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Effect of *Hedyotis diffusa* Polysaccharide on Apoptosis of Thyroid Cancer Cells

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ABSTRACT

The objective of this study was to investigate the effect and mechanism of *Hedyotis diffusa* polysaccharide (HDP) on apoptosis of thyroid cancer cell line. TPC-1 cells were treated with HDP (final concentration 1 mg/mL, 2 mg/mL and 4 mg/mL), PI3K/AKT signal pathway inhibitor LY294002 (20 μmol/L) and LY294002 + HDP (4 mg/ml HDP and 20 μmol/L LY294002). Cell viability and apoptosis rate were detected by MTT and flow cytometry, respectively. The expression of Cyt-C, Bcl-2, Bax, cleaved Caspase-3, PI3K, AKT and p-AKT were detected by Western blotting. We found that activity of TPC-1 cells was inhibited by different concentrations of Polysaccharide from *H. diffusa*, which was time and concentration dependent ($P<0.05$). When TPC-1 cells were treated with polysaccharide of *H. diffusa* for 48 h, the apoptosis rate increased, the expression of Bcl-2, PI3K and p-AKT decreased, the expression of Cyt-C, Bax and cleaved caspase 3 increased significantly ($P<0.05$). LY294002 could significantly inhibit the activity of TPC-1 cells, promote apoptosis, down regulate the expression of Bcl-2, PI3K and p-AKT, up regulate the expression of Cyt-C, Bax and cleaved caspase 3, and significantly enhance the effect of Polysaccharide from *H. diffusa* on the activity, apoptosis of TPC-1 cells and the expression of Cyt-C, Bcl-2, Bax, cleaved caspase 3, PI3K, AKT and p-AKT ($P < 0.05$). We concluded HDP can promote the apoptosis of thyroid cancer cells by inhibiting the PI3K/AKT signal pathway.

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Thyroid cancer, Hedyotis diffusa polysaccharide, Apoptosis, PI3K/AKT signal pathway

INTRUDUCTION

Thyroid cancer is a common malignant tumor of the head, neck and endocrine system, whose incidence rate has been increasing year by year in recent years (Gong *et al*., 2018). The traditional treatment for thyroid cancer is total thyroidectomy, followed by radioiodine therapy in some cases. Differentiated thyroid cancer, undifferentiated thyroid cancer, and poorly differentiated thyroid cancer that are unsuitable for surgery and refractory for radioiodine are the main cause of thyroid cancer death (Chang *et al*., 2018). Therefore, it is particularly important to find effective drugs for thyroid cancer treatment. *Hedyotis diffusa* is a dried whole herb (Family: Rubiaceae) which is mainly composed of polysaccharides, flavonoids, anthraquinones, organic acids and other active ingredients. *Hedyotis diffusa* polysaccharide (HDP) has anti-tumor, immune regulation effects (Chen *et al*., 2012). Studies have shown that HDP can inhibit the growth of tumor cells such as nasopharyngeal cancer and gastric cancer (Zhang *et al*., 2015; Liuet al., 2018). The effect and mechanism of HDP on the growth of thyroid cancer cells are not yet clear. PI3K/AKT signaling pathway is an important signaling pathway in cells, which participates in the processes of cell growth, apoptosis, invasion and metastasis in a way closely related to the occurrence and development of human tumors (Li *et al*., 2018c; Zhang *et al*., 2018). A large number of studies have shown that PI3K/AKT signaling pathway is involved in the occurrence and development of thyroid cancer (Xu *et al*., 2017). Studies have found that active ingredients of *H. diffusa* will affect the growth of colorectal cancer, liver cancer and other tumors by regulating PI3K/AKT signaling pathway (Li *et al*., 2018b; Yue *et al*., 2015). Therefore, this study aimed to study the effect of HDP on apoptosis of thyroid cancer cells via PI3K/AKT signaling pathway, further explore the possible mechanism of action to provide theoretical basis for clinical anticancer and cancer prevention applications of HDP.

MATERIALS AND METHODS

Reagents and instruments

RPMI 1640 medium and FBS were purchased from HyClone, USA; trypsin-EDTA digestive juice was purchased from Gibco, USA; *Hedyotis diffusa* polysaccharide (HDP) was purchased from Nanjing Zelang Pharmaceutical Technology Co., Ltd.; LY294002 was purchased from Cayman, USA; MTT, DMSO were purchased from Sigma, USA; Annexin V-FITC/PI Apoptosis Double Staining

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Kit, RIPA Lysate, BCA Protein Quantification Kit, PVDF Membrane, ECL Kit were purchased from Beyotime, China; Cyt-C, Bcl-2, Bax, cleared caspase 3, PI3K, AKT, p-AKT antibody and HRP-labeled secondary antibody were all purchased from Abcam, USA; microplate reader was purchased from Thermo, USA; flow cytometer was purchased from BD, USA.

Cell culture

Human thyroid cancer TPC-1 cells were purchased from ATCC, USA. TPC-1 cells were cultured using RPMI 1640 medium, the culture solution contained 10% FBS and 1% penicillin, and the culture was undertaken in a sterile constant temperature incubator at 37°C under CO2 volume fraction of 5% and saturated humidity. Depending on the cell growth state and color change of the culture medium, change the medium every 2-3 days. When the cells reached 90% fusion, trypsin digestion and passage were performed. The experiment was performed on cells of logarithmic growth phase.

Detection of cell proliferation by MTT method

After trypsin-EDTA digestion of TPC-1 cells in the logarithmic growth phase, the cell suspension was collected, centrifuged, resuspended in complete medium. The cell density was adjusted to 4×10^4/mL after cell counting, and the cell suspension was inoculated in a sterile 96-well plate at 200 μL/well, and cultured in 37°C incubator with 5% volume fraction CO_2 and saturated humidity. After the cell adherence, the medium was changed, and the cells were treated with HDP (with final concentration of 1 mg/mL, 2 mg/mL and 4 mg/mL), LY294002 (20 μmol/L) and LY294002 + HDP(4 mg / mL HDP and 20 μmol/L LY294002), while the control group was only added with culture medium. Set up with 5 replicates, each group was routinely incubated in an incubator. Cells cultured for 24h and 48h were collected, added with MTT solution (5mg/mL) according to 20μL/ well, followed by continued incubation for 4h in the incubator. Culture medium was discarded from each well. DMSO solution was added according to 200μL/well, shaken at low speed for 10min in a shaker. After the color crystalline material was fully dissolved, absorbance value (OD) of each well was measured with microplate reader at a wavelength of 490 nm. The experiment was repeated three times.

Apoptosis rate detection by flow cytometry

Logarithmic growth phase TPC-1 cells were inoculated at 1×105 cells/well in a 6-well plate. Following cell adherence at 24h after inoculation, the cells were treated with HDP (with final concentration of 1 mg/mL, 2 mg/mL and 4 mg/mL), LY294002 (20 μmol/L) and LY294002 + HDP, while the control group was only added with culture medium, and each group was set up with 3 replicates. After dosing, the cells were placed in a 37°C incubator with 5% volume fraction CO2 for 48h. The cells were collected, centrifuged, washed twice with PBS, centrifuged, added with 500μL Binding Buffer for resuspension. 5μL of Annexin V-FITC was added, mixed well, and incubated at 4°C for 10 min. Then, 5μL PI was added, mixed well, and incubated at 4°C for 10 min. 300μL of Binding Buffer was added before testing on the machine. Within 1 h, flow cytometry detection was performed. The experiment was repeated three times.

Detection of Cyt-C, Bcl-2, Bax, cleaved caspase3, PI3K, AKT and p-AKT expression by western blotting

After adherent cell reached logarithmic growth phase, the cells were treated with HDP (with final concentration of 1 mg/mL, 2 mg/mL and 4 mg/mL), LY294002 (20 μmol/L) and LY294002+ HDP, incubated for 48h and then culture solution was discarded. The cells were washed twice with pre-chilled PBS, added with appropriate amount of RIPA cell lysate, followed by reaction on ice for 30min, centrifugation to collect the supernatant, i.e. extracted protein. The protein was quantified using BCA protein quantification kit. Before loading, appropriate amount of loading buffer was added to the protein to be tested, mixed well, and boiled for 5 min in boiling water at 100°C to denature the protein. Denatured protein was added at 40μg/well. After SDS-PAGE electrophoresis and transfer to PVDF membrane, the membrane was washed with TBST buffer, put in 5% skimmed milk powder, and sealed for 1.5h in a shaker at room temperature. Remove the PVDF membrane from the blocking solution, let the protein-containing surface of the membrane fully contact with the configured primary antibody working solution (1:1000 diluted Cyt-C, Bcl-2, Bax, cleared caspase3, PI3K, AKT, and p-AKT antibodies), incubate at 4°C overnight, wash the membrane, add secondary antibody working solution (1:2000 diluted HRP labeled antibody), incubate at 37°C for 2h, and wash the membrane. Add ECL color developing solution on the PVDF membrane to develop color and take pictures. Gray value of each protein band was analyzed using Image J software. The experiment was repeated three times.

Statistical analysis

All experimental data were analyzed by SPSS21.0 software, measurement data was expressed by $(\bar{x}\pm s)$. One-way analysis of variance was taken for comparison among multiple groups, and SNK-q test was used for pairwise comparison. $P<0.05$ indicates statistically significant difference.

RESULTS

HDP can inhibit TPC-1 cell proliferation and promote apoptosis

MTT results showed that HDP of different concentrations could inhibit the viability of TPC-1 cells, showing time-dependent concentration ($P<0.05$). After TPC-1 cells are treated with HDP for 48h, the flow cytometry results indicate that different concentrations of HDP can significantly promote cell apoptosis in concentration-dependent manner ($P<0.05$) (Fig. 1, Table I).

Table I. Effect of different concentrations of *Hedyotis diffusa* polysaccharide (HDP) on TPC-1 cell proliferation and apoptosis.

Group	OD value		Apoptosis rate (%)
	24h	48h	
Control group	0.54±0.05	0.84±0.07	2.23±0.21
HDP (1 mg/Ml)	0.40±0.04*	0.72±0.07*	10.54±0.56*
HDP (2 mg/mL)	0.32±0.04*	0.59±0.06*	16.77±1.01*
HDP (4 mg/mL)	0.25±0.03*	0.46±0.05*	24.35±1.43*
F	97.99	63.36	926.24
P	0.00	0.00	0.00

Note: Compared with the control group, *$P<0.05$

Activating the mitochondrial apoptosis pathway of TPC-1 cells

After TPC-1 cells were treated with different concentrations of HDP for 48h, Western blotting was used to detect Cyt-C, Bcl-2, Bax and cleared caspase 3 protein expressions related to the mitochondrial apoptosis pathway. The results indicate that HDP group can significantly inhibit Bcl-2 expression, promote Cyt-C, Bax and cleared caspase3 expression in a dose-dependent manner ($P<0.05$) (Fig. 2, Table II).

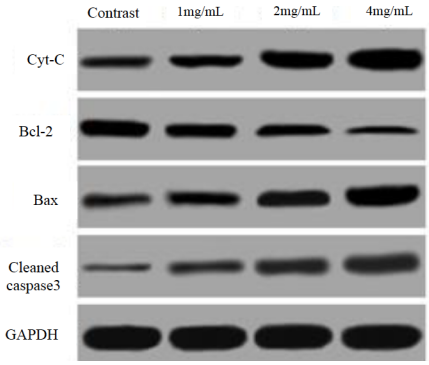

Fig. 2. Effect of different concentrations of Hedyotis diffusa polysaccharide (HDP) on Western blotting detection of Cyt-C, Bcl-2, Bax, and cleaved caspase3 protein expression in TPC-1 cells.

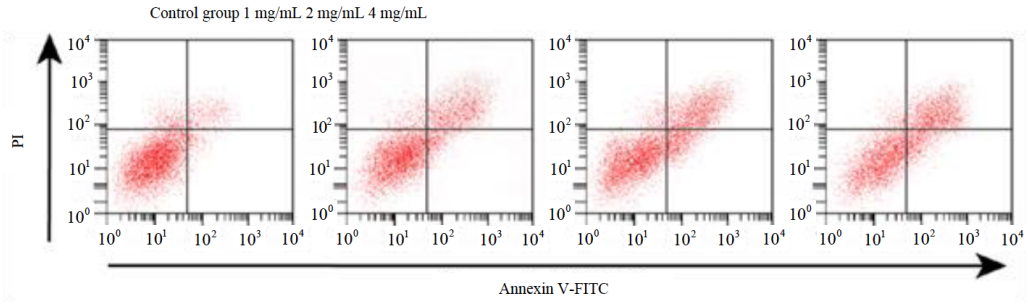

Fig. 1. Effect of different concentrations of *Hedyotis diffusa* polysaccharide (HDP) on apoptosis of TPC-1 cells.

Table II. Effect of different concentrations of *Hedyotis diffusa* polysaccharide (HDP) on the relative protein expression of Cyt-C, Bcl-2, Bax and cleared caspase3 in TPC-1 cells.

Group	Cyt-C	Bcl-2	Bax	Cleaved caspase3
Control group	0.07±0.01	0.36±0.01	0.07±0.01	0.03±0.005
HDP (1 mg/mL)	0.16±0.02*	0.25±0.03*	0.13±0.04*	0.06±0.007*
HDP (2 mg/mL)	0.49±0.05*	0.14±0.02*	0.33±0.04*	0.09±0.01*
HDP (4 mg/mL)	0.73±0.07*	0.07±0.01*	0.62±0.06*	0.13±0.02*
F	425.28	226.38	388.97	173.86
P	0.000	0.000	0.000	0.000

Note: Compared with the control group, *$P<0.05$.

Inhibiting PI3K/AKT signaling pathway in TPC-1 cells

Western blotting detection of the expression of PI3K, AKT and p-AKT in TPC-1 cells treated with HDP for 48h shows that HDP of different concentrations can significantly inhibit the expression of PI3K and p-AKT in a dose-dependent manner (P<0.05) (Fig. 3, Table III).

Table III. Effect of different concentrations of HDP on the relative protein expression of PI3K, AKT and p-AKT in TPC-1 cells.

Group	PI3K	AKT	p-AKT
Control group	0.44±0.05	0.33±0.03	0.203±0.023
HDP (1 mg/mL)	0.31±0.03*	0.35±0.04	0.136±0.017*
HDP (2 mg/mL)	0.13±0.02*	0.33±0.03	0.071±0.010*
HDP (4 mg/mL)	0.06±0.007*	0.34±0.03	0.039±0.005*
F	360.88	0.46	201.91
P	0.000	0.711	0.000

Note: Compared with the control group, *P<0.05

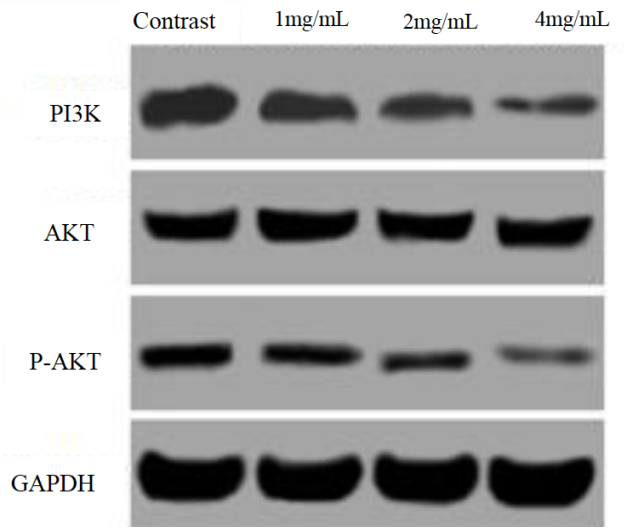

Fig. 3. Effect of different concentrations of Hedyotis diffusa polysaccharide (HDP) on Western blotting detection of PI3K, AKT and p-AKT expression in TPC-1 cells.

LY294002 can enhance the growth inhibition and apoptosis promotion effect of HDP on TPC-1 cells

The results of cell proliferation and apoptosis in each group show that LY294002 can significantly inhibit the viability of TPC-1 cells and promote its apoptosis. The combined use of LY294002 and HDP produces more significant effect in inhibiting TPC-1 cell viability and promoting apoptosis (P<0.05) (Fig. 4, Table IV).

LY294002 can enhance the effect of HDP on mitochondrial apoptosis pathway of TPC-1 cells

Western blotting detection of the expression of Cyt-C, Bcl-2, Bax and cleared caspase3 in each group of cells indicates that compared with the control group, LY294002 group has significantly reduced Bcl-2 expression, and significantly increased expression of Cyt-C, Bax and cleared caspase 3 (P<0.05). The combined use of LY294002 and HDP can enhance the effect of LY294002 on the expression of Cyt-C, Bcl-2, Bax and cleared caspase3 (Fig. 5, Table V).

LY294002 can enhance the inhibition of PI3K/AKT signaling pathway in TPC-1 cells by HDP

PI3K, AKT and p-AKT expression detection results of each group indicate that compared with the control group, LY294002 group has significantly reduced PI3K and p-AKT expression (P<0.05). The combined use of LY294002 and HDP can enhance the inhibitory effect of LY294002 on PI3K and p-AKT expression (Fig. 6, Table VI).

Table IV. Effect of LY294002 on proliferation and apoptosis of normal and HDP treated TPC-1 cells.

Group	OD value	Apoptosis rate (%)
Control group	0.83±0.07	2.41±0.26
LY294002 group	0.55±0.06*	20.12±2.21*
LY294002+HDP group	0.32±0.04#	32.45±3.67#
F	179.37	334.21
P	0.000	0.000

Note: Compared with the control group; *P<0.05; compared with LY294002 group, #P<0.05

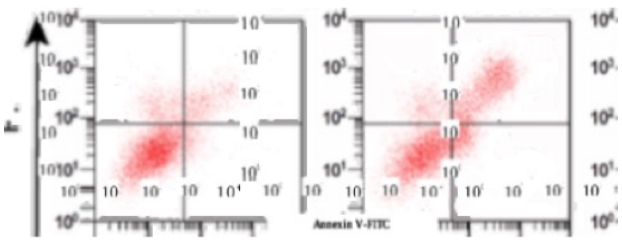

Fig. 4. Effect of LY294002 on apoptosis of TPC-1 cells.

Table V. Effect of LY294002 on the relative protein expression of Cyt-C, Bcl-2, Bax and cleared caspase3 in of normal and HDP treated TPC-1 cells.

Group	Cyt-C	Bcl-2	Bax	Cleaved caspase 3
Control group	0.14±0.02	0.32±0.04	0.07±0.007	0.06±0.01
LY294002 group	0.31±0.03*	0.17±0.02*	0.12±0.02*	0.09±0.01*
LY294002+HDP group	0.52±0.06#	0.10±0.01#	0.19±0.02#	0.14±0.02#
F	239.54	291.66	152.65	124.99
P	0.000	0.000	0.000	0.000

Note: Compared with the control group, *P<0.05; compared with LY294002 group, #P<0.05

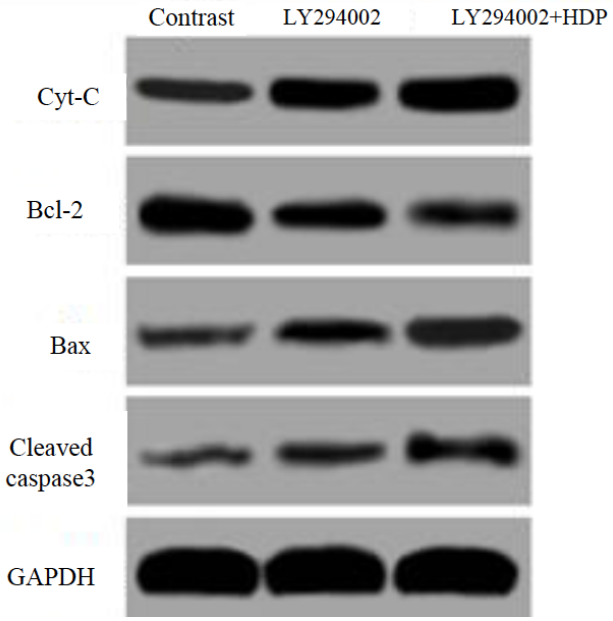

Fig. 5. Effect of LY294002 on Western blotting detection of Cyt-C, Bcl-2, Bax and cleared caspase3 protein expression of normal and HDP treated TPC-1 cells.

Table VI. Effect of LY294002 on the relative protein expression of PI3K, AKT and p-AKT in of normal and HDP treated TPC-1 cells.

Group	PI3K	AKT	p-AKT
Control group	0.64±0.06	0.32±0.04	0.13±0.02
LY294002 group	0.31±0.03*	0.33±0.04	0.07±0.008*
LY294002+HDP group	0.12±0.02#	0.33±0.03	0.03±0.005#
F	370.297	0.120	201.522
P	0.000	0.888	0.000

Note: Compared with the control group, *P<0.05; compared with LY294002 group, #P<0.05

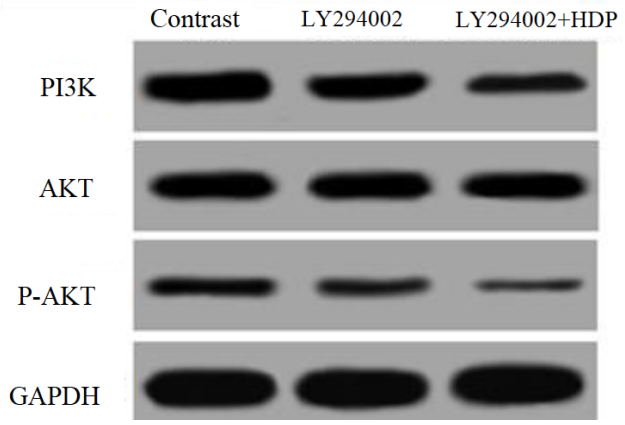

Fig. 6. Effect of LY294002 on Western blotting detection of PI3K, AKT and p-AKT expression in of normal and HDP treated TPC-1 cells.

DISCUSSION

Chinese forage has complex components, multiple targets, wide biological activity, which also features rich resources, low price and low toxicity. The use of forage to develop low-toxic and efficient anti-tumor therapy and adjuvant therapy drugs has become a hot spot in cancer drug research and development. *H. diffusa* is an important Chinese herbal medicine with good anti-tumor effect. Yue *et al*. (2015) have shown that ethanol extract of *H. diffusa* will block the leukemia cell cycle and promote apoptosis. Hu *et al*. (2015) have shown that *H. diffusa* extract will inhibit the proliferation and migration of prostate cancer cells and promote its apoptosis. Li *et al*. (2015) have shown that *H. diffusa* can reduce 5-fluorouracil resistance in colorectal cancer HCT-8/5- FU cells. Our results indicate that HDP can inhibit the proliferation of TPC-1 cells in a concentration-dependent manner and promote its apoptosis in a concentration-dependent manner. Similar type of results have been reported for multiple myeloma

cells (Lin *et al*., 2013).

Mitochondrial pathway, endoplasmic reticulum pathway and death receptor pathway are currently recognized as apoptosis pathways. Mitochondrial pathway can be divided into caspase-dependent and non-caspase-dependent pathways. In the caspase-dependent pathway, Cyt-C and some apoptotic factors are released into the cytoplasm, which in turn activates caspase9 and caspase3, causing a caspase cascade reaction and thereby cell apoptosis (Song *et al*., 2017). The Bcl-2 protein family can be divided into proteins that inhibit apoptosis (Bcl-2, Bcl-XL, etc.) and proteins that promote apoptosis (Bax, Bid, etc.). The Bcl-2/Bax ratio has close relation to cell apoptosis. When Bax in the cell has greater amount than Bcl-2, the two form a homodimer, which can induce apoptosis, otherwise it inhibits apoptosis (Huang *et al*., 2018; Li *et al*., 2018a). Studies have shown that Bcl-2/Bax homodimer can inhibit mitochondrial membrane potential and reduce membrane permeability, thereby promoting the release of Cyt-C which activates caspase9, caspse3 in turn and eventually cause cell apoptosis (Shen *et al*., 2016). Studies have shown that the apoptosis of various tumor cells, including thyroid cancer, is related to the mitochondrial apoptosis pathway (Zhu and Li, 2017; Hu *et al*., 2018). The results of this study suggest that HDP can obviously upregulate the expression of Cyt-C, Bax and cleared caspase3, and downregulate Bcl-2 expression. Studies have shown that *H. diffusa* extract can induce apoptosis by activating the mitochondrial-dependent pathway of human colon cancer cells (Lin *et al*., 2010). The results of this study suggest that HDP may induce thyroid cancer cell apoptosis by activating the mitochondrial pathway. PI3K/AKT signaling pathway is abnormally activated in most human tumors. Multiple studies have shown that inhibition of PI3K/AKT signaling pathway can reduce the growth of thyroid cancer cells (Zhang *et al*., 2016). LY294002 is an inhibitor of PI3K/AKT signaling pathway, which can specifically inhibit the phosphorylation of AKT. Several studies have shown that LY294002 can inhibit the proliferation, invasion and migration of thyroid cancer cells and promote its apoptosis (Duan *et al*., 2019). The results of this study suggest that LY294002 can upregulate the expression of Cyt-C, Bax and cleaved caspase3, and downregulate the expression of Bcl-2. Both LY294002 and HDP can downregulate the expression of PI3K and p-AKT, and LY294002 can enhance HDP's role in TPC-1 cell viability, apoptosis and expression of Cyt-C, Bcl-2, Bax, cleared caspase3, PI3K, AKT and p-AKT. Studies have shown that *H. diffusa* combined with *Scutellaria barbata* can inhibit AKT signaling pathway to induce bladder cancer cell apoptosis by down-regulating miR-155 expression (Pan *et al*., 2016). The results of this study suggest that HDP can inhibit the apoptosis of thyroid cancer cells by inhibiting the PI3K/AKT signaling pathway (Yang *et al*., 2020).

CONCLUSION

To conclude, this study found that HDP can induce thyroid cancer cell apoptosis, whose mechanism may be related to its activation of mitochondrial apoptosis pathway and inhibition of PI3K/AKT signaling pathway. These results provide a theoretical basis for studying new drugs for thyroid cancer treatment. The effect and mechanism of *H. diffusa* extract on thyroid cancer is worthy of in-depth exploration, which is expected to provide new ideas for the treatment of malignant tumors.

Statement of conflict of interest

The authors have declared no conflict of interest.

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