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As a novel anticancer candidate, ether extract of *Dendrobium nobile* overstimulates cellular protein biosynthesis to induce cell stress and autophagy

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Abstract
Increasing data has confirmed the potential anticancer properties of *Dendrobium*, a traditional Chinese herb. However, most anticancer compositions from the plant of *Dendrobium* were usually extracted by high polar solvent, while weak polar compositions with excellent anticancer activity remained largely unexplored. In this study, the differences between ether extract and ethanol extract of *Dendrobium nobile* Lindl. on chemical components and anticancer activities were investigated, as well as the anticancer mechanisms among different extracts. The results demonstrated that the ether extract exhibited a stronger anticancer effect than ethanol extract, and its anticancer effect was mainly due to weak polar compounds rather than polysaccharides and alkaloids. Quantitative proteomics suggested that the ether extract significantly stimulated the over-expression of immature proteins, the endoplasmic reticulum stress and unfolded protein response were subsequently induced, the intracellular reactive oxygen species level was seriously elevated, and oxidative stress occurred in the meanwhile. Eventually, autophagy and apoptosis were activated to cause cell death. Our findings demonstrate that the ether extract of *D. nobile* is a potential candidate for anticancer drug development, and that future research on anticancer drugs derived from medicinal plants should also concentrate on weak polar compounds.

Keywords: Anticancer; Autophagy; Cell stress; *Dendrobium nobile*; Ether extract

Highlights:
- Ether extract of *Dendrobium nobile* exhibited stronger anticancer ability than ethanol extract.
- Weak polar compounds were primarily responsible for the anticancer impact of ether extract.
- Ether extract of *D. nobile* could overstimulate the protein biosynthesis of cancer cells to induce cell stress and death.
- The future research of anticancer drugs from medicinal plants should focus more attention on weak polar compounds.

Abbreviations:
BCA, bicinchoninic acid; Bip, immunoglobulin binding protein; CCK-8, cell counting kit-8; *D., Dendrobium*; DMSO, dimethyl sulfoxide; DTGS, deuterated triglycine sulfate; DTT, dithiothreitol; ECL, efficient chemiluminescence; ER, endoplasmic reticulum; FBS, fetal bovine serum; FDR, false discovery rate; FTIR, fourier transform infrared spectroscopy; GO, gene ontology; HCD, normalized collision energy; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; IAA, iodoacetamide; PBS, phosphate buffer saline; PIG3, quinone oxidoreductase PIG3; PVDF, polyvinylidene fluoride; RAB7A, ras-related protein Rab-7a; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SILAC, stable isotope labeling with amino acids in cell culture; SQSTM, Sequestosome-1; TBST, tris buffered saline with Tween 20; UPR, unfolded protein response.

Introduction

Cancer is one of the leading causes of death globally, and cancer therapy medications are frequently in the spotlight (Prasad and Koch, 2014). Drugs derived from medicinal plants have played an essential role in illness therapy, and many herbal products used in traditional Chinese medicine can exert anticancer effects (Parekh et al., 2009; Zhao et al., 2016). Natural products from herb have also been deemed effective for boosting the efficacy and/or alleviating adverse effects of popular therapeutic medicines (Smith et al., 2014; Wang et al., 2015). As a result, discovering novel natural components for cancer therapy is always a hot issue for herb research.

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Dendrobium is the second largest genus in *Orchidaceae* (Lam et al., 2015), with 74 species and 2 variants discovered in China (Luo et al., 2013). In traditional Chinese medicine, some *Dendrobium* plants are considered as medicinal plants. As early as 200 B.C., *Dendrobium* was described as a source of tonic, astringent, analgesic, and anti-inflammatory substances in the Chinese pharmacopoeia "The Sang Nung Pen Tsao Ching" (Lam et al., 2015). Many bioactive constituents of *Dendrobium* species have been identified in current herb research, and the anticancer components and activity have received substantial attention in recent years (Ng et al., 2012). Some *Dendrobium* species such as *D. catenatum* (Zheng et al., 2015), *D. draconis* (Charoenrungruang et al., 2014), *D. candidum* (Guo et al., 2019), *D. formosum* (Prasad and Koch, 2014), and *D. nobile* (Lu et al., 2014), have been identified as resource candidates for anticancer drug development.

In traditional Chinese medicine, decoctions (boiled water extracts) are the most frequently utilized pharmaceutical type for disease treatment (Yip et al., 2016). Chinese people also soak *Dendrobium* in Chinese liquor (which contains 52% ethanol) as a healthy herbal drink. In modern studies, a high polar solvent such as methanol and ethanol are usually used to extract anticancer constituents from *Dendrobium* (Guo et al., 2019; Prasad and Koch, 2014; Wang et al., 2014; Zhao et al., 2014). However, the high polar solvent may not be adequate for extracting the weak polar anti-tumor components, resulting in the absence of weak polar components with anticancer activity.

Dendrobium nobile Lindl. (*D. nobile*) is a representative species for "Dendrobii Caulis" in Chinese Pharmacopoeia (2020 Edition). Many kinds of crude plant extracts, such as polysaccharide, denbinobin and dendrobine extracted from *D. nobile*, exhibited anticancer efficacy. Water, methanol, ethanol, and even ethylacetate were commonly used as extraction solvents (Lee et al., 1995; Song et al., 2012; 2019; Wang et al., 2010; Yang et al., 2012; Zhang et al., 2019). However, in our study we found that the weak polar solvent ether was more efficient at extracting anticancer components from *D. nobile*, and substantial changes in extract compositions were discovered. The results suggested that ether extract of *Dendrobium* might be a superior source for developing novel anticancer drugs. We studied the constituent difference between ether extract and ethanol extract, as well as the association between the extract composition and anticancer efficacy. The anticancer mechanism of *D. nobile* ether extract was also investigated. This work provides a new perspective for the development of anticancer drugs from medicinal plants.

Materials and methods

Preparation of extracts of D. nobile

The *D. nobile* was purchased from the Miaoling Chinese Medicinal Materials Co., Ltd. (Hejiang County, Sichuan Province, China). It was authenticated by Prof. Ze Chun (Chengdu Institute of Biology, Chinese Academy of Sciences). The stems of *D. nobile* were washed and dried at 50 °C. It was then grinded to powder and sieved using a 200 mesh sieve. Dried powder (10 g) was suspended in 200 ml solvent (ether, absolute ethanol and 52% ethanol) respectively, with occasional stirring at room temperature for 12 h, and then heated to reflux for 6 h. The solvent was filtered twice with filter paper, and then the filtered solvent was evaporated to residue. The residue was collected and stored at 4 °C.

Cell culture and SILAC labeling

Human lung cancer cells A549 and liver cancer cells HepG2 were obtained from Dr. Haiteng Deng (Tsinghua University, Beijing, China). Human gastric cancer cell line MGC-803 was obtained from Dr. Caixia Li (Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China). Human lung primary cell MRC-5 was obtained from Shanghai Institute of Cell Biology (Shanghai, China). The methods of cell culture and SILAC labeling mainly referred to previous reports (Hu et al., 2014), and the SILAC A549 cells were labeled by L-$^{13}C_6$Lysine.

CCK-8 assay

The *D. nobile* extract was dissolved with DMSO. To avoid interference of DMSO, the concentration of DMSO in cell culture medium was less than 0.2%. The inhibition rate of cells with different extracts of *D. nobile* were analyzed with the CCK-8 kit. Cells (3000 each) were seeded into wells of 96-well culture microplate and incubated for 48 h prior to treatment. Cells were then treated with extracts at different concentrations in triplicates for 24 h. The CCK-8 reagent (Dojindo Laboratories, Japan) was added to wells and incubated with cells at 37 °C for 3 h. Optical density (OD) was measured at 450 nm with a microplate reader. The experiments were repeated three times.

Detection of polysaccharides and total alkaloid

The detection of polysaccharides and total alkaloid were performed following the previously described procedure (Chen et al., 2019; Zheng et al., 2020). For the detection of polysaccharides, each of 0.25 g sample powder was briefly used for extraction in 100 ml 80% ethanol solution. The filtered residue was used for extracting in 100 ml pure water once more. The extract was mixed with phenol solution and H_2SO_4, kept in a bath of boiling water, and then detected at absorbance of A_{490}. Glucose was used to calculate the content of polysaccharides. For the detection of total alkaloid, the dry extract of *D. nobile* was briefly dissolved in chloroform and then filtered to obtain a filtrate. The diluted filtrate was transferred to a separatory funnel, and then pH 4.5 buffer solution and 0.04% bromocresol green solution were added to the separatory funnel. The mixture was given a strong shake and the lower chloroform layer solution removed. Subsequently, 0.01 M NaOH was mixed with the filtrate. The total alkaloid content was detected with a UV-visible spectrophotometer at 620 nm, and *Dendrobine* was used to calculate the total alkaloid content. The experiments were repeated three times.

Fourier transform infrared spectroscopy (FTIR) analysis

According to Luo et al. (2013), the dry extract of *D. nobile* was mixed uniformly with spectroscopic grade potassium bromide powder (1% w/w) and the Spectrum GX FTIR spectrometer (PerkinElmer) – which, equipped with a deuterated triglycine sulfate detector was used to record the FTIR spectra in the region of 4000–400 cm^{-1}.

The experiments were repeated three times.

High performance liquid chromatography and HPLC fingerprints

According to Zheng et al. (2020), the dry extract of *D. nobile* was dissolved in methanol at a concentration of 1 mg/ml. Then, 10 µl of sample was used for HPLC analysis with C_{18} chromatographic column (Agilent, USA). In the analysis of HPLC, the acetonitrile concentration increased from 5% to 100%. The specific procedures were as follows: 0 min, 5%;

10 min, 10%; 35 min, 20%; 55 min, 28%; 60 min, 80%; 75 min, 85%; 77 min, 100%; 80 min, 100%; 85 min, 5%. The flow rate was 0.6 ml/min, and the detection wavelength was 270 nm. The fingerprint of each sample was generated with three independent chromatograms. The Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012, Chinese Pharmacopoeia Commission) was used for the construction and comparison of HPLC fingerprints. The experiments were repeated three times.

Protein sample preparation and quantitative proteomics analysis

The A549 cells cultured with normal RPMI 1640 medium were treated with 100 μM extract of *D. nobile* for 12 h. The A549 cells cultured in SILAC PRMI 1640 medium were treated with an equal amount of DMSO as the control. For the ethanol extract group, the treated cells were designated as A549(+) cells, while the control cells were designated as A549(–) cells. For the ether extract group, the treated cells were designated as A549(++) cells, while the control cells were designated as A549(– –) cells. The protein sample preparation was performed according to the description by Hu et al. (2014). Briefly, an equal amount of proteins from A549(+)/A549(++) cells and A549(–)/A549(– –) cells were mixed together and separated by 1D SDS-PAGE. Then the gel bands were excised from the gel, and the proteins in the gel bands were reduced with DTT and alkylated with IAA. After gel digestion, the proteins were extracted from gel bands.

The LC-MS/MS analysis was performed following the previously described procedure (Hu et al., 2014; Liu et al., 2016b). Briefly, the peptides were separated by nano-HPLC system (Proxeon, Denmark) which directly interfaced with Thermo Q Exactive mass spectrometer. For MS/MS spectra from each LC-MS/MS run, database searching and protein quantification were carried out with uniprot human database (version 20150110) and in-house Proteome Discoverer Searching Algorithm. The ratios of lysine-containing peptide were calculated using the peak area of Lys6 and Lys0, and the relative expression ratios of proteins were calculated by averaging all peptide ratios.

Western blotting analysis

According to Hu et al. (2014), A549 cells treated with *D. nobile* extract and DMSO respectively for 12 h were harvested and lysed on ice. After centrifugation, the supernatants were collected and the protein concentrations measured. The proteins were then separated with SDS-PAGE gel and transferred onto PVDF transfer membrane. After blocking with skimmed milk, the membrane was incubated with primary antibody and secondary antibody labeled with HRP. Finally, the membrane was developed with ECL reagents, and β-actin was detected as an internal control.

Intracellular reactive oxygen species (ROS) level

Total intracellular ROS level was determined by Reactive Oxygen Species Assay Kit (Beyotime, China). The cells were briefly treated with ether extract of *D. nobile*, while DMSO and Rosup were utilized as negative and positive controls. The cells were then washed with PBS and incubated with 10 μM DCFH-DA at 37 °C for 30 minutes. After being washed twice with PBS, pictures of the cells were taken with fluorescence microscope. The intracellular ROS level was proportional to the fluorescence intensity (Wang et al., 2018).

Statistical analysis

The statistical analysis of data was performed using GraphPad Prism software (Version 5.0). All data were expressed as mean ± standard deviation (SD), $n = 3$, and p values of < 0.05 were considered significant.

Results

Cell susceptibility to various D. nobile *extracts*

The extracts from *D. nobile* were dissolved in DMSO. Human lung primary cells MRC-5 and three other kinds of cancer cells were treated with various extracts at different concentrations for 24 h. The cytotoxicity of extracts to A549, HepG2, MGC-803, and MRC-5 were measured with CCK-8 assay. The results are shown in Fig. 1.

Despite the inhibitory rate of the extracts on cells varying, ether extract demonstrated the strongest cytotoxicity to three cancer cell lines when compared with ethanol extract and 52% ethanol extract (Fig. 1A–C). At a concentration of 100 μg/ml, ether extract inhibited A549, HepG2, and MGC-803 cells by 52.67%, 29.98%, and 23.32%, respectively. Meanwhile, the 52% ethanol extract showed the weakest inhibitory effect on cancer cells, and the inhibitory rates on A549, HepG2, and MGC-803 cells were only 11.25%, 3.59% and 2.58% respectively. The inhibitory effect of ethanol extract on cancer cells was slightly stronger than that of 52% extract but much less than that of ether extract.

At a concentration of 100 μg/ml, ether extract presented weak cell cytotoxicity and the cell viability was 97.12% in non-tumor cells MRC-5. The ethanol extract did not show obvious inhibitory effect on MRC-5 cells at a concentration of 100 μg/ml. On the contrary, the 52% ethanol extracts with concentrations ranging from 50 to 400 μg/ml all modestly improved MRC-5 cell survival (Fig. 1D).

Different chemical compositions of ether extract, ethanol extract and 52% ethanol extract

Each chemical compound in the *D. nobile* extract contributes to the absorbance spectrum. Thus the spectrum of the extract reflects its overall chemical composition, allowing the extraction to be characterized as a fingerprint (Aouidi et al., 2012). The FTIR spectra obtained from different extracts demonstrated significant differences (Fig. 2A). For example, the transmittance of 52% ethanol extraction was about zero, while the transmittance of ethanol extract was about 26.56% at the wavenumber of 3417 cm^{-1}. As for ether extract, the data was about 59.60%.

Polysaccharides and alkaloids in *Dendrobium* have usually been studied and thought of as active ingredients. To assess the difference of compositions among different extracts, we tested the content of polysaccharides and total alkaloid (Fig. 2B and 2C). Because polysaccharides are highly soluble in water, the content of polysaccharides was highest in 52% ethanol extract, up to 36.44%. For ethanol extract, the content of polysaccharides was 27.82%. Additionally, polysaccharides had the lowest solubility in ether, thus the content of polysaccharides in the ether extract was only 4.24%. However, the contents of total alkaloids in three extracts were not significantly different. Total alkaloid concentration in 52% ethanol extract, ethanol extract, and ether extract were 6.33%, 6.12%, and 6.77%, respectively.

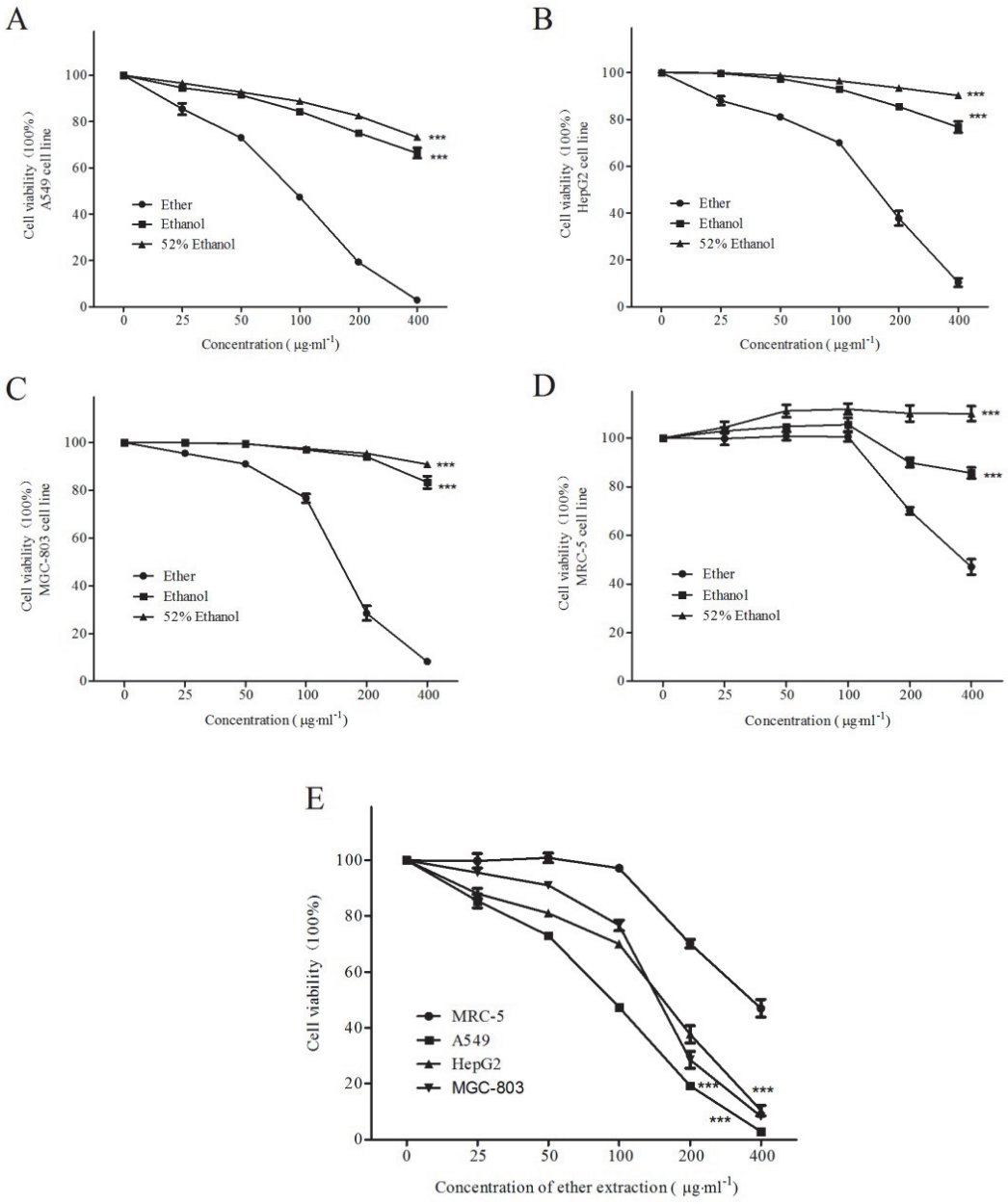

Fig. 1. (**A–D**) The viability of cells treated with different extracts of *D. nobile* determined with the CCK-8 assay. (**E**) The viability of cells treated with ether extract at different concentrations. *** $p < 0.001$; $n = 3$.

The polarities of water, ethanol, and ether solutions are different. HPLC fingerprints were applied to detect differences in the content of compounds with different polarities in three extracts. As shown in Fig. 2D, the HPLC fingerprint of ethanol extract was similar to that of 52% ethanol extract, while the difference between it and ether extract was obvious. When retention times were 3–9 min and 25–46 min, the quantity of compounds detected in ether extract was lower than that of ethanol extract. However, when the retention time was between 64–74 min, more compounds were detected in ether extract. In this HPLC experiment, the high polar compounds were easily eluted and identified from the C_{18} chromatographic column, while the weak polar compounds had a longer retention time in the HPLC spectrum. Therefore, there were more weak polar compounds in the ether extract.

All the results indicated that the ether extract possessed different compositions to the ethanol extract and 52% ethanol extract, leading to distinct inhibitory effects on cancer cells.

Quantitative proteomic analysis of cells treated with ethanol extract and ether extract

Proteomic analysis was carried out on A549 cells to study the inhibition mechanisms of ethanol and ether extracts. Differentially expressed proteins (DEPs) were identified and quantified using SILAC quantitation three times, and the SILAC ratio (>1.5 or <0.67) and protein score (>50) were set as the threshold for DEPs.

In the experiment, 5,596 proteins were identified after being treated with ethanol extract. 440 proteins were found to be differentially expressed between A549(+) and A549(–)

cells, of which 10 proteins were up-regulated and 430 proteins down-regulated (Suppl. Table 1 and 2, Fig. 3A). The results indicated that up to 98% of the DEPs were down-regulated, which suggests that cellular protein expression levels were suppressed on a large scale after the ethanol extract treatment.

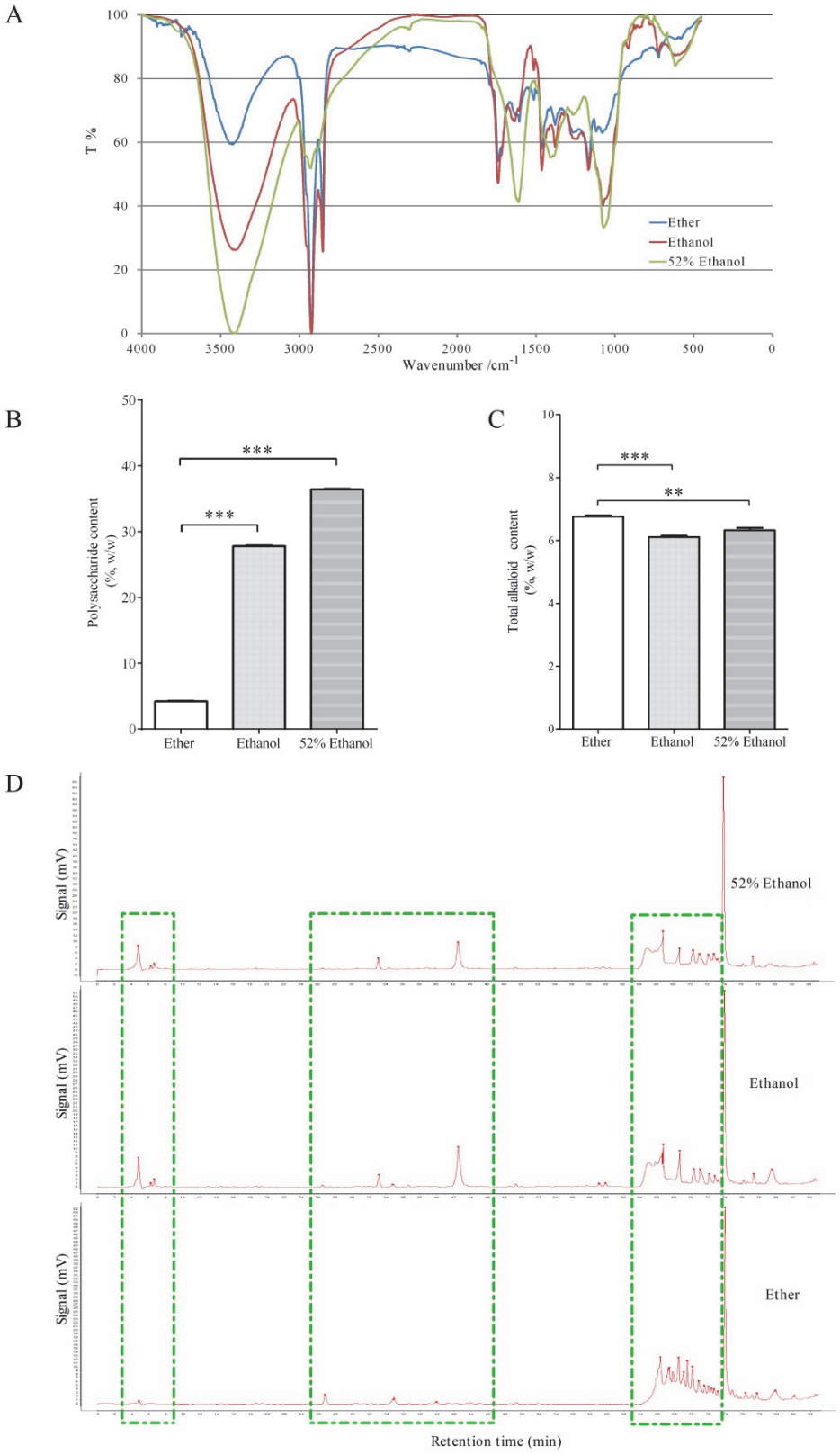

Fig. 2. Differences in chemical components of different extracts. (**A**) The FTIR spectra of different extracts of *D. nobile*. (**B**) The polysaccharide contents of different extracts of *D. nobile*. (**C**) Total alkaloid contents of different extracts of *D. nobile*. (**D**) HPLC fingerprints of different extracts of *D. nobile*. ** $p < 0.01$; *** $p < 0.001$; $n = 3$.

In the ether extract treatment, a total of 5,387 proteins were identified, of which 291 were DEPs. In contrast to the ethanol extract treatment, 278 proteins were up-regulated while only 13 proteins were down-regulated (Suppl. Table 3 and 4, Fig. 3B). This demonstrated that cellular protein expression levels were elevated on a large scale after the ether treatment. Obviously, ethanol extract and ether extract inhibited the viability of lung cancer cells in quite distinct ways.

To understand the biological significance of the DEPs, GO analysis via the PANTHER bioinformatics platform and UNIPROT database were used to cluster proteins based on the biological processes in which they involved. The annotations of the down-regulated 430 proteins of ethanol extract group and the up-regulated 278 proteins of ether extract group are summarized in Fig. 3.

The down-regulated proteins of ethanol extract group were classified into several significant groups including protein biosynthesis, mRNA and protein transport, carbohydrate, lipid, nucleotide biosynthesis and metabolism, ion transport, electron transport, lipid transport, glycolysis, tricarboxylic acid cycle, cell adhesion, cell migration, cell shape, protein folding, proteolysis, DNA damage, DNA repair, response to oxidative stress, DNA replication, cell cycle, cell division, and apoptosis. Meanwhile, the protein biosynthesis component can be divided into translation, translation regulation, post-translational protein modification, mRNA processing, mRNA splicing, transcription, transcription regulation, rRNA processing, and tRNA processing (Fig. 3A).

The up-regulated proteins of the ether extract group can be classified into protein biosynthesis, mRNA and protein transport, DNA replication, cell cycle, cell division, carbohydrate, lipid and nucleotide metabolic process, cell adhesion, cell shape, cell migration, protein folding and proteolysis, cellular stress response, apoptosis, and autophagy (Fig. 3B).

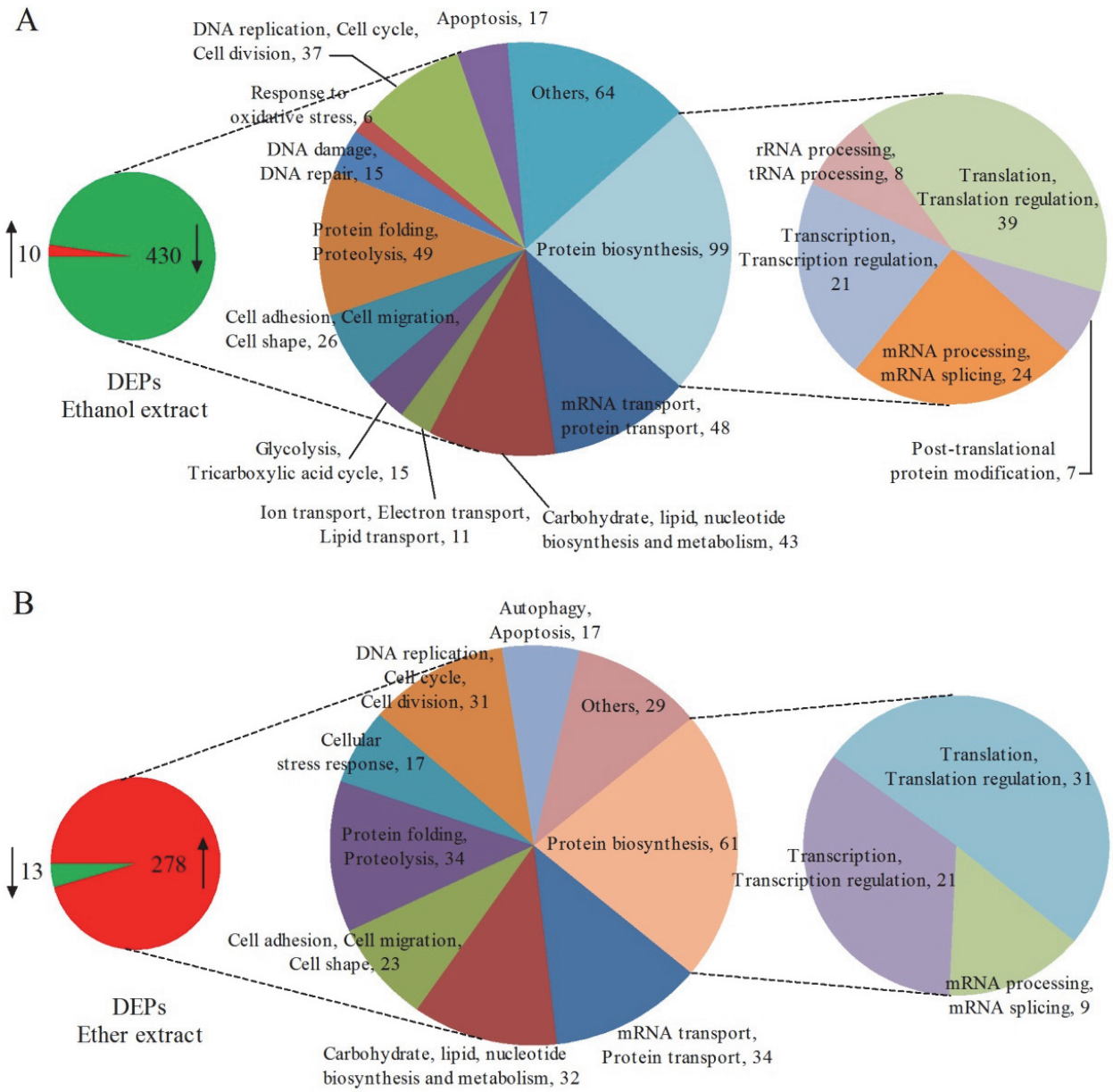

Fig. 3. The differentially expression proteins (DEPs) of A549 cells caused by *D. nobile* extracts. (**A**) Functional classification of DEPs caused by ethanol extract in A549(+) cells based on UniProt database (https://www.uniprot.org/). (**B**) Functional classification of DEPs caused by ether extract in A549(++) cells based on UniProt database.

To further investigate the influence of ether extract on A549 cells, we carried out the network analysis on 139 DEPs with SILAC ratio (>1.5 or <0.67) and higher protein score (>100) using the STRING database. The protein-protein interactions generated by ether extract are shown in Fig. 4. Most of the up-regulated proteins related to protein biosynthesis and protein folding, as indicated by the red circle in Fig. 4. This demonstrated that large amounts of ribosomal proteins, elongation factors, and chaperones were up-regulated by ether extract, and that the proteins clearly interacted. We also found that many ras-related proteins involved in cell growth and cell death were up-regulated, and there were obvious interactions among them (Green circle in Fig. 4). In total, eleven ras-related proteins were found to be up-regulated by ether extract (Table 1).

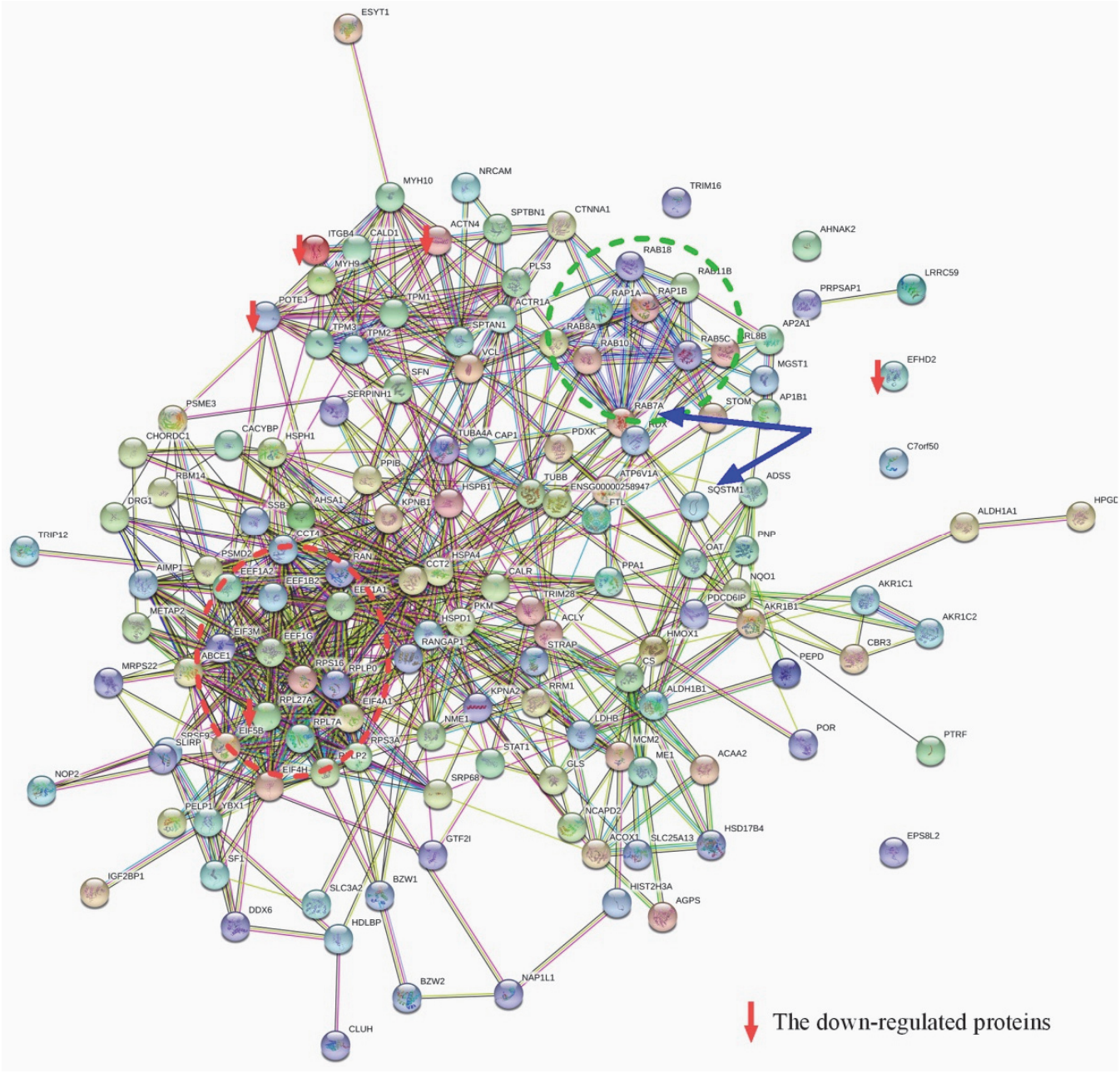

Fig. 4. Interaction network of differentially expressed proteins in A549(++) cells based on STRING database (https://string-db.org/cgi/input.pl). Red circle, proteins associated with protein biosynthesis and protein folding; Green circle, ras-related proteins; Red arrows, the down-regulated proteins; Blue arrows, the interaction of protein RAB7A and protein SQSTM1 (p62).

Table 1. The differentially expressed ras-related proteins caused by ether extract

UniProt accession	Protein name	Score	Sequence coverage	Unique peptides	PSMs	Mean ratio: A549(++)/A549(– –)
Q6IQ22	Ras-related protein Rab-12	53.27	17.21	2	20	2.74
P51149	Ras-related protein Rab-7a	293.96	84.54	19	93	1.81
Q15907	Ras-related protein Rab-11B	193.22	51.38	1	65	1.72
P51148	Ras-related protein Rab-5C	239.56	58.33	9	75	1.66
Q9NP72	Ras-related protein Rab-18	100.66	66.02	11	34	1.63
C9J6B1	Ras-related protein Ral-B	76.00	25.75	4	23	1.62
P61026	Ras-related protein Rab-10	334.70	61.50	10	121	1.60
P61006	Ras-related protein Rab-8A	161.58	35.27	2	58	1.58
P62834	Ras-related protein Rap-1A	104.48	53.26	2	36	1.56
H7C3P7	Ras-related protein Ral-A (Fragment)	66.65	34.76	4	21	1.55
P61224	Ras-related protein Rap-1b	148.85	53.26	3	52	1.54

The proteins Pyruvate kinase PKM (PKM2), Peroxiredoxin-1 (PRDX1), and Actin were selected to verify the quantitative proteomics of ethanol extract group by western blotting (Fig. 5A). For the ether extract group, Quinone oxidoreductase PIG3 (PIG3), Sequestosome-1 (SQSTM) and Actin were selected to verify the results of quantitative proteomic with western blotting (Fig. 5B). The results of western blotting showed a consistent trend with the result of quantitative proteomic (Suppl. Table 5).

ROS generation caused by ether extraction

The treatment of A549 cells with ether extract resulted in the overexpression of a large number of proteins. The over-expressed proteins may overload the endoplasmic reticulum, which is involved in protein folding and transporting. Although proteins involved in protein folding and proteolysis were up-regulated, the appearance of cell death meant the endoplasmic reticulum stress (ER stress) still occurred in cells. Western blot was performed on BiP, a protein as a marker for unfolded protein response (UPR), and the up-regulated of Bip confirmed the occurrence of ER stress (Fig. 5D).

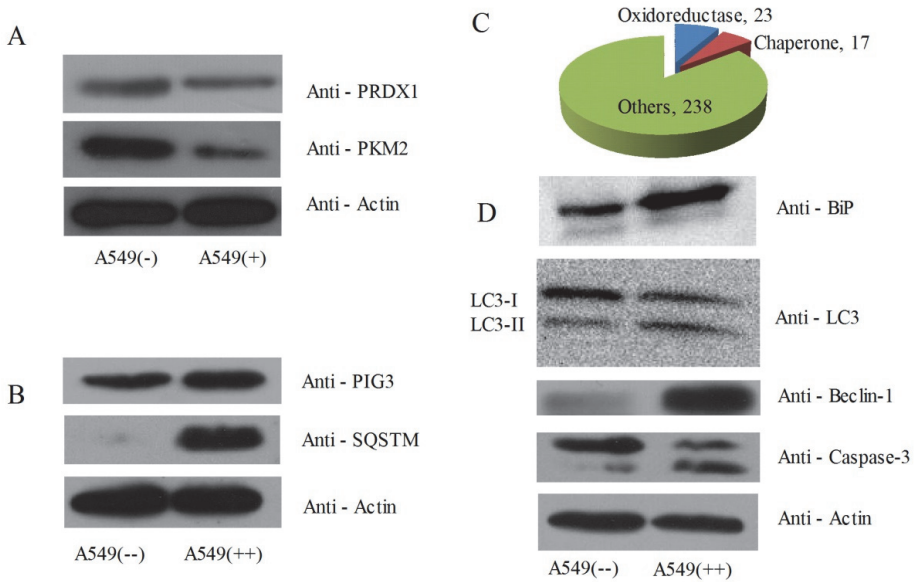

Fig. 5. (**A**) Western blotting analysis of selected differentially expressed proteins in A549(–) and A549(+) cells. (**B**) and (**D**) Western blotting analysis of selected differentially expressed proteins in A549(– –) and A549(++) cells. (**C**) The number of oxidoreductases and chaperones in up-regulated proteins caused by ether extract of *D. nobile*.

Based on UNIPROT database, the analysis of DEPs caused by ether extract demonstrated that 23 oxidoreductase and 17 chaperones were up-regulated (Fig. 5C and Table 2). This indicated that the cellular ROS level of A549(++) cells were severely altered by ER stress. The cellular ROS level was measured and the results are shown in Fig. 6. From the photomicrographs of A549, we found that the intracellular ROS level of A549(++) cells greatly increased. We measured cellular ROS levels at 4, 12, and 24 hours. The level of cellular ROS was the highest at 12 hours. The cellular ROS level caused by ether extract was also substantially greater than the positive control, indicating that ether extract could strongly elevate the intracellular ROS level.

Table 2. The up-regulated chaperone proteins caused by ether extract

UniProt accession	Protein name	Score	Sequence coverage	Unique peptides	PSMs	Mean ratio: A549(++)/A549(– –)
P04792	Heat shock protein beta-1	778.27	90.73	14	230	1.96
P50454	Serpin H1	177.99	43.30	12	51	1.88
Q9UDY4	DnaJ homolog subfamily B member 4	59.94	22.85	4	18	1.83
Q99615	DnaJ homolog subfamily C member 7	74.84	28.74	13	25	1.59
P34932	Heat shock 70 kDa protein 4	291.18	42.14	28	93	1.59
P50502	Hsc70-interacting protein	74.09	21.68	9	27	1.58
P27797	Calreticulin	243.14	60.67	18	78	1.57
Q16543	Hsp90 co-chaperone Cdc37	62.65	28.84	8	20	1.57
P78371	T-complex protein 1 subunit beta	784.96	67.66	31	230	1.56
Q7KZ85	Transcription elongation factor SPT6	83.28	17.21	18	26	1.54
P61221	ATP-binding cassette sub-family E member 1	164.21	46.58	18	52	1.53
O75880	Protein SCO1 homolog, mitochondrial	69.59	22.92	5	21	1.52
P78318	Immunoglobulin-binding protein 1	52.24	43.66	10	17	1.52
Q9UHD1	Cysteine and histidine-rich domain-containing protein 1	107.96	36.14	11	38	1.51
O95433	Activator of 90 kDa heat shock protein ATPase homolog 1	211.87	49.11	12	55	1.51
P50991	T-complex protein 1 subunit delta	459.74	64.75	28	149	1.51

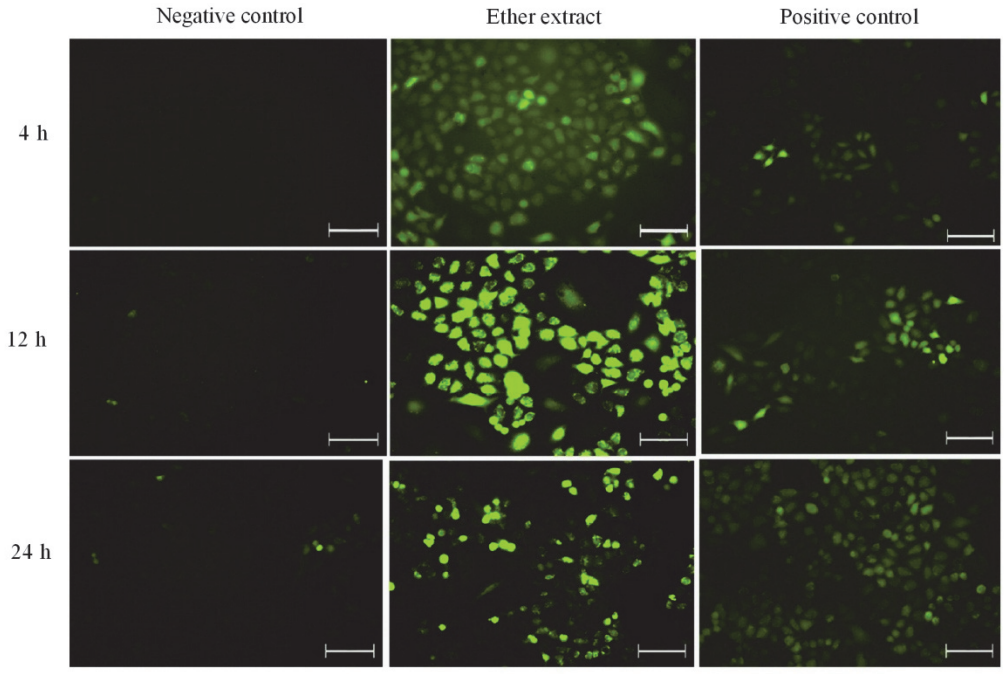

Fig. 6. Fluorescence photograph of A549 cells treated with ether *D. nobile* extract. Scale bars, 100 μm.

Discussion

As high polar solvents, ethanol and methanol are usually used to extract anticancer ingredients from plants (Liu et al., 2016a; Wang et al., 2014). Thus the compounds with high polarity were typically chosen as anticancer medication candidates. However, our previous research showed considerable variations in anticancer properties across different extracts of *Dendrobium* extracted using different solvents (Li et al., 2018). To study the inhibitory effect of ether extract of *D. nobile*, several types of cancer cells were employed as cell models. A549, HepG2, and MGC-803 were shown to be sensitive to ether extract. We selected three kinds of cells and human lung primary cells MRC-5 for further study. The results demonstrated that the weak polar solvent ether extracted the anticancer ingredients from *D. nobile* better than ethanol and water (Fig. 1A–C). This suggests that ethanol and water are not a suitable solvent for anticancer ingredients extracting from *Dendrobium*, despite there being several reports which use methanol/ethanol or water as an extraction solvent for anticancer activity studies of *Dendrobium* (Guo et al., 2019; Liu et al., 2016a; Prasad et al., 2017; Zhao et al., 2014; 2016). Also, the cytotoxicity of ether extract to cancer cells was greater than that of non-tumor cells MRC-5, suggesting that the ether extract exhibited some degree of selectivity towards cancer cells (Fig. 1E).

Distinct extracts had different anticancer properties, indicating that different extracts include specific components. As shown in Fig. 2A, the results of fourier transform infrared spectroscopy analysis showed the obvious difference in chemical composition among ether extract, ethanol extracts, and 52% ethanol extract. As representative active ingredients, polysaccharides and alkaloid have exhibited certain anticancer effects (Song et al., 2019; Wang et al., 2010; Xing et al., 2018). However, we found that the anticancer ability of the *Dendrobium* extract was inversely related to the polysaccharide level (Fig. 1 and Fig. 2B). Although the levels of total alkaloid in various extracts were statistically different, the difference in their inhibitory effects on cancer cells was not proportionate (Fig. 1 and Fig. 2C). This demonstrated that the unknown part in the extracts determined the anticancer ability of the extracts. On the other hand, the difference in HPLC fingerprint results of different extracts clearly showed that the content of weak polar compounds in ether extract was significantly higher than in ethanol extract (Fig. 2D). It indicated that most compounds with anticancer activities were soluble in weak polar solvent ether, and their content was proportional to the anticancer ability of the extract. Thus the ether extract of *D. nobile* would be a more promising candidate for natural anticancer drug development. Furthermore, research for anticancer drugs derived from *Dendrobium* should focus more attention on the weak polar compounds.

Lung cancer is a common disease throughout the world, particularly in China. In our previous studies, the ether extract of *D. denneanum* also presented a stronger inhibitory effect on lung cancer cells A549. Thus A549 cells were selected for the anticancer mechanism study of ether extract of *D. nobile*. To understand the difference in anticancer mechanisms between ethanol and ether extract, we scanned the proteomic changes of the A549 cells after they were treated with different extracts. We found that the two kinds of extract had completely different effects on the protein expressions of lung cancer cells based on the results of quantitative proteomics. When the cells were treated with ethanol extract, most of the DEPs were down-regulated. On the contrary, most of the DEPs caused by ether extract were up-regulated.

Under ethanol extract treatment, up to 98% of DEPs were down-regulated. This was mainly related to cellular material synthesis, energy supply, and cell stress response (Fig. 3A). Most of the down-regulated proteins were associated with protein biosynthesis and transport, although several proteins involved in carbohydrate, lipid, nucleotide biosynthesis and metabolism were also down-regulated. It was discovered that treatment with ethanol extract inhibited the synthesis and transport of bio-macromolecules in lung cancer cells. Meanwhile, fifteen proteins associated with glycolysis and tricarboxylic acid cycle were down-regulated. Among them, twelve down-regulated proteins were involved in the bioprocess of glycolysis (Table 3). Although the energy provided by glycolysis is limited, studies have found that the intensity of glycolysis is significantly stronger in cancer cells than in normal cells, indicating that glycolysis is a more important energy supply method in cancer cells (Christofk et al., 2008). Therefore, treatment with ethanol extract could severely weaken the energy supply capability in lung cancer cells.

On the other hand, lots of proteins involved in protein folding and proteolysis were down-regulated by the treatment of ethanol extract, preventing mis-folded proteins from being removed in time. Down-regulation of proteins associated with DNA damage, DNA repair, and response to oxidative stress revealed that the capability of cells to resist endogenous and exogenous damage was weakened.

When lung cancer cells were treated with ether extract, we found that up to 95% of DEPs were up-regulated proteins. It was hypothesized that the ether extract might stimulate the rapid increase in the amount of various proteins in cancer cells. Based on biological process analysis, 61 of the 278 up-regulated proteins were associated with protein biosynthesis, including transcription, transcription regulation, mRNA processing, and translation (Fig. 3B). In addition, 33 proteins associated with mRNA and protein transport were up-regulated. In total, up to 35% of the up-regulated proteins were involved in the protein biosynthesis and transport. This demonstrated that ether extract could strongly stimulate the biosynthesis and transport of cellular proteins. Many proteins associated with carbohydrate, lipid and nucleotide metabolic, cell adhesion, cell shape, cell migration, cell cycle and cell division were also up-regulated. Obviously, the up-regulated proteins were a stock preparation for cell proliferation. Hence the cells presented a state of preparation of materials for over-proliferation.

As cancer cells, the lung cancer cell line A549 is already subjected to significantly faster rates of protein synthesis/folding and higher ROS levels than normal cells (Grek and Tew, 2010). In this study, the overexpression of large amounts of proteins was bound to further increase the burden of the endoplasmic reticulum, resulting in the overloading of endoplasmic reticulum, and lots of proteins could not be folded appropriately. Although several proteins associated with protein folding and proteolysis, such as chaperones (Table 2), were up-regulated, the large amount of unfolded proteins might still not be eliminated in time. Immunoglobulin binding protein (BiP) is a member of the HSP70 chaperones, which participates in protein folding in the endoplasmic reticulum (Ramirez et al., 2017). BiP protein is usually a marker for ER stress to indicate the unfolded protein response (UPR) (Roth et al., 2018). In Fig. 5D, the up-regulation of BiP protein level proved the activation of UPR in A549(++) cells. It suggested that the overexpression of proteins caused by the treatment of ether ex-

Table 3. Down-regulated proteins involved in glycolysis bioprocess caused by ethanol extract

UniProt accession	Protein name	Score	Sequence coverage	Unique peptides	PSMs	Mean ratio: A549(++)/A549(– –)
P06733-1	alpha-enolase	468.20	81.57	15	1287	0.66
P18669	Phosphoglycerate mutase 1	206.49	63.78	16	220	0.62
P60174	Triosephosphate isomerase	186.13	77.27	21	206	0.62
P00338-3	Isoform 3 of L-lactate dehydrogenase A chain	214.36	69.81	25	316	0.62
P17858-1	ATP-dependent 6-phosphofructokinase, liver type	104.02	34.74	16	54	0.59
P14618	Pyruvate kinase PKM	401.07	71.56	40	668	0.58
P06744	glucose-6-phosphate isomerase	243.39	61.11	30	193	0.58
P00558	phosphoglycerate kinase 1	291.51	81.53	36	365	0.57
Q01813	ATP-dependent 6-phosphofructokinase, platelet type	113.84	37.63	23	62	0.54
P09972	Fructose-bisphosphate aldolase C	143.79	52.75	18	123	0.53
P04075-2	Isoform 2 of Fructose-bisphosphate aldolase A	298.55	71.53	31	551	0.53
P08237-3	Isoform 3 of ATP-dependent 6-phosphofructokinase, muscle type	94.63	26.32	13	29	0.23

tract exceeded the protein processing capacity of endoplasmic reticulum, resulting in ER stress and cell death.

ER stress is most commonly inferred by activation of the UPR, while the production of ROS is an essential part of ER stress response (Ochoa et al., 2018). In the quantitative proteomic results, many kinds of oxidoreductase were up-regulated, which suggested the disorder of redox reaction had happened (Fig. 5C). The results indicated that cellular ROS levels might be greatly altered by ether extract. Indeed, the treatment of ether extract seriously increased the level of cellular ROS, especially 12 hours after treatment (Fig. 6). Obviously, the increase in the level of cellular ROS was another injury to cells that would cause cell death (Xin et al., 2020).

Ras proteins are associated with cell growth, differentiation, survival, and human cancer (Simanshu et al., 2017). Treatment of ether extract stimulated the overexpression of large amounts of ras-related proteins (Fig. 4 and Table 1). It suggested the ether extract may regulate the intercellular signal transduction via ras-related proteins, and the RAS proteins or ras-related signal transduction pathway may be one target for ether extract. Furthermore, some ras-related proteins, such as ras-related protein Rab-8A, participated in the biological process of autophagy (Sellier et al., 2016), indicating that cell death caused by ether extract can occur through autophagy. SQSTM1 (also known as p62) is a multifunctional receptor protein involved in biological processes such as autophagy, apoptosis, and tumorigenesis (Sánchez-Martín and Komatsu, 2018). Fig. 4 shows an interaction between SQSTM1 and RAB7A (Fig. 4, blue arrow). It suggests that the level change of ras-related proteins play an important role in the process of apoptosis or autophagy caused by ether extract.

SQSTM1, LC3 and Beclin-1 are commonly used as markers to detect cell autophagy (Al-Shenawy, 2016; Xu et al., 2019; Zheng et al., 2017), thus the levels of these proteins were detected using western blotting. As shown in Fig. 5B and 5D, the levels of SQSTM1 and Beclin-1 protein were both up-regulated strongly by ether extract, and the LC3-I/LC3-II ratio was obviously decreased. The findings demonstrated that the administration of ether extract induced autophagy in cells. The apoptosis marker caspase-3 was detected and most of the proteins were cleaved in A549(++) cells, indicating that the apoptosis process was also activated by ether extract. Thus, the cell death of A549(++) cells caused by ether extract occurred through both apoptosis and autophagy.

Taken together, the ethanol extract suppressed the material biosynthesis and energy supply of cancer cells, while the cellular self-repair ability was weakened. On the contrary, the ether extract stimulated the over-expression of more proteins to induce ER stress and UPR. It also accelerated the cell growth speed that the cells could not endure. Meanwhile, the cellular ROS increased and oxidative stress occurred with the treatment of ether extract. Autophagy and apoptosis were also activated – thus cell death was inevitable.

Conclusions

In the present study, we found that the ether extract of *D. nobile* exerted stronger anticancer ability than ethanol extract. It was found that the anticancer effect of ether extract was mainly produced by weak polar compounds, rather than high polar compounds such as polysaccharides and alkaloids. Proteomics results revealed that ethanol extract and ether extract had completely different inhibitory mechanisms on cancer cells. Our data suggests that the ether extract of *D. nobile* significantly stimulated the over-expression of immature proteins, which the cell could not process in time. Under cell stress, the autophagy and apoptosis were activated to cause cell death. Collectively, the findings indicate that ether extract of *D. nobile* is a better candidate for anticancer drug development. Future research on anticancer drugs from medicinal plants should focus more attention on weak polar compounds.

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Ethical aspects and conflict of interests

The authors have no conflict of interests to declare.

Author's contributions

RZ: Investigation, Formal analysis, Visualization. SZ: Investigation, Formal analysis, Visualization. YL: Investigation, Formal analysis. XZ: Investigation, Visualization. DR: Investigation. ZC: Resources. YH: Conceptualization, Supervision, Validation, Writing-Review and Editing.

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