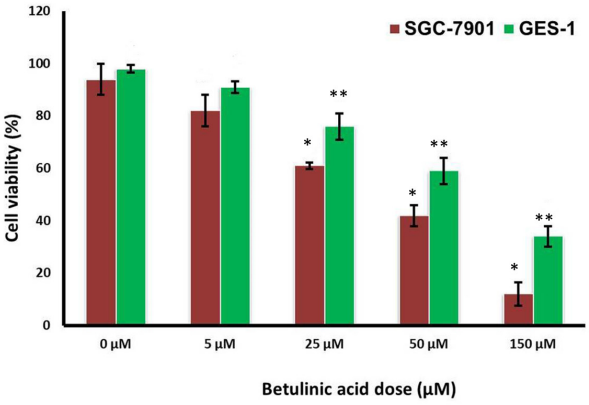

Figure 2. Viabilities of gastric cancer SGC-7901 and normal GES-1 cells as obtained from execution of the MTT assay after exposure to the indicated betulinic acid doses.
Both cell lines were treated for 48 h and viability was assessed by optical density calculations. Results revealed that the viability of SGC-7901 was reduced to a larger extent than that of normal GES-1 cell line, in a dose-dependent fashion. All the data are shown as mean ±S.D. of three independent replicates (*p<0.05 for SGC-7901 cell line, and **p<0.01 for GES-1 cell line).

Statistical analysis was performed by using a two-way ANOVA (post hoc: tukey test), along with regression and correlation analysis. Statistically significant value was taken as p<0.05.

RESULTS

Betulinic acid revealed anti-viability effects against the SGC-7901 cells

The viability of SGC-7901 and GES-1 cells was estimated after 48 h of betulinic acid exposure at altering doses (0–150 μM) (Fig. 1). Uncontrolled multiplication of cancer cells remains a fundamental cause of disease origination and spread. Thus, targeting of uncontrolled proliferation in cancer cells remains a primary target of anticancer drugs. Betulinic acid induced significant antiproliferation effects in SGC-7901 cells, in comparison to the GES-1 cells. The viability of normal GES-1 cells was reduced only by an insignificant margin, which showed specificity of betulinic acid toxicity against the SGC-7901 cells. In case of SGC-7901 cells, the viability were reduced to nearly 10% at 150 μM, considering controls as 100% viable cells (Fig. 2). The colony establishing tendency of SGC-7901 cells was reduced to a large extent by their treatment with betulinic acid (Fig. 3A). In comparison to control group, a significant (*p<0.05) decrease in the number of SGC-7901 colonies was observed post betulinic acid treatment. About only 50 colonies of SGC-7901 cells were remained at 150 μM of the drug, in contrast to 350 colonies at controls (Fig. 3B). Thus, betulinic acid produced remarkable anti-viability effects in the SGC-7901 cells.

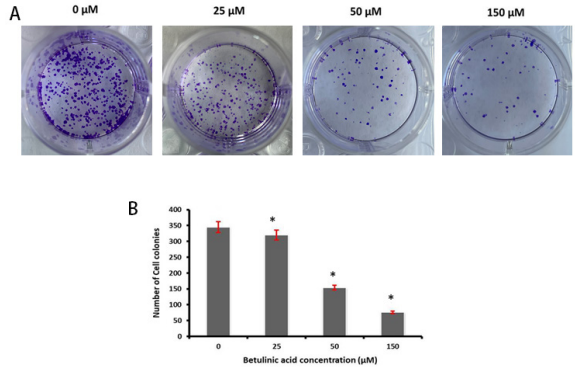

Figure 3. (A) Clonogenic assay was implemented to determine the colony generation potential of the SGC-7901 cells.
Results revealed significant inhibition of colony proliferation after betulinic acid treatment, at indicated doses.
(B) Graphical presentation of the SGC-7901 cell colony number at indicated concentrations of betulinic acid, in the treated and control groups.
Data of triplicate experiments for each betulinic acid concentration is presented as mean ±S.E., with *p<0.05 considered as statistically significant, in comparison to control group.

Betulinic acid caused autophagic cell death in the SGC-7901 cells

Till date, a number of targets have been explored in cancer cells besides the programmed cell death which remains one of the primary goals. Transmission electron microscopy was used to investigate morphological modifications displayed by the SGC-7901 cells after being exposed to betulinic acid. Results revealed presence of autophagosomes in case of the drug treated cells, in comparison to controls. Upon increasing the doses of betulinic acid to 150 μM, autolysosomes were also observed (Fig. 4A). These results hallmark that betulinic acid stimulates autophagic cell death in the SGC-7901 cells. Further, western blotting suggested that the levels of p62 gene expression were reduced along with the LC3-II and LC3-I levels, in the betulinic acid treated cells (Fig. 4B). In different cancer cells, p62 silencing exhibited antiproliferative and autophagic effects, hence it is considered as an autophagy inhibitor gene (Nihira *et*

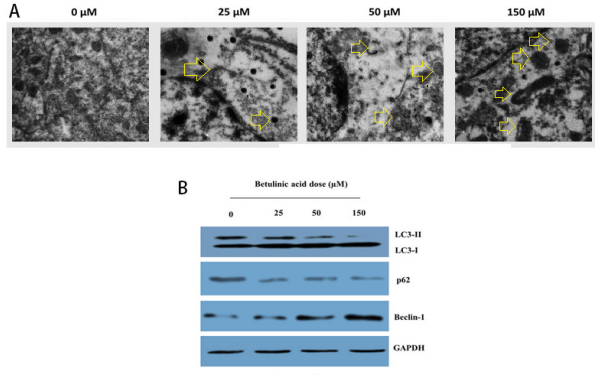

Figure 4. (A) Transmission electron microscopy was performed for examination of intracellular morphology of the SGC-7901 cells after betulinic acid treatment.
Results showed presence of autophagosomes and autolysosomes in the treated cells, which suggested autophagic antiproliferative effects of betulinic acid.
(B) Western blotting analysis of autophagy associated proteins in betulinic acid treated SGC-7901 cells.
Results showed decreased levels of p62 and LC3-II, and increased levels of the LC3-I proteins. Experiments for each betulinic acid concentration were repeated thrice.

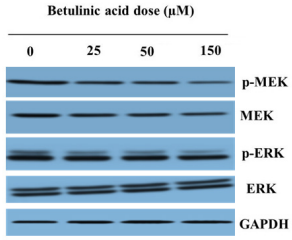

Figure 5. Western blotting assay was performed to assess the levels of expressions of the ERK/MEK signaling pathway allied proteins.
Results revealed that after exposure to indicated betulinic acid concentrations, expressions of phosphorylated MEK and ERK went down significantly. Experiments for each betulinic acid concentration were repeated thrice.

al., 2014). Herein, we found reduced expressions of the p62 gene in the treated SGC-7901 cells when compared to controls, showing autophagy promotion effects of betulinic acid. The Beclin-1 gene is a key autophagy regulator gene in mammalian cells. Herein, we found that betulinic acid exerted regulatory effects on Beclin-1 and enhanced its expressions in the SGC-7901 cells. Hence, western blotting strengthened the results obtained from transmission electron microscopy showing formation of autophagosomes in the SGC-7901 cells, indicating autophagic death.

Betulinic acid blocked the ERK/MEK signaling pathway in the SGC-7901 cells

The ERK/MEK signaling pathway is a key survival signaling pathway. The effect of betulinic acid on this pathway in the SGC-7901 cells was assessed by western blotting. Results showed that betulinic acid could potentially inhibit the ERK/MEK signaling pathway. The expression levels of phosphorylated MEK and ERK proteins were markedly reduced in betulinic acid treated SGC-7901 cells, in comparison to controls (Fig. 5). This indicated that antiproliferative effects of betulinic acid could be due to downregulation of the ERK/MEK signaling pathway by betulinic acid.

Betulinic acid suppressed the SGC-7901 cells' migratory and invasive potential

One of the lethal features of malignant cancer cells is to migrate and invade to distant places, leading to cancer metastasis. Herein, betulinic acid was examined for its anti-migratory and anti-invasive potential in SGC-7901 cells via a transwell chamber assay. The transwell chamber migration assay results showed a remarkable anti-migratory potential of betulinic acid in SGC-7901 cells, which reduced the number of migrated cells in comparison to controls (Fig. 6A and 6B). The transwell chamber invasion assay outcomes also revealed a remarkable downfall in the number of invasive cells in comparison to controls (Fig. 7A and 7B). Therefore, the transwell chamber migration and invasion assays collectively showed that betulinic acid could significantly inhibit the malignant metastatic feature of the SGC-7901 cells.

DISCUSSION

Gastric cancer is a highly destructive and malignant disorder associated with the human digestive tract. Gastric cancer (including adenocarcinoma of the stomach and gastro-esophageal junction) has been ranked as

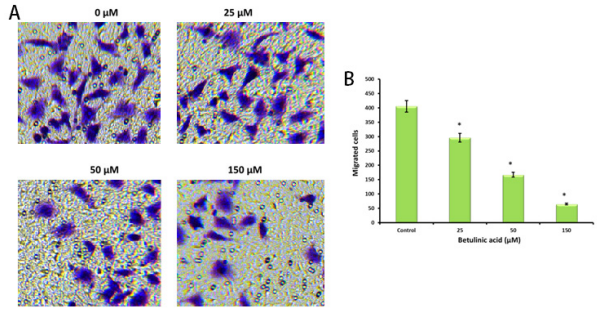

Figure 6. (A) Transwell chamber migration assay was used to estimate the migratory potential of SGC-7901 cells after betulinic acid exposure at indicated concentrations.
Results showed reduced number of migrated cells in case of betulinic acid treatment, as compared to controls. Experiments for each betulinic acid concentration were repeated thrice.
(B) Graphical representation of the effect of various doses of betulinic acid on cell migration.
Data were presented as mean ±S.E. with *p<0.05 considered as statistically significant, in comparison to control group.

the second in terms of cancer mortality and prevalence worldwide (Machlowska *et al.,* 2020). Gastric cancer incidences are prevailing at higher frequency in South America, Eastern Asia and Eastern Europe. In most cases, gastric cancer diagnosis is very poor until later stages due to its asymptomatic behavior. The cytotoxic chemotherapy being the lone backbone for advanced gastric cancer systemic treatment leads to very low 5-year survival chances of >10% (Jim *et al.,* 2017). For advanced gastric cancer, fluoropyrimidine and platinum based combination therapy is used as the first line treatment (Takahari, 2017). Herein, this research was designed to estimate the anticancer potency of betulinic acid against gastric cancer SGC-7901 cells. Results indicated that betulinic acid has remarkably inhibited the proliferation of gastric cancer SGC-7901 cells, in a dose-dependent manner. In order to determine the toxicity of betulinic acid against normal gastric cells, we evaluated its effects on proliferation of normal GES-1 gastric cells. Results confirmed that betulinic acid induced minuscule effects on proliferation of the GES-1 cells in comparison to the SGC-7901 cells. The ability of SGC-7901 cells to establish colonies was significantly reduced upon exposure to betulinic acid. Chemopreventive drugs are designed in such a manner that they could choose specific therapeutic targets within

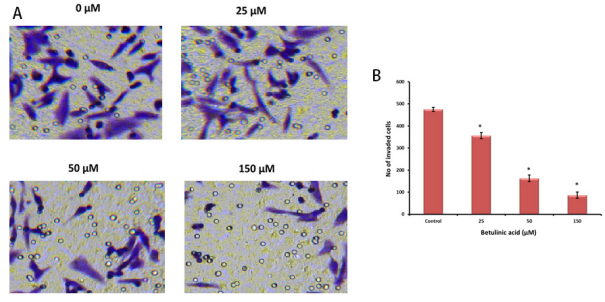

Figure 7. (A) Transwell chamber invasion assay was implemented to estimate the invasive potential of SGC-7901 cells after betulinic acid exposure at indicated concentrations.
Results showed reduced number of invasive cells in case of betulinic acid treatment, as compared to controls.
(B) Graphical representation of the effect of various doses of betulinic acid on cell invasion.
Data were presented as mean ±S.E. with *p<0.05 considered as statistically significant, in comparison to control group.

cancer cells (Klampfer, 2006). There have been numerous therapeutic targets identified till date, with naturally occurring programmed cell death (PCD) being the primary once. Autophagy is a leading target of chemopreventives and a type-II PCD (Mizushima, 2007). Betulinic acid induced autophagic cell death in the SGC-7901 cells, as was evident by generation of autophagosomes, and enhanced expressions of LC3-I and LC3-II and reduced expressions of LC3-I and p62. Metastatic feature of cancer cells is another major target for chemopreventives (Andreasen *et al.,* 1997). Cancer cells undergo uncontrolled frequent proliferation with migration and invasion to distant places, wherein leading to spreading of the disease away from source (Walter *et al.,* 2011). Betulinic acid retarded the potency of migration and invasion of the SGC-7901 cells, thereby inhibiting the metastatic feature of gastric cancer. The pathway of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MEK) is a modulatory pathway that regulates several fundamental cellular processes (Mochizuki *et al.,* 1999; Forshew *et al.,* 2009). Recently, various endogenous inhibitors and scaffolding proteins have been recognized, and their vital functions in modulating signaling via this pathway are emerging at present (Kolch, 2005). Herein, we found that betulinic acid treated SGC-7901 cells showed lower expressions of p-MEK and p-ERK, indicating inhibition of phosphorylation of MEK and ERK by betulinic acid. This suggested that betulinic acid blocked ERK/MEK signaling in the SGC-7901 cells.

Betulinic acid was observed to have significant suppressive effects against the ERK/MEK signaling pathway in gastric cancer SGC-7901 cells.

CONCLUSION

The results of investigation presented here revealed that betulinic acid possesses a striking anti-gastric cancer activity. The effects of cancer suppression were observed to operate via induction of autophagic cell death, suppression of cell migration and invasion, and modulation of the ERK/MEK signaling pathway.

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