Ethanol extract of *Patrinia scabiosaefolia* induces the death of human renal cell carcinoma 786-O cells via SIRT-1 and mTOR signaling-mediated metabolic disruptions

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Abstract. Recently, natural plant extracts have shown tremendous potential as novel antitumor drugs. *Patrinia scabiosaefolia*, a traditional prescription for inflammatory diseases, has been reported to effectively suppress various types of cancers. However, the mechanisms underlying its antitumor properties remain elusive. In the present study, we investigated the antitumor effects of an ethanol extract of *Patrinia scabiosaefolia* (EPS) on human renal cell carcinoma 786-O cells. After 24 h of incubation with EPS, the cell viability and colony number of 786-O cells were significantly decreased in a concentration-dependent manner as compared to the control group as determined by MTT and colony formation assays, respectively. The necrotic rate and apoptotic rate in the EPS exposure group were significantly higher than these rates noted in the control group as revealed by LDH release assay and Hoechst 33342/ PI double staining, respectively. At the concentration of 1.0 mg/ml, the necrotic and apoptotic rates reached 41.7±6.6 and 7.8±1.4%, respectively ($P<0.01$). However, the fluorescence intensity of intracellular calcium concentration ($[Ca^{2+}]_i$) was markedly elevated from 0.029±0.0007 to 0.060±0.003 ($P<0.001$) after the intervention of EPS. Moreover, the fluorescence intensity of intracellular ROS in the EPS exposure group was significantly higher (0.074±0.005) compared to that observed in the control group (0.033±0.001, $P<0.001$), which was partly attenuated by the specific antioxidant *N*-acetylcysteine (NAC). Furthermore, our results demonstrated that EPS significantly downregulated the expression of SIRT-1 and obviously induced the dephosphorylation of mTOR. Moreover, combined treatment with the SIRT-1 inhibitor nicotinamide and EPS was able to significantly enhance the induction of necrosis and reduction in cell viability of 786-O cells noted following treatment with EPS alone. In summary, we conclude that EPS induced the death of 786-O cells via SIRT-1 and mTOR signaling-mediated metabolic disruptions, which provide novel insight into the application of natural plant extracts for the treatment of cancers.

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Abbreviations: EPS, ethanol extract of *Patrinia scabiosaefolia*; ROS, reactive oxygen species; SIRT-1, sirtuin-1; mTOR, mammalian target of rapamycin

Key words: Patrinia scabiosaefolia, 786-O cells, antitumor, metabolic disruptions, SIRT-1, mTOR

Introduction

Traditional Chinese medicine (TCM), including various natural plant extracts, has shown tremendous potential for the discovery of alternative drugs for the treatment of cancer (1,2). Fewer adverse effects and cost effectiveness endow TCM great advantages over Western chemotherapeutics (3). *Patrinia scabiosaefolia*, a perennial plant mainly distributed in East Asia, is extensively prescribed for various diseases, such as oxidative damage (4), inflammation (5), tumors (6) and edema (7). Of all the above-mentioned effects, antitumor activity is a prominent characteristic of *Patrinia scabiosaefolia*. Peng *et al* reported the *Patrinia scabiosaefolia* inhibited the growth of different cancer cell models including colorectal cancer mouse tumor tissues, HT-29 and U266 through G1/S cell cycle arrest, suppression of tumor angiogenesis and the STAT3 pathway, respectively (6,8,9). A previous study demonstrated that *Patrinia scabiosaefolia* induced the apoptosis of breast carcinoma MCF-7 cells without caspase-9 activation (10). However, the mechanisms underlying its antitumor properties emain elusive, and the antitumor effects of *Patrinia scabiosaefolia* deserve increased attention.

Recently, research which has focused on exploiting metabolic perturbations in the area of cancer therapy has been extensively investigated (11). ROS homeostasis serves as a critical factor for the survival of cancer cells. There is

evidence indicating that cancer cells are more vulnerable to high levels of ROS (12), which further evokes a series of metabolic dysfunction, such as apoptosis, calcium overload and bio-macromolecule degradation (13-15). The calcium ion, as a ubiquitous secondary messenger, induces intracellular ROS generation. In turn, ROS can regulate the Ca^{2+} signaling pathway (14). Excessive accumulation of ROS and calcium overload can initiate the apoptotic pathway (16). The reciprocal interactions of the above three alterations intensively promote the death processes of cancer cells. Thus, manipulation of metabolic disruptions of cancer cells may be a promising target for cancer treatment.

SIRT-1, as an NAD^+-dependent deacetylase, has been demonstrated to regulate extensive cellular metabolic processes including cell stress response, apoptosis and lifespan extension (17-19). A previous study indicated that excessive expression of SIRT-1 in large B-cell lymphoma was closely related to poor patient prognosis (20). Recently, SIRT-1 has been demonstrated to function as an oncoprotein and tumor promoter in various types of cancer cells (21,22). Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, has been widely accepted to regulate cell growth and proliferation (23). Various chronic diseases, such as cancer, ageing and diabetes, are closely correlated with mTOR (24). There is evidence indicating that mTOR-dependent mechanisms are responsible for the tumorigenesis of many types of cancers (25). Thus, we speculated that SIRT-1 and mTOR may be involved in the EPS-induced antitumor effects.

In the present study, we investigated the antitumor effects of an ethanol extract of *Patrinia scabiosaefolia* (EPS) on 786-O cells. MTT assay, colony formation assay and Hoechst/propidium iodide (PI) staining were performed to detect the inhibition of proliferation and the pro-apoptotic effects of EPS. Intracellular ROS and Ca^{2+} were examined to identify the metabolic disruptions induced by EPS. However, we conducted western blotting to explore the expression of SIRT-1 and mTOR after EPS stimulation. Furthermore, the above antitumor effects were found to be augmented by the SIRT-1 inhibitor nicotinamide. Thus, we conclude that EPS induced the death of 786-O cells via SIRT-1 and mTOR signaling-mediated metabolic disruptions.

Materials and methods

Cell culture and reagents. Human renal cell carcinoma 786-O cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), and human proximal tubular cells (HK-2) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) supplemented with 10% FBS (all from HyClone, Logan, UT, USA). Both types of cells were cultured under the condition of 95% humidity and 5% CO_2 at 37˚C. After passaging, the cells were cultured for 24 h and then treated with different concentrations of EPS for 24 h. MTT was provided by Solarbio (Beijing, China). Hoechst 33342 and PI, reactive oxygen species assay kit, Fluo-3 AM probe, *N*-acetylcysteine (NAC) and LDH cytotoxicity assay kit were purchased from Beyotime Biotechnology (Shanghai, China). Antibodies for SIRT-1, p-mTOR, mTOR and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). All other reagents used were commercially available.

Preparation of an ethanol extract of Patrinia scabiosaefolia. *Patrinia scabiosaefolia* Link (also named *Patrinia hispida* Bunge and *Patrinia scabiosaefolia* Fisch. ex Trev (Caprifoliaceae family) was provided by Shennong Bencao Pharmacy (Shandong, China) and authenticated by Professor Aidong Lang, the analyst of the School of Pharmacy at Shandong University. Dry herbs of *Patrinia scabiosaefolia* (the plant name has already been verified with http://www.theplantlist.org) of 1 kg were drenched in 95% ethanol (10 l) for 3 days, and then filtered. The filtrates were evaporated in a vacuum evaporator. The relative density was determined as 1.05. The EPS powder was obtained by a freeze-dryer. Subsequently, EPS was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution with the concentration of 300 mg/ml. The final concentrations of DMSO in all the culture medium were <0.5%. *Patrinia scabiosaefolia* specimen was deposited at the Urology Laboratory of Cardiovascular Research Center in Qilu Hospital of Shandong University.

MTT assay. MTT assay was performed to evaluate the cell viability of 786-O and HK-2 cells. 786-O and HK-2 cells were seeded into 96-well plates at a density of $5x10^3$/well, and incubated with different concentrations of EPS for 24 h. Subsequently, MTT (0.5 mg/ml) was added into each well and the cells were incubated for 4 h under conditions of 37˚C and 5% CO_2. Then, the supernatant was removed and the formazan crystals were dissolved in 100 μl DMSO. The absorption value was measured at 570 nm by an ELISA reader (Thermo Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA).

Colony formation assay. 786-O cells were seeded in 6-well plates at the density of $1x10^3$/well, and were then cultured for 10-14 days after stimulations. When colonies were visible, the incubation was terminated. Subsequently, the cells were rinsed with phosphate-buffered saline (PBS) and stained with crystal violet after being fixed with methanol. Colonies >50 cells were counted under a microscope with low magnification.

Hoechst 33342 and PI double staining. Hoechst/PI double staining was conducted to examine the apoptotic rate of 786-O cells. In brief, 786-O cells were cultured with 10 μg/ml Hoechst 33342 and PI at 37˚C for 15 min and then rinsed with PBS three times after stimulation. Morphological alterations of 786-O cells were observed by fluorescence microscope (Carl Zeiss SAS, Jena, Germany). Hoechst 33342 can penetrate the normal cell membrane, whereas PI merely penetrates the damaged cell membrane. Cell staining with Hoechst 33342 manifests blue fluorescence, whereas cells stained with PI manifest red fluorescence. The apoptotic rate and necrotic rate of 786-O cells were identified as the percentages of apoptotic cell nuclei and necrotic cell nuclei in five random fields.

LDH release assay. To further verify the necrosis promoting effect of EPS, LDH release assay was conducted. 786-O cells were plated in a 96-well at the density of $5x10^3$/well. After a 24-h treatment with EPS, 100 μl of the supernatant was transferred to a clear 96-well plate, and then 50 μl LDH reaction working solution was added to each well for 30 min at room temperature. Subsequently, the absorption value was measured

at 490 nm by an ELISA reader (Thermo Multiskan MK3). The relative LDH activity was represented by the following equation: $(OD_{sample} - OD_{blank})/(OD_{max} - OD_{blank})$.

ROS assay. ROS assay was performed to investigate the level of intracellular ROS. 786-O cells were seeded into a 24-well at the density of $3x10^4$/well. The cells were co-incubated with 250 μl DCFH-DA probe (10 μM, 1:1,000 diluted with RPMI-1640 medium) at 37˚C for 20 min after stimulation. Then, the cells were rinsed with medium for three times and 200 μl of the medium was added into every well. All the images were captured by fluorescence microscopy and the fluorescent intensity was analyzed by ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA].

$[Ca^{2+}]_i$ measurement. 786-O cells were plated into a 24-well plate at the density of $3x10^4$/well. After EPS stimulation, the cells were washed by PBS, and then incubated with 5 μM Fluo-3 AM probe for 30 min at 37˚C. Subsequently, the cells were washed with PBS and then observed by fluorescence microscopy. The fluorescence intensity analysis was performed by ImageJ software.

Western blotting. 786-O cells were harvested by RIPA buffer plus phenylmethylsulfonyl fluoride (PMSF), and then total proteins were collected after stimulation. The concentrations of proteins were measured using the BCA method. Protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands of interest were subsequently electrotransferred to a nitrocellulose (NC) membrane and blocked with 5% skim milk for 1 h at room temperature. Incubations were conducted with primary antibodies (1:1,000) against GAPDH, p-mTOR, mTOR and SIRT-1 at 4˚C overnight and subsequently secondary antibodies (1:5000) under room temperature for 1 h. After that, the gray scale values of the bands were detected using western immunoblotting detection (ECL system) reagents and then semi-quantitative analyzed by ImageJ software.

Statistical analysis. All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were conducted with one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis and unpaired t-test. The value of $P<0.05$ was considered as statistically significant. In addition, GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used in all processes of the statistical analysis.

Results

EPS significantly inhibits the proliferation and growth of 786-O and HK-2 cells. The ethanol extract of *Patrinia scabiosaefolia* (EPS) exhibited significant antiproliferation effects on the 786-O cells. Under the stimulation of EPS, cell viability of the cells was markedly decreased in both concentration-dependent and time-dependent manners compared to the control group. At the concentration of 0.6 mg/ml, 786-O cells exposed to EPS had a cell viability of 0.49±0.12 ($P<0.05$), whereas, there was only 0.14±0.01 ($P<0.001$) viability when the concentration reached 1.0 mg/ml (Fig. 1B). Morphological alterations were consistent with the above results. The cells shrunk into a bright-round shape and less number of cells were noted with shorter processes following treatment with EPS. These damages were enhanced with the increasing EPS concentration (Fig. 1A). The time-dependent manner of the antiproliferation effects was also determined by MTT assay (Fig. 1C). Furthermore, as shown in Fig. 1D and E, the colony count significantly decreased from 100.0±1.5 to 20.3±0.9 when the cells were co-incubated with EPS at 0.2 mg/ml for 24 h ($P<0.001$). Whereas, almost no colonies could be observed under the concentration of 0.6 mg/ml EPS ($P<0.001$).

Human proximal tubular cells (HK-2) were also used to investigate the effects of EPS on normal cells. As shown in Fig. 1D, the cell viability of the HK-2 cells was significantly decreased in a concentration-dependent manner. Cell viabilities of 0.793±0.062 ($P<001$) and 0.257±0.013 ($P<0.001$), respectively were noted at concentrations of 0.6 and 1.0 mg/ml.

EPS significantly promotes apoptosis and necrosis of 786-O cells. Apoptosis, which is characterized by chromatin condensation and nuclear shrinkage, is identified by bright blue fluorescence following staining with Hoechst 33342/PI. In contrast, red fluorescence indicates necrotic cells due to the penetration of PI and viable cells show dull blue fluorescence. As shown in Fig. 2, more necrotic and apoptotic cells were detected in the EPS stimulation group as compared to the control group. At the concentration of 0.6 mg/ml, the apoptotic rate was 8.3±0.7% and the necrotic rate was 21.2±4.6% compared to 0.4±0.2% for apoptosis and 0.4±0.4% for necrosis in the control group. When the concentration of EPS increased to 1.0 mg/ml, the necrotic rate of cells was markedly increased from 21.2±4.6 to 41.7±6.6% ($P<0.05$). However, there was no significant difference regarding the apoptotic rate between treatment with EPS concentrations of 0.6 and 1.0 mg/ml ($P>0.05$).

To further verify the promotion effect of EPS on necrosis, LDH release assay was performed. There is a positive linear relationship between the level of LDH release and the necrotic rate. As shown in Fig. 2L, as the EPS concentration increased, the level of LDH release was significantly elevated in a concentration-dependent manner.

At the concentration of 1.0 mg/ml, the LDH release rate was 0.455±0.021 ($P<0.001$), which was consistent with the necrotic rate determined by Hoechst 33342/PI staining.

Effects of EPS on the intracellular ROS level. To investigate the oxidative damage effects of EPS on 786-O cells, ROS assay was performed to measure the intracellular ROS level. H_2DCFDA can be hydrolyzed by intracellular esterases, whereas ROS oxidates non-fluorescent H_2DCFDA to convert to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Thus the level of green fluorescence can indicate the intracellular ROS level. As shown in Fig. 3, the intracellular ROS level was significantly elevated compared to that noted in the control group after EPS exposure ($P<0.01$). However, in the presence of 10 mM NAC, the level of intracellular ROS was significantly decreased compared to that following treatment with 0.6 mg/ml EPS alone ($P<0.05$). However, there was no obvious concentration-dependent trend in the EPS-induced ROS increase ($P>0.05$).

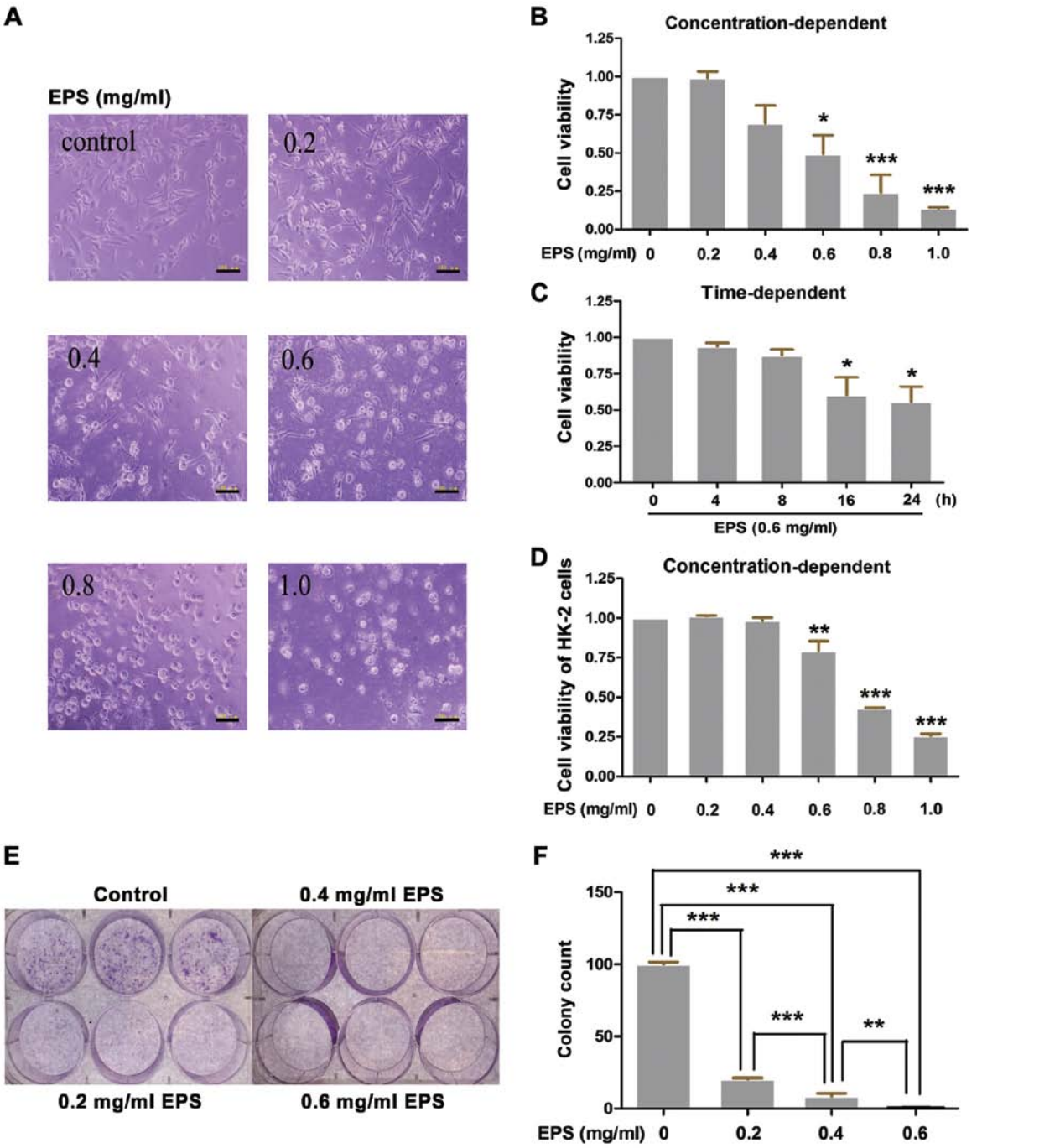

Figure 1. Ethanol extract of *Patrinia scabiosaefolia* (EPS) significantly inhibits the proliferation and growth of 786-O and HK-2 cells. (A and B) 786-O cells were cultured with different concentrations of EPS (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) for 24 h. (B) Then, cell viability was measured by MTT assay and (A) images of morphological alterations were captured by microscopy; *P<0.05, ***p<0.001, vs. 0 mg/ml. (C) 786-O cells were incubated with EPS at 0.6 mg/ml, and then cell viability was measured at 0, 4, 8, 16 and 24 h, respectively; *P<0.05, vs. 0 h. (D) HK-2 cells were cultured with different concentrations of EPS (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) for 24 h and then cell viability was measured by MTT assay; **P<0.01, ***p<0.001, vs. 0 mg/ml. (E and F) Colony formation assay was performed. After incubation with different concentrations of EPS (0, 0.2, 0.4 and 0.6 mg/ml) for 24 h, 786-O cells were cultured for 10-14 days, and then the colony formation was determined. (E) The difference in the colony count was analyzed; **P<0.01, ***p<0.001. All data were analyzed by one-way ANOVA with post hoc test (n=3). Scale bars in A, 100 μm.

Effects of EPS on the intracellular calcium concentration. To explore the metabolic perturbation effects of EPS on 786-O cells, we detected the intracellular calcium concentration by Fluo-3 AM probe. These probes specifically bind to $[Ca^{2+}]_i$ and emit green fluorescence. As shown in Fig. 4, the mean fluorescence intensity of the EPS exposure group was significantly higher than that of the control group (P<0.01). In addition, the above effects were significantly enhanced with the increase in EPS concentration (P<0.001).

EPS downregulates the expression of SIRT-1 and induces the dephosphorylation of mTOR. Western blotting was performed to investigate the involvement of SIRT-1 and mTOR signaling in the antitumor effects of EPS. As shown

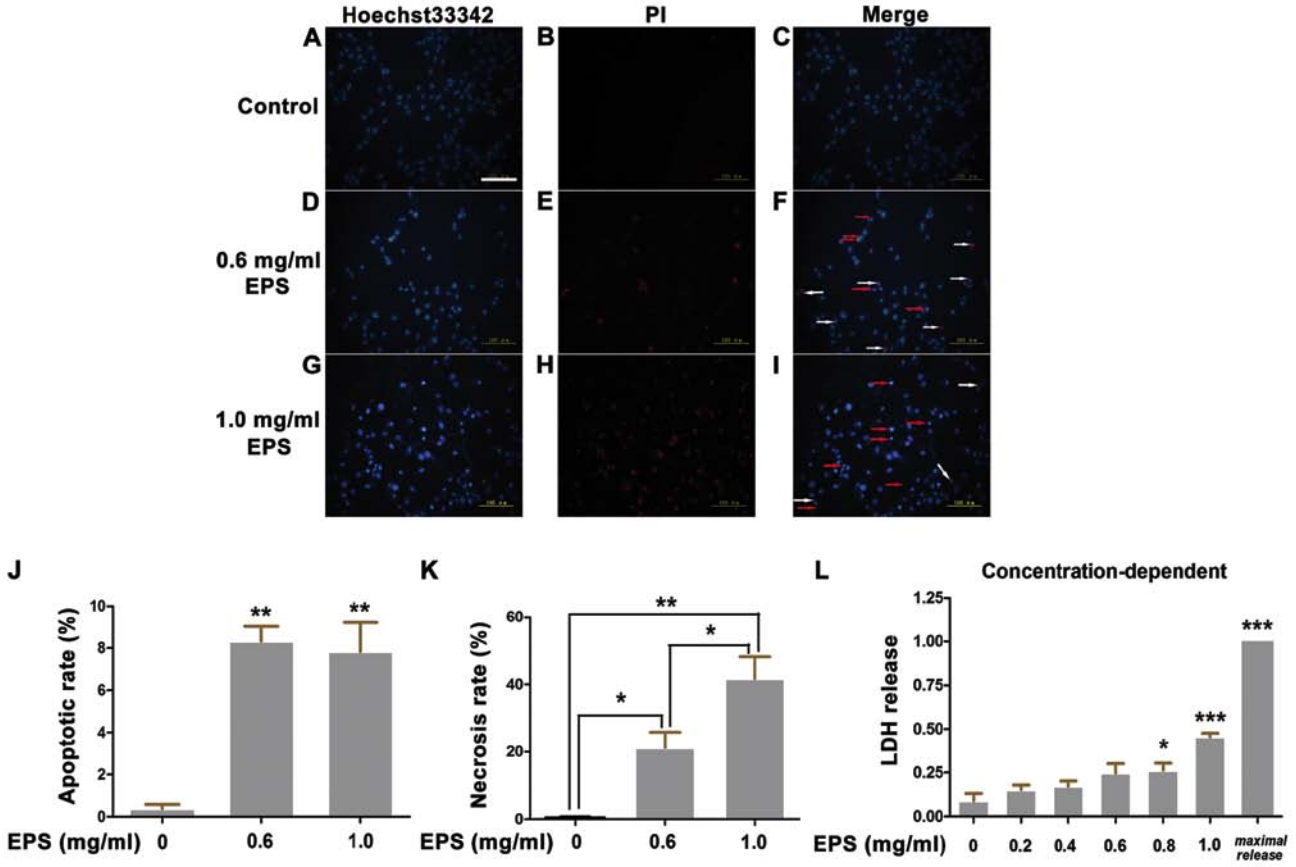

Figure 2. Ethanol extract of *Patrinia scabiosaefolia* (EPS) markedly promotes the apoptosis and necrosis of 786-O cells. 786-O cells were cultured with different concentrations of EPS (0, 0.6 and 1.0 mg/ml) for 24 h before Hoechst/PI staining and LDH release measurement. (A-I) Morphological changes were observed by fluorescence microcopy. Red fluorescence indicates necrotic cells whereas bright blue fluorescence indicates apoptotic cells. White arrows indicate necrotic cells whereas yellow arrows indicate apoptotic cells. (J) Apoptotic rate; **P<0.01, vs. 0 mg/ml. (K) Necrosis rate; *P<0.05, 0.6 mg/ml vs. 0 mg/ml and 1.0 vs. 0.6 mg/ml; **P<0.01, 0 mg/ml vs. 1.0 mg/ml. (L) LDH release; *P<0.05, vs. 0 mg/ml; ***P<0.01, vs. 0 mg/ml. All data were analyzed by one-way ANOVA with post hoc test (n=3). Scale bars: A-I, 100 μm.

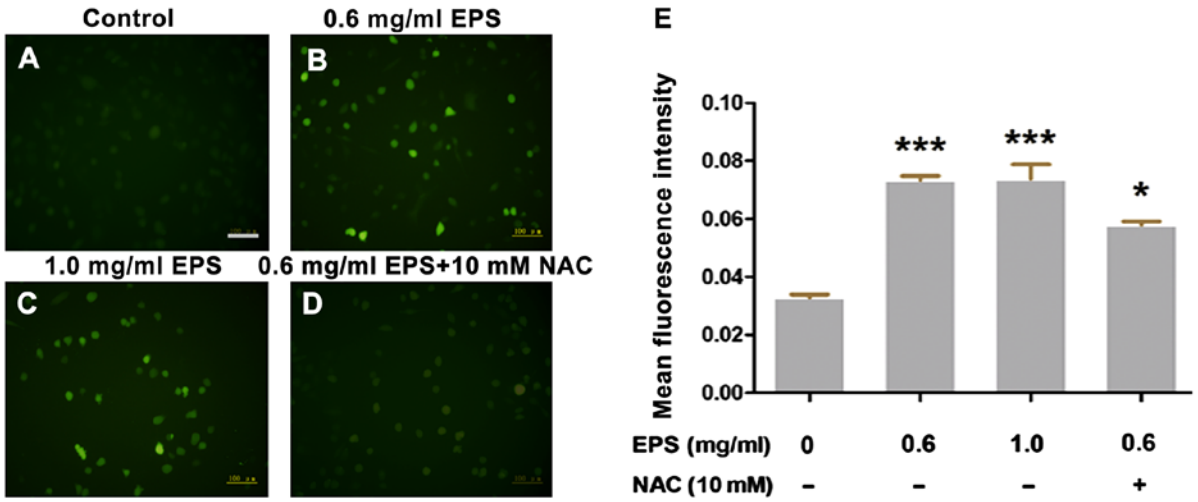

Figure 3. Ethanol extract of *Patrinia scabiosaefolia* (EPS) significantly induces intracellular ROS increase in 786-O cells. 786-O cells were incubated with different concentrations of EPS (0, 0.6 and 1.0 mg/ml) for 24 h (A-C) and with the presence of 10 m NAC (D) before staining. The intensity of green fluorescence indicates the intracellular ROS level. (A-D) The morphological alterations were captured by fluorescence microcopy. (E) The mean fluorescent intensity was analyzed by ImageJ; *P<0.05, vs. 0.6 mg/ml EPS only; ***P<0.001, vs. 0 mg/ml). All data were analyzed by one-way ANOVA with post hoc test (n=3). Scale bars: A-D, 100 μm.

in Fig. 5, EPS significantly downregulated the expression of SIRT-1 (P<0.05) and decreased the ratio of p-mTOR/mTOR (P<0.001) in the 786-O cells. Nevertheless, there was no statistically significant difference regarding the ratio of

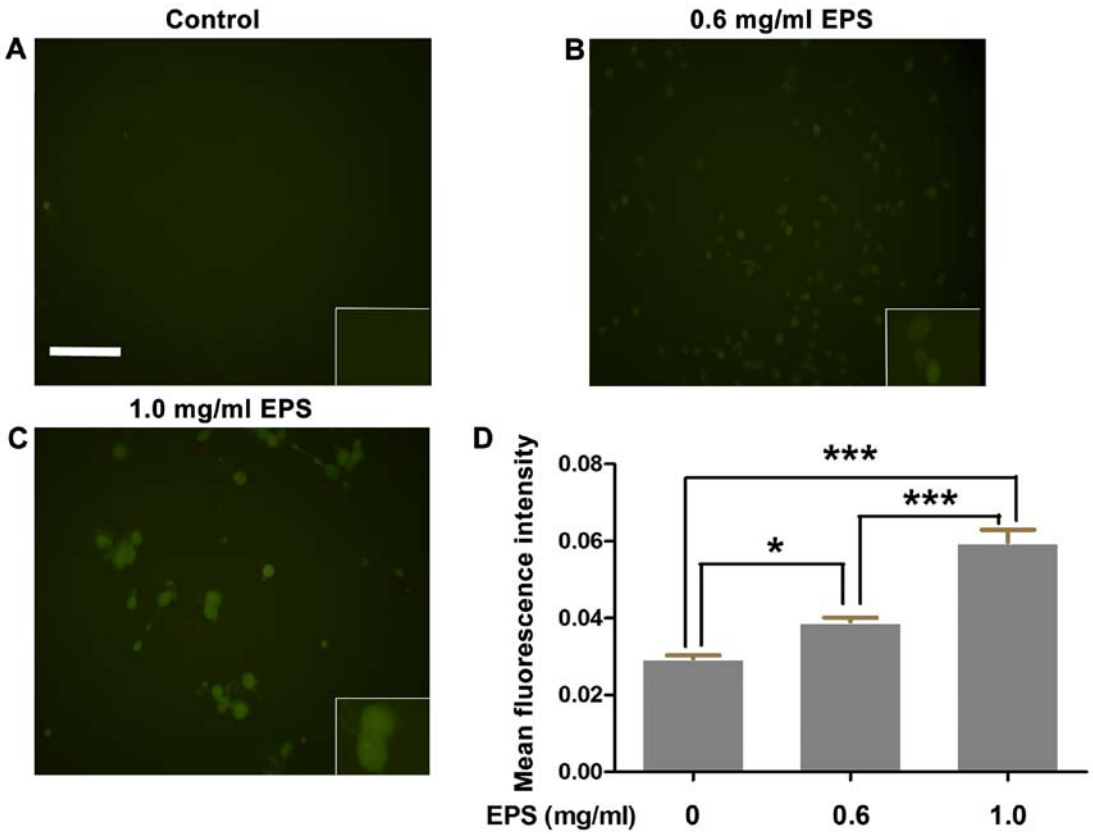

Figure 4. Effects of an ethanol extract of *Patrinia scabiosaefolia* (EPS) on intracellular calcium concentration. After incubation with different concentrations of EPS (0, 0.6 and 1.0 mg/ml), 786-O cells were stained for $[Ca^{2+}]_i$. The intensity of green fluorescence represents the intracellular calcium concentration. (A-C) The morphological alterations were captured by fluorescence microcopy. Scale bars: A-C, 100 μm. (D) The mean fluorescent intensity was analyzed by ImageJ. All data were analyzed by one-way ANOVA with post hoc test (n=3); *P<0.05, ***p<0.001.

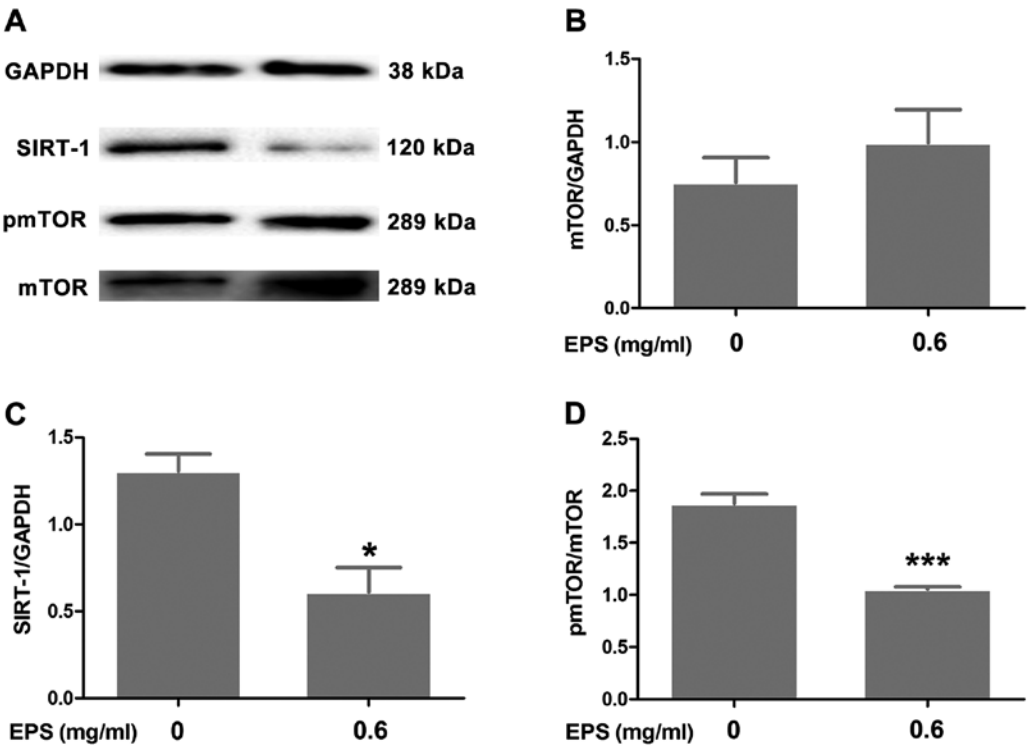

Figure 5. Ethanol extract of *Patrinia scabiosaefolia* (EPS) significantly suppresses the expression of SIRT-1 and induces the dephosphorylation of mTOR. 786-O cells were cultured with different concentrations of EPS (0 and 0.6 mg/ml) for 24 h and then the proteins were collected for western blotting. GAPDH was used as a loading control. (A) The representative bands of interest and (B-D) the statistical analyses of the gray scale are represented. SIRT-1 was normalized to GAPDH. Phosphorylated mTOR was normalized to total mTOR. Total mTOR was normalized to GAPDH; *P<0.05, ***P<0.001, vs. 0 mg/ml. All data were analyzed by unpaired t-test (n=3).

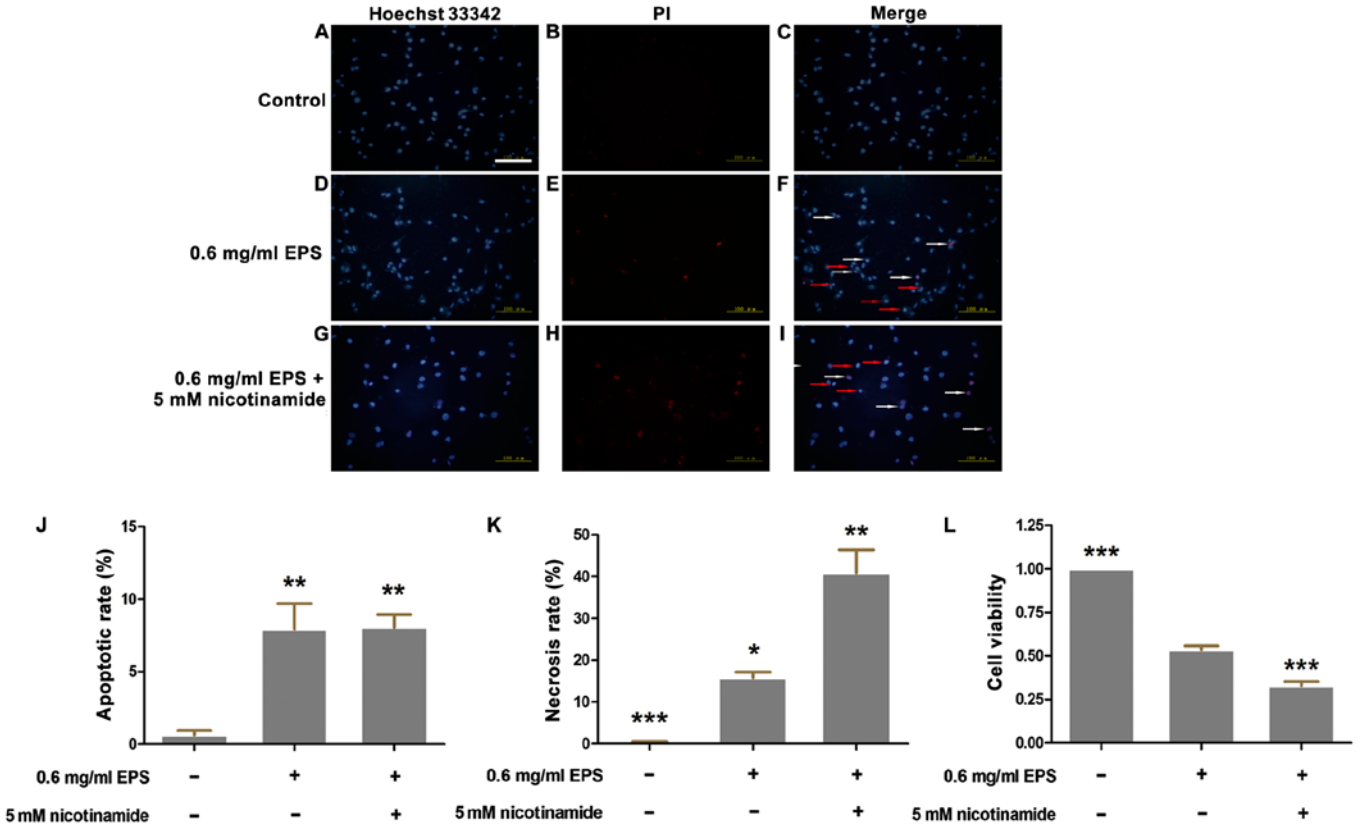

Figure 6. Nicotinamide partly enhances the antitumor effects of EPS. 786-O cells were incubated at different concentrations (0 and 0.6 mg/ml) of EPS with or without the presence of 5 mM nicotinamide for 24 h. (A-I) The morphological changes were observed by fluorescent microcopy. White arrows indicate necrotic cells whereas yellow arrows indicate apoptotic cells. Scale bars: A-I, 100 μm. (J) Apoptotic rate as detected by Hoechst 33342/PI staining; **P<0.01 vs. 0 mg/ml. (K) Necrotic rate as detected by Hoechst 33342/PI staining; *P<0.05, vs. 0 mg/ml; **P<0.01, vs. 0.6 mg/ml EPS; ***P<0.001 vs. 0.6 mg/ml EPS + 5 mM nicotinamide. (L) MTT assay was performed to measure the cell viability; ***P<0.001 vs. 0.6 mg/ml EPS. All data were analyzed by one-way ANOVA with post hoc test (n=3).

mTOR/GAPDH between the EPS exposure group and the control group (P>0.05).

SIRT-1 inhibitor nicotinamide partly enhances the antitumor effects of EPS. To further corroborate the involvement of SIRT-1 signaling in the antitumor effects of EPS, nicotinamide, a specific SIRT-1 inhibitor, was adopted for co-incubation with EPS. Cell viability assay and Hoechst 33342/PI double staining were carried out to explore the synergetic effects of nicotinamide. As shown in Fig. 6, the cells co-cultured with EPS plus nicotinamide had a significantly higher necrotic rate of 40.9±5.5% than that of 15.8±1.3% in the EPS group (without nicotinamide, P<0.01). The results of the MTT assay verified the above phenomena. The cell viability of the nicotinamide and EPS exposure group was significantly lower (0.33±0.02) compared to the EPS exposure group (0.54±0.02) (P<0.001). However, there was no significant difference regarding the apoptotic rate between the nicotinamide and EPS exposure group and the EPS exposure only group (P>0.05).

Discussion

In the present study, we initially investigated the antitumor effects of EPS on 786-O cells *in vitro*. Our results demonstrated that EPS showed vigorous antiproliferation and growth inhibitory effects even at a low concentration of 0.6 mg/ml, which needed a x500 dilution of the stock solution. Certain fluorescence staining assays were performed to identify the manner of cell death and the involvement of metabolic disruption. The results indicated that an increase in ROS and calcium overload may be responsible for the processes of metabolic perturbations. Furthermore, the underlying molecular mechanism was explored and we found that SIRT-1 and mTOR signaling were involved in the antitumor effects of EPS.

Previous studies have reported that *Patrinia scabiosaefolia* showed marked antiproliferation effects on various types of tumor cells, including HT-29, MCF-7 and HeLa cells (4,10,26). Our results demonstrated that EPS significantly decreased the cell viability of 786-O cells in concentration- and time-dependent manners. As shown in Fig. 1A, the effects of EPS on cells involved not only antiproliferation, but also death-promotion based on morphological observation. Moreover, EPS markedly impeded the formation of cell colonies. However, the concentration-dependent tendency in the colony formation assay was not in accordance with the MTT results. We speculated that the lower initial cell seeding number may be responsible for this inconsistency. The interactions of cells are vital to their survival. Thus, a lower cell number made the cells more vulnerable to EPS. As shown in Fig. 1D, EPS showed significant antiproliferation effects on HK-2 cells. However, at the concentration of 0.6 and 1.0 mg/ml, the cell viabilities of HK-2 cells were 0.793±0.062 and 0.257±0.013, while cell

viabilities of 786-O were 0.493±0.124 and 0.136±0.008. This result indicated that EPS exhibited, at least partly, specificity in regards to its antitumor effects.

Metabolic reprogramming, which refers to a series of metabolic alterations that stem from the molecular level, is a distinguishing characteristic of cancer cells (11). Changes in metabolism are critical for malignant proliferation and metastasis. However, these alterations also make the cells more susceptible to disruptions due to its metabolic dependency (27). As an essential role of metabolic regulation, ROS can affect the metabolism of cancer cells (12), and further cause cascaded reactions of metabolic perturbations including Ca^{2+} overload and apoptosis (14,16). Our results showed that EPS significantly induced apoptosis and necrosis of 786-O cells. However, intracellular Ca^{2+} and ROS levels were markedly elevated after the exposure of EPS, while the antioxidant NAC partly reversed the increase in ROS induced by EPS. Therefore, metabolic disruptions may mediate the antitumor effects of EPS, which indicate a new target for cancer intervention.

SIRT-1, as an NAD^+-dependent deacetylase, has been demonstrated to mediate extensive cellular metabolic processes including cell stress response, apoptosis and cell cycle arrest (17-19). SIRT-1 deacetylates a series of downstream molecules and induces extensive alterations, including an increase in ROS and apoptosis (28,29). Previous research has reported that MHY2256, an SIRT inhibitor, showed significant anticancer effects on MCF-7 and SKOV-3 cells through p53 acetylation (30). Accumulated evidence has demonstrated that overexpression of SIRT-1 is found in many malignant tumors including gastric cancer, hepatocellular carcinoma tissues, ovarian epithelial tumors and is closely linked with poor survival outcomes (21,31,32). Our results showed that the expression of SIRT-1 was significantly downregulated after EPS exposure, which was consistent with the above literature. Mammalian target of rapamycin (mTOR), formed by mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), has been implicated in metabolism, tumorigenesis and aging (24,33,34). The mTOR signaling pathway is involved in energy conservation, growth and division based on its protein kinase characteristics. PI3K/Akt/mTOR and mTOR/P53/P21 pathways have been demonstrated to be involved in antitumor effects by induction of autophagy and inhibition of proliferation (35,36). Wu *et al* reported that the Akt/GSK3β/mTOR pathway executes the effects of neuroprotection through an antioxidative mechanism (37). However, previous studies and clinical practice have also demonstrated that rapamycin, a specific inhibitor of mTOR, shows marked antitumor effects (24). Our results indicated that EPS obviously decreased the ratio of pmTOR/mTOR compared to the control group, which suggests that dephosphorylation of mTOR may induce the antitumor effects of EPS.

SIRT-1 and mTOR both mediate crucial molecular pathways in the signaling transduction network. However, there is no direct and explicit link between the two regulators to date (17). Previous study has reported that SIRT-1 negatively regulated mTOR signaling potentially mediated by sclerosis complex 2 (TSC2) (17). Guo *et al* reported that SIRT-1 mediated neuron regeneration by suppressing mTOR signaling (38). Nevertheless, there is evidence indicating that plumbagin induced the apoptosis and autophagy in prostate cancer cells by inhibition of both SIRT-1 and mTOR signaling (39). Therefore, to further substantiate the involvement of SIRT-1 signaling, we employed nicotinamide, an inhibitor of SIRT-1, to block expression of SIRT-1 signaling. The results demonstrated that nicotinamide significantly enhanced the antitumor effects of EPS, which matched our original inference.

However, the role of SIRT-1 and mTOR regarding tumorigenesis is still controversial and there must be several intermediate molecules between SIRT-1 and mTOR. Zhang *et al* reported that the behaviors of SIRT-1 in tumorigenesis depend on p53 mutations (18). Thus, the alterations of P53 in cancer tissue, along with SIRT-1 and mTOR signaling, deserve much attention in future experiments. Otherwise, given to the involvement of intracellular ROS, the role of MAPKs needs to be further investigated. The MAPK cascade can be activated by ROS via various mechanisms (40). Thus, elucidation of MAPK alterations may provide new insight into the mechanism underlying the antitumor effects of EPS.

Based on previous literature, 10 bioactive agents have been identified (41), including iridoids (42), saponins (41,43), triterpenes (44,45) and lactones (46). Detailed chemical fingerprint of NMR can be reviewed in the above literature. Forty-four essential oils of *Patrinia scabiosaefolia* have been identified by chemical fingerprinting of GC (Gas Chromatography) (4). Thus, identification of these bioactive components can provide valuable directions for further research.

In summary, the present study investigated the effects of EPS on 786-O cells and partly validated the involvement of ROS and Ca^{2+}-mediated SIRT-1 and mTOR signaling. We highlighted the markedly antitumor effects of EPS and provide novel insight in drug discovery for cancer treatments.

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