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ORIGINAL ARTICLE

Determination of Three Active Components in *Euphorbia humifusa* Willd. Using High-performance Liquid Chromatography with Diode-array Detection and Autophagy and Apoptosis Analysis of Normal Rat Kidney and HeLa Cells

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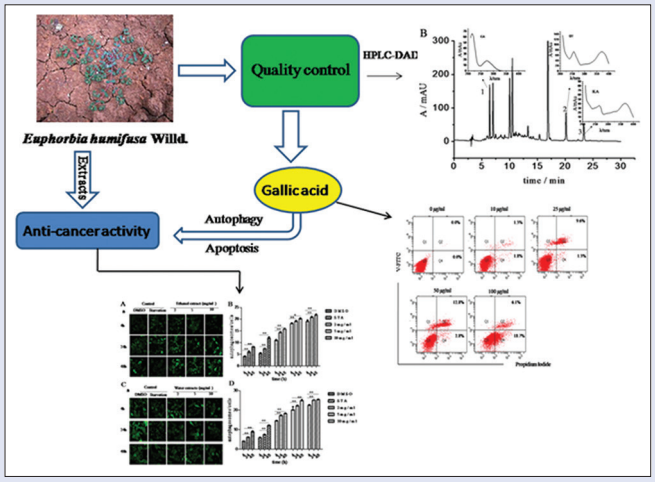
ABSTRACT

Background: *Euphorbia humifusa* Willd. (EH) is a kind of Chinese medicinal plant belonging to the family *Euphorbiaceae,* which is traditionally used for influenza, jaundice, hepatitis B *virus*, hypotension, inflammation, etc. In a modern study, active ingredients of EH reported to exhibit antioxidant, anticancer, and other properties. However, there are few reports showing that EH and its active compounds have inhibitory effects on cervical cancer through autophagy and apoptosis. **Objective:** To evaluate the anticervical cancer activity of EH and its main active ingredient Gallic acid (GA) by studying on autophagy and apoptosis of normal rat kidney (NRK) and HeLa cell lines, respectively. **Materials and Methods:** GA, kaempferol (KA), and quercetin (QU) are the main active compounds of EH. Identification and quantification of three substances from four batch samples obtained from four areas in Xinjiang were analyzed by high-performance liquid chromatography with diode-array detection. The potent effects of EH on anticervical cancer were investigated through autophagy and apoptosis in HeLa and NRK cells with varying concentrations of extracts and active compound GA treatment. The antiproliferation activity against HeLa cells was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Autophagy and apoptosis in NRK and HeLa cells were observed by laser scanning confocal microscope. Quantitative data of apoptosis were estimated by Hoechst staining and Annexin-V binding assay using flow cytometry. The expression levels of autophagy-related protein (LC_3 and P_{62}) were subjected to western blot. Moreover, the autophagic vacuoles and other ultrastructures of cells were observed under transmission electron microscopy. **Results:** The contents of GA, KA, and QU were measured as 2.3342–3.4688, 0.4636–1.5922, and 0.9349–3.1500 mg/g, respectively. We used different concentrations of GA and the extracts of EH to treat with the line of cells, respectively. The best concentration of GA, water, and ethanol extracts inducing autophagy was 25 μg/ml, 10 mg/ml, and 10 mg/ml, respectively. The autophagy mediated with EH induced the accumulation of autophagosome, and even resulted in apoptosis. **Conclusion:** From our study, these results indicated that EH and GA may induce both autophagy and apoptosis in NRK and HeLa cells. The activity we studied on autophagy and apoptosis of HeLa cells may provide a new foundation for cervical cancer therapy or other related applications.

Key words: Apoptosis, autophagy, cervical cancer, *Euphorbia humifusa* Willd., HeLa cells, high-performance liquid chromatography with diode-array detection

SUMMARY

- In this study, high-performance liquid chromatography with diode-array detection (HPLC-DAD) was performed for quantitative analyses of three active components, namely, gallic acid (GA), kaempferol (KA), and quercetin (QU), of E. humifusa Willd. And we mainly studied anticancer effects of GA and extracts of E. humifusa Willd. in inducing autophagy and apoptosis in normal rat kidney and Hela cells. Those studies may provide new foundations for cancer therapy or other related applications.



Abbreviations used: EH: *Euphorbia humifusa* Willd.; HPLC-DAD: High-performance liquid chromatography with diode-array detection; NRK: Normal rat kidney; GA: Gallic acid; FC: Flow cytometry; LSCM: Laser scanning confocal microscope; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; TEM: Transmission electron microscopy; KA: Kaempferol; QU: Quercetin; LC3: The light chain 3; DMEM: Dulbecco's Modified Eagle's medium; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; PBS: Phosphate-buffered saline; PI: Propidium iodide; IC_{50}: Half-maximal inhibitory concentration; BFA: Bafilomycin A1.

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INTRODUCTION

Euphorbia humifusa Willd. (EH) belongs to the family *Euphorbiaceae* and is an annual creeping herb[1] distributed in various places such as fields, roadside, and wastelands. The clinical applications of EH became increasingly widespread, and it is now frequently used for dysentery, enteritis, and hemoptysis and owns antioxidant and anticancer properties.[2] EH is also a type of traditional Uyghur medicine commonly used in clinics for skin disease therapy. Some chemical studies showed that phenolic acids, flavonols, sesquiterpenoids, and triterpenoids are the main bioactive ingredients found in EH.[3-5]

Gallic acid (GA) is a bioactive phenolic compound, which exists in various plants such as grains, tea, and fruits and features various applications, including human cosmetics, food, pharmaceutical formulation, and juice production.[6] GA was reported to possess remarkable effects on some pharmacological activities such as antioxidant,[7] antiviral, antimicrobial,[8] and anticancer properties.[9] GA also demonstrates anticancer activity by inducing autophagy and apoptosis in some cancer cells.[10,11] Kaempferol (KA) is a kind of flavonoid that is widely used as antibacterial,[12] anticancer, and antioxidant agent and as treatment for inflammatory disorders.[13,14] Quercetin (QU) is an important flavonoid exhibiting vast pharmacological properties including antitumor, antioxidant, anti-inflammatory,[15] and cardioprotective effects.[16,17]

Autophagy corresponds to a major degradation pathway of highly conserved lysosomal-dependent eukaryotic cells, a protective mechanism that protects tumor cells from various stimulations such as low nutrition, hypoxia, starvation, radiation, and treatment damage.[18,19] Autophagy maintains intracellular homeostasis by eliminating intracellularly error-folded aggregated proteins, long-lived proteins, or damaged organelles.[20] Thus far, data showed that autophagy plays a significant role in various physiological conditions and pathophysiological processes to regulate cell growth, apoptosis, survival, and cure cancer.[21] The cervical cancer is one of the most common cancers in women globally, which is a cause of cancer death.[22,23] The anticancer plant of the world would be an important source to be anticancer drugs.[24] Moreover, autophagy and apoptosis will also be the main focus of cancer research in the future.[25-28]

In this study, KA, QU, and GA were main active substances which were analyzed through high-performance liquid chromatography with diode-array detection (HPLC-DAD), which is a most efficient and useful analytical technique for identification and quantification of sensitive compounds in extracts of Chinese herbal medicines with its less expensive, simple, rapid, sensitive, and accurate characteristics.[29-31] Moreover, we also demonstrated that the extract and main active compound (GA) of EH can induce dose-dependent autophagy and apoptosis in NRK cells and HeLa cells, which may provide a new foundation for cervical cancer therapy or other related applications.

MATERIALS AND METHODS

Preparation of extractions and high-performance liquid chromatography analysis

Four batches of samples of EH (whole plant) were collected from Ciconhabo Uighur Medicine Co., Ltd., Hetian, Fukang, Kashi, Xinjiang, China and labeled S1 (100701-1), S2 (130117-1), S3 (160816-1), and S4 (160817-1), respectively, which all were identified by Yonghe Li, a chief pharmacist of the Chinese Medicine Hospital of Xinjiang. Whole plants were powdered and passed through a 50-mesh sieve. Powdered samples weighed 1.5 g and were subjected to extraction at 75°C in a reflux device for 1.5 h with 50 ml of 80% methanol (Fuyu Fine Chemical Company, China) and weighted timely. Then, 80% methanol was added to complement loss after cooling. Samples were shacked and then filtered. A total of 7 ml HCl solution (Fuyu Fine Chemical Company, China) was added to 20 ml of residue; resulting mixture was measured, heated, and then hydrolyzed in water bath for 30 min before immediate cooling.[32] Afterward, the solution was transferred to a 50 ml volumetric flask and diluted to 50 ml by addition of methanol. All sample solutions were shaken and filtered through a 0.22 μm membrane filter for HPLC analysis. The extracts were freeze-dried to powder and stored at 4°C for the next study. Qualitative and quantitative analyses were performed using Agilent 1220 LC-Agilent 1220 DAD equipped with Agilent ZORBAX SB-C18 column (4.6 mm × 250 mm, 5 μm USA) with a flow rate of 0.8 ml/min and injection volume of 8.0 μl. Column temperature measured 25°C. Mobile phase was 0.4% phosphoric acid (Chengdu Kelong Chemical Reagents Company, China) in pure water (A) and methanol (B) (Fisher Scientific, USA) filtered through a 0.45 μm membrane filter and then denaturized ultrasonically before use with the following gradient elution: 0–2 min, 10%–28% B; 2–7 min, 28%–40% B; 7–10 min, 40%–50% B; 10–15 min, 50%–60% B; 15–18 min, 60%–63% B; 18–20 min, 63%–65% B; and 20–25 min, 65% B. Detection wavelength was set at 270 nm. Active compounds, GA, KA, and QU were simultaneously identified by comparing retention time with standards and quantified with chromatogram peak areas. And six different concentrations of standard solutions were injected for constructing the linear standard curves to obtain linear regression equations with the peak area as the longitudinal coordinate (Y) and the quality of the corresponding reference as the cross coordinate (X).

Preparation of standard solutions for high-performance liquid chromatography analysis

Stock standard solutions of GA, QU, and KA (the National Institutes for Food and Drug Control, Beijing, China)) were dissolved in methanol. These compounds were prepared at concentrations of 0.57 mg/ml for GA, 0.11 mg/ml for QU, and 0.75 mg/ml for KA. Standard solutions were filtered through a 0.45 μm membrane filter for further study.

Cell culture

NRK cell lines constructed with the eukaryotic expression vector of GFP-LC_3 were obtained from Tsinghua University (Beijing, China). HeLa cell lines were obtained from Xinjiang Medical University. Cell lines were cultured in DMEM (Hycolon, USA) supplemented with 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin), 10% fetal bovine serum (FBS) (Hycolon, USA), and 1% glutamine at 37°C in a 5% CO_2 humidified atmosphere. Medium of NRK cell lines was refreshed every day during cultivation and every 24 h in the experimental setting. The medium of HeLa cell lines was refreshed every 2 days during cultivation and every 36 h in the experimental setting.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay

An 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to the effect on cell proliferation in HeLa cells. We cultured and collected during logarithmic growth phase. And then, the cells were seeded in 96-well plates at a density of 6×10^4 cells/mL with a volume of 100 μL each well and incubated for 24 h. The cells were treated with GA (1–100 μg/mL) and extracts (1–10 mg/mL) with different concentrations for 24 h; dimethyl sulfoxide (DMSO) (Sigma, USA) treatment was taken as a control. After the above treatment, 0.5 mg/mL of MTT was added with 20 μL to each well, and then, the cells were incubated for 4 h at 5% CO_2 and 37°C. After 4 h, the supernatants of wells were removed, followed by 150 μL of DMSO being added into each well to dissolve the formazan crystals. The result was measured by a microplate reader at 490 nm.

Treatment with extracts and Gallic acid

S2 powder sample was dissolved with distilled water and ethanol at different concentrations (2, 5, and 10 mg/ml) for subsequent cell experiments. After treatment with designed concentrations of extracts for 4, 24, and 48 h, samples were prepared for related analyses.

GA was dissolved in DMSO as stock solutions (50 μg/μl) and frozen at −20°C until use. Stocks were diluted to determined concentrations using DMEM when needed. The cells were treated with GA at different concentrations for 4, 24, and 48 h.

Laser scanning confocal microscope

The NRK cells were treated with GA (10, 25, and 50 μg/ml) and coincubated for 4, 24, and 48 h. Then, laser scanning confocal microscope (LSCM) (Nikon, Japan) was performed to observe expressions of GFP-LC_3, lysosome, autophagosomes, and mitophagy in NRK cells at 60 magnification with 10 single confocal sections of 0.7 μm. Extracts with different concentrations were also added to NRK cells for related observation using the same method. Experiments were repeated thrice for each concentration group and sample.

Hoechst 33342 staining

As mentioned above, HeLa cells were exposed to different concentrations of GA for 4, 24, and 48 h. After incubation, cells were washed twice with PBS, and then stained with 1 μg/ml Hoechst 33342 and diluted with PBS for 30 min in incubator. Then, LSCM was employed to observe nuclear morphology of cells.

Transmission electron microscopy

Cells were treated with different concentrations of GA, washed thrice with PBS, trypsinized with trypsin solution, neutralized with DMEM, and then centrifuged. Then, supernatant was removed, and 200 μl FBS was added. Resulting mixture was centrifuged, and supernatant was removed again. The final product was fixed with 4% paraformaldehyde and 2.5% glutaraldehyde for 30 min and then postfixed with 1% osmium tetroxide. Dehydration was performed in graded alcohol, and samples were embedded in Epon. Ultrathin sections corresponded to representative areas. Transmission electron microscopy (TEM) (Hitachi, Tokyo, Japan) was used to visualize changes in ultrastructure of cells to examine autophagic vacuoles to evaluate autophagy and to observe nuclear condensation to assess apoptosis.

Flow cytometry

FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, Shanghai, China) was applied to test cell apoptosis according to its protocol. The cells incubated for 24h and 48h were exposed to GA (25μg/ml, 50μg/ml, and 100μg/ml) for 24 h and 48 h, respectively; and then washed with PBS, trypsinized and centrifuged. And then washed cells twice with cold PBS, resuspended cells in 1X Binding Buffer at a concentration of $1 \times 10^{\wedge 6}$ cells/ml. The cells were transferred to 100 μl of the solution ($1 \times 10^{\wedge 5}$ cells) to a 5 ml culture tube, added 5 μl of FITC Annexin V and 5 μl PI. And then gently vortexed the cells and incubated for 15 min at room temperature in the dark. Finally, added 400 μl of 1X Binding Buffer to each tube. After samples collected, a flow cytometry machine (BD Bioscience, Shanghai, China) was performed to detect and analyze cell apoptosis percentage which was evaluated with Annexin V/PI ratio.

Western blot

Harvested NRK and HeLa cells were rinsed with PBS. Protein lysis buffer was used to lyse cells. Protein samples were denatured by boiling for 15 min at 100°C and then separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein samples were transferred into nitrocellulose filter membrane using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA). Proteins were blocked in 5% blocking solution for 1 h at room temperature. The NC membrane was incubated with primary antibodies (Sigma, USA), including p62 (1: 1000), β-acting (1: 50,000), and LC_3 I/II (1: 1000) for 1 h at room temperature and then incubated with secondary antibody goat anti-rabbit IgG (H + L) (1: 5000) (Southern Biotech, USA). Enhanced chemiluminescence western blot detection reagent was used for visualizing results with Simon Western Blot automatic detection analyzer (Protein Simple, USA) to analyze band results.

Statistical analysis

All the experiments were performed in three independent times. The results were expressed as the mean ± standard deviation. We used Student's test to evaluate the differences between the control and drug-treated groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Method validation

The method was validated for linearity, precision, stability, repeatability, recovery, and robustness.

Linearity

The calibration curves were constructed by analyzing three standard solutions at six different concentrations ($n = 3$). The results presented good linearity. Moreover, the linear standard curves of the analytes and the calculated correlation coefficients (r^2) are shown in Table 1.

Stability

The same sample solution was analyzed to assess the stability of this method at 0, 2, 4, 6, 8, 10, 12, and 24 h. The relative standard deviation (RSD) value of the peak areas of this sample was in the range of 0.90%–1.39%. The result indicated that sample solution of EH was stable within 24 h [Table 2].

Precision

The interday precision was analyzed by determining the mixture of three standard solutions.

The RSD values of the peak area for six times are given in Table 3. These results demonstrated the suitable precisions of the proposed analytical method.

Recovery

The recovery was determined according to the standard addition method. The experiments were performed with adding standards of GA, QU, and KA to extracts of EH prepared according to the method of "*Preparation of extractions*" for six time, and the results were provided in Table 4.

Identification and quantification of *Euphorbia humifusa* Willd.

Active compounds were identified and quantified by HPLC-DAD at 270 nm. Results of HPLC showed that retention times of three

Table 1: Statistical performances of linear regression equation analysis

Compound	Regression equation	r^2	Linear range (μg/μl)
GA	Y=6112.6X + 13.2400	0.999	0.02828-0.28810
KA	Y=5412.5X − 0.6133	0.999	0.00548-0.00562
QU	Y=3674.7X + 8.7267	0.999	0.03733-0.03811

GA: Gallic acid; KA: Kaempferol; QU: Quercetin

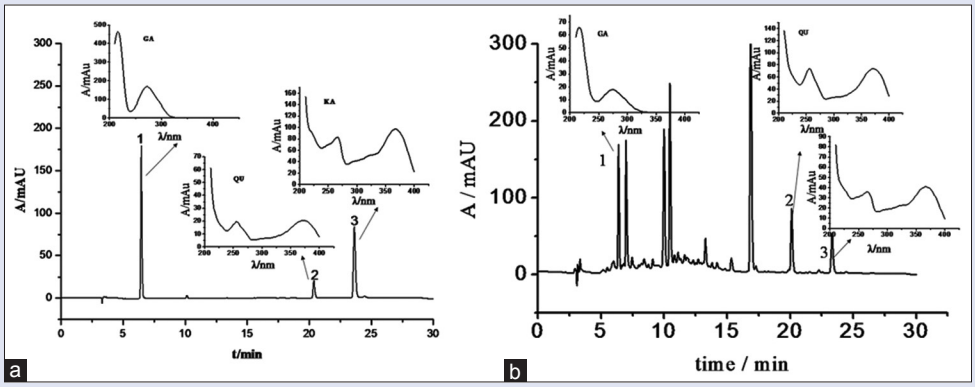

Figure 1: The typical rapid resolution liquid chromatography chromatograms and UV spectra. (a) Standards; (b) Samples; (1) GA: Gallic acid; (2) QU: Quercetin; (3) KA: Kaempferol

Table 2: The results of the stability test

Compound	Time (h)/peak area (mAU)						Average (peak area)	RSD (%)
	0	2	4	6	8	10		
GA	501.7	502.8	503.9	499.2	505.6	498.4	501.9	0.5498
QU	420.9	418.8	418.1	407.4	411.4	407.5	414.0	1.4493
KA	553.7	559.6	561.4	555.0	561.2	555.5	557.7	0.6089

RSD: Relative standard deviation; GA: Gallic acid; KA: Kaempferol; QU: Quercetin

Table 3: The results of the precision test (n=6)

Compound	Peak area (mAU)						RSD (%)
GA	1396.0	1411.1	1413.4	1416.4	1420.5	1422.2	0.6764
QU	239.8	236.6	240.1	240.6	242.6	241.7	0.8764
KA	1104.6	1116.2	1117.0	1126.3	1129.1	1125.4	0.8191

RSD: Relative standard deviation; GA: Gallic acid; KA: Kaempferol; QU: Quercetin

components [Figure 1a] measured 6.408 (GA), 20.38 (QU), and 23.64 min (KA), which agree with retention time of standards [Figure 1b]. As shown in Table 5, analyzed contents of GA, QU, and KA in four batches of EHW reached 2.3342–3.4688, 0.4636–1.5922, and 0.9349–3.1500 mg/g, respectively.

Effect of Gallic acid and extracts on cell proliferation in HeLa cells

We examined the effect of GA and extracts on the proliferation of HeLa cells by MTT assay [Figure 2]. We observed that GA exerted cytotoxicity on HeLa cells after treatment with various concentrations for 24 h. They also exhibited the antiproliferative activity against HeLa cells in a dose-dependent manner. The IC_{50} of GA, water extract, and ethanol extract was approaching 25 μg/ml, 4.5 mg/ml, and 4.5 mg/ml, respectively.

Observation of autophagy and apoptosis by laser scanning confocal microscope

We estimated expression of autophagy and apoptosis through LSCM with observing the incidence of GFP-LC_3, lysosome, autophagosomes, and mitophagy in NRK cells with extracts and GA by capturing 10 photos in at least 10 different views. As shown in Figure 3a-d, cells were incubated with extracts which showed promoted autophagy at 2–10 mg/ml in dose- and time-dependent manners. Autophagy was triggered by GA for 4, 24, and 48 h. Fluorescent puncta of autophagic vacuole of cells treated with increasing concentrations of GA showed a upregulation as shown in Figure 3e and f. At 50 μg/ml concentration, GA treatment for 4, 24, and 48 h possibly induced apoptosis in NRK cells. We inferred that apoptosis is more possible at a concentration over 50 μg/ml.

Staining with Hoechst 33342

As mentioned above, aside from those observed at 4 h, cell lines under GA treatment also showed remarkable incidence of autophagy and apoptosis at 24 and 48 h. We examined nuclear morphological changes in HeLa cells treated with GA for 4, 24, and 48 h with LSCM by staining with Hoechst 33342. Compared with the control group, GA-treated cells showed brighter staining and densely stained or fragmented nuclei of apoptotic cells in dose- and time-dependent manners [Figure 4]. Some turgescent and enlarged nuclei were observed after staining.

The quantitative data of cell death induced by Gallic acid

We determined the quantitative data of cell death induced by GA through analyzing changes in apoptosis marker with flow cytometric technique after staining with Annexin V-FITC/PI. The apoptosis of cells showed a dose-dependent manner after incubated with GA [Figure 5].

Gallic acid-enhanced autophagy in normal rat kidney and HeLa cells

Results of LSCM showed that GA and water extracts of EH dose dependently upregulated expression of autophagy. Western blot analyses were performed to detect LC_3 levels to further identify GA-induced autophagy in cells. Results showed that conversion rates of autophagy-activated LC_3-I to LC_3-II were expressed highly after increasing concentrations of GA [Figure 6Aa and Ab] and extract [Figure 6Ba and Bb] administrations in cells, respectively. Drug-induced conversion of LC_3-I to LC_3-II is the most conclusive and characteristic indicator of autophagic process with autophagosome formation; thus, results were subjected to LSCM analyses. Bafilomycin A1 (BFA),

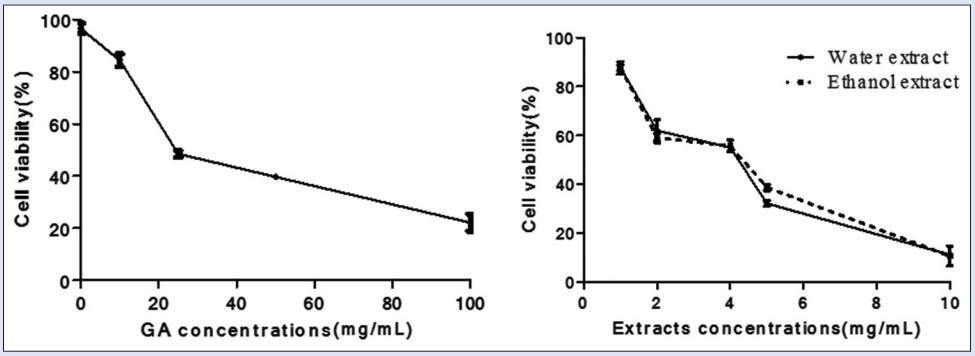

Figure 2: The viability of HeLa cells treated with Gallic acid and extracts for 24h was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. The data are obtained with mean ± standard deviation of results from three independent experiments

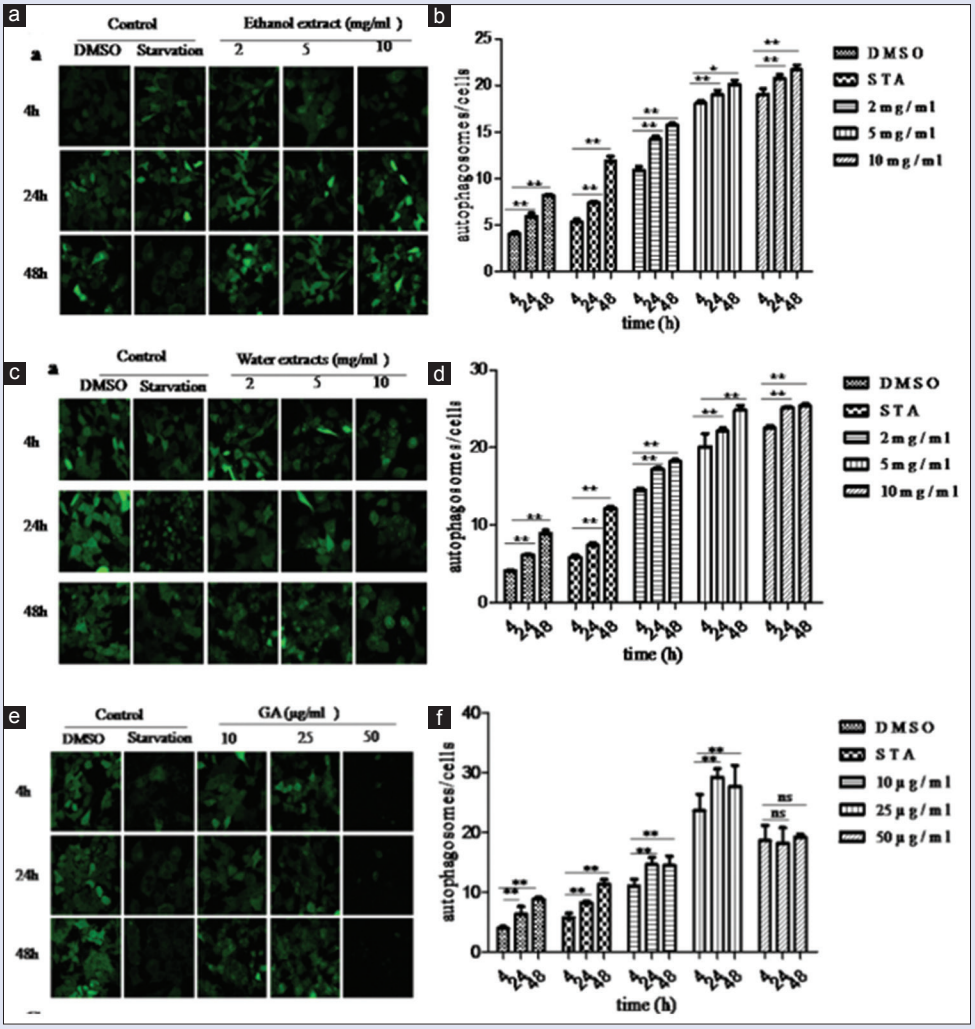

Figure 3: Water, 95% ethanol extracts, and Gallic acid increase induces autophagy and apoptosis observed by CLSM. NRK-GFP-LC3 cells were incubated with the indicated concentrations of ethanol extract (a and b) and water extract (c and d) of *Euphorbia humifusa* Willd. and increasing concentrations of Gallic acid (e) for 4, 24 and 48 h. Autophagosomes were analyzed by the presence of GFP-LC3 puncta under CLSM. DPBS starvation was used as a positive control for inducing autophagy. b, d, and f were evaluated by GraphPad Prism, mean ± standard deviation were presented (*$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$; Student's test)

belonging to vacuolar H + ATPase (V-ATPases) macrolide inhibitors, inhibits the formation of autophagosomes by inhibiting vacuolar V-ATPase activity, resulting in increased autophagy and decrease in autophagosomes.[33] Expression of LC3-II significantly increased, and we used BFA as a stronger positive control. The p62 is a multifunctional protein generated under stress, sequestered in autophagosomes, and

Table 4: Recoveries of the three compounds (*n*=6)

Compound	Amount of sample (mg)	Amount of standard (mg)	Amount of found (mg)	Recovery (%)	Average (%)	RSD (%)
GA	1.4098	1.50	2.8097	93.33	97.68	2.31
	1.5932		3.0542	97.40		
	1.4936		2.9815	99.19		
	1.5240		3.0065	98.83		
	1.5110		3.0012	99.35		
	1.5097		2.9791	98.00		
QU	0.3476	0.30	0.6365	96.30	98.47	1.95
	0.3325		0.6331	100.20		
	0.3181		0.6209	100.93		
	0.3047		0.5937	96.33		
	0.3045		0.6003	98.60		
	0.3123		0.5198	98.48		
KA	1.9321	1.91	3.7240	93.82	96.37	1.74
	1.9045		3.7324	96.21		
	1.9037		3.7365	96.46		
	1.9208		3.7401	95.75		
	1.8976		3.7411	97.03		
	1.8616		3.7520	98.97		

RSD: Relative standard deviation; GA: Gallic acid; KA: Kaempferol; QU: Quercetin

Table 5: Quantification of gallic acid, quercetin, and kaempferol in four batches of *Euphorbia humifusa* Willd. (*n*=3)

Batches	GA (mg/g)	QU (mg/g)	KA (mg/g)
S1	2.3865±0.1678	0.4636±0.1237	3.1500±0.1910
S2	3.4688±0.1325	0.8240±0.2143	2.5658±0.2013
S3	2.3342±0.1140	1.5922±0.1705	0.9349±0.0795
S4	2.7621±0.2280	1.1086±0.1815	2.1897±0.1437

GA: Gallic acid; KA: Kaempferol; QU: Quercetin

degraded in autolysosomes. The p62 level is an important indicator of impaired autophagic flux.[34] Compared with positive and negative controls, GA and extract treatments decreased expression of p62 in NRK [Figure 6Ac and Ad] and HeLa cells [Figure 6Bc and Bd] indicating that such treatments failed to block autophagy flux. These results agree with those of the above experiments.

Ultrastructural analysis by transmission electron microscopy

TEM analyses were performed to observe morphological and ultrastructural changes in cells. The number of lysosomes promoted by GA administration in NRK cells was also analyzed. As shown in Figure 7, numerous double-membrane vacuolar structures in the cytoplasm of NRK cells were affected with GA, indicating dose-dependent changes in morphological characteristics of autophagosomes and the number of lysosomes. Apoptotic cells with typical apoptotic characteristics showed chromatin condensation or margination.

DISCUSSION

EH, a traditional Chinese plant and Uyghur medicine, showed its effect on inducing autophagy and apoptosis in HeLa and NRK cells because of its own features and active ingredients, such as GA. EH is usually exerted in clinical practice to cure dysentery, enteritis, hemoptysis, traumatic bleeding, and hemafecia. In Xinjiang, Uyghur and traditional Chinese medicine physicians usually use it for skin disease therapy. The pharmacological effects of EH were antioxidant, anti-inflammatory, and anticancer properties. In this study, we determined three active ingredients of EH by HPLC-DAD. We studied the relationship between anticancer activity and cell autophagy treated with EH and GA, respectively. Among three compounds, GA may play an important role in anticancer therapy.

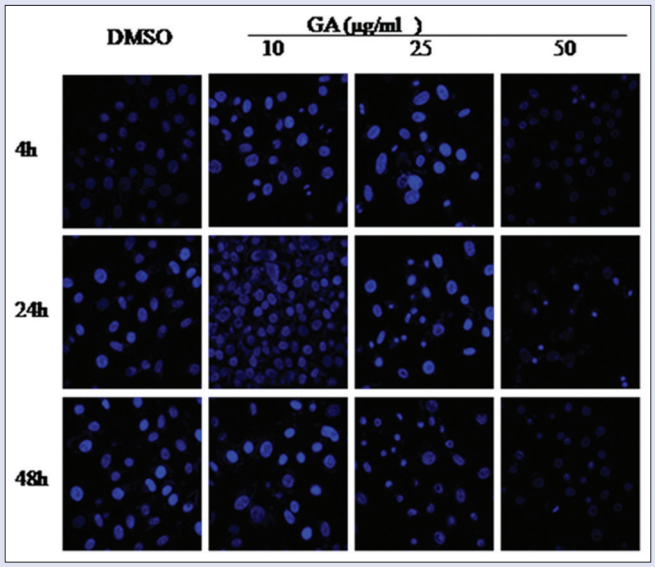

Figure 4: Morphology changes in HeLa cells. The cells were treated with increasing concentrations of Gallic acid for 4 h, 24 h and 48 h. And the morphologic changes of Gallic acid-induced autophagy in HeLa cells were observed under LSCM after Hoechst 33342 staining

The result of HPLC analyses showed good linearity (r^2 = 0.9999), precision, stability, repeatability, recovery, and robustness. Moreover, HPLC data showed that GA, KA, and QU of S2 sample are generally higher in contents. We choose S2 as the main research sample which was extracted into different concentrations using distilled water and ethanol for quality control using HPLC-DAD and anticancer study by observing autophagy and apoptosis levels in NRK and HeLa cells.

We found that extracts and GA may have an effect on cervical cancer through inducing autophagy and apoptosis in HeLa cells. Moreover, morphological changes were observed using LSCM, ultrastructural changes investigated with TEM, and LC_3 and p62 levels detected by western blot. In the cytoplasm, autophagosomes (autophagy generates vacuoles) can be assessed by determining levels of LC_3. The ratio of LC_3-II to LC_3-I can directly express autophagosome formation levels. After

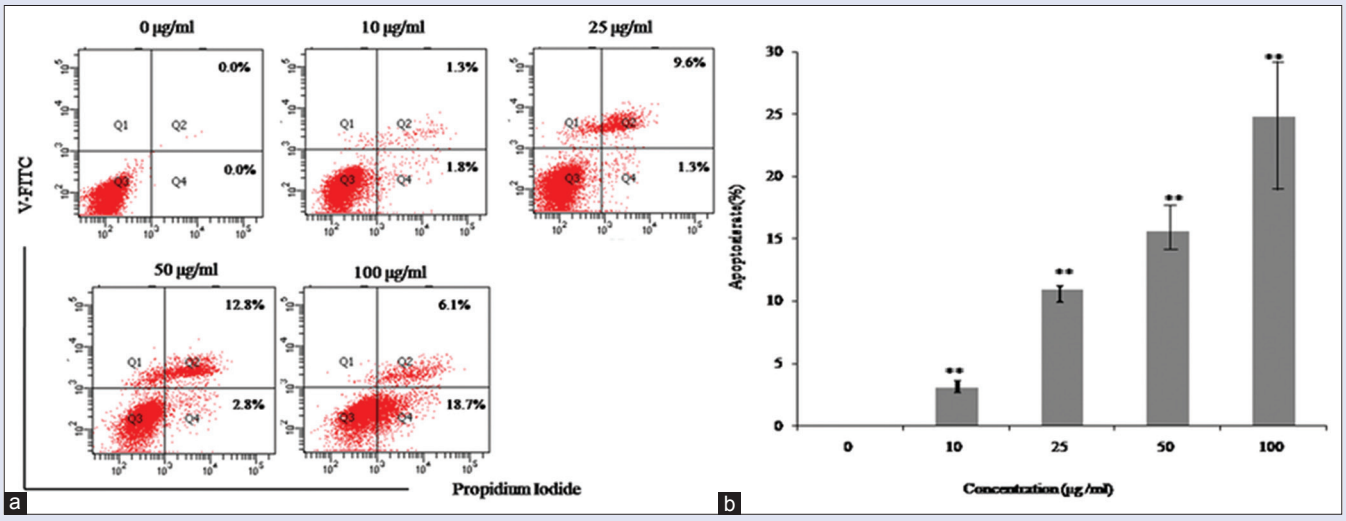

Figure 5: The apoptosis detection of HeLa cells exposed to Gallic acid with various concentrations for 24 h. (a) The representative dot plots of data by Annexin V-FITC/PI staining using flow cytometry analysis. (b) Is quantification of apoptosis rate data from figure 5a. The data were presented as the mean ± standard deviation of three independent experiments (**$P < 0.01$; Student's test)

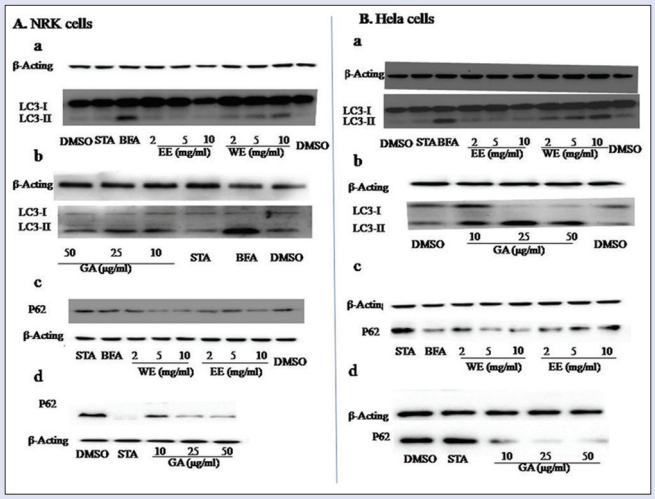

Figure 6: Western blot for detecting LC3 conversion and the level of P62. (A) Normal rat kidney cells and (B) HeLa cells were treated with increasing concentrations of Gallic acid (10, 25, 50 μg/ml), ethanol extract (EE; 2, 5, 10 mg/ml), and water extract (WE; 2, 5, 10 mg/ml) for 4 h to analyze the expression levels of LC3-I, LC3-II, and P62 in normal rat kidney and HeLa cells by the representative blots. β-Acting was used as a loading control. The bafilomycin A1 and serum starvation group in western blotting were used to be positive control

studying GA-treated NRK and HeLa cells with increasing concentrations for 4, 24, and 48 h, LC_3-II levels increase depended on time and dosage. We screened concentrations of GA and extracts of EH. Results showed that 25 μg/ml of GA and water extract treatment in cells significantly affected autophagy, and GA exerted apoptotic effects over 50 μg/ml in cells.

In this study, the anticancer effect of EH and GA were screened *in vitro* against HeLa cells. We demonstrated that EH induced autophagy and apoptosis in a time-dependent and concentration-dependent manner, which may show its anticancer activity.

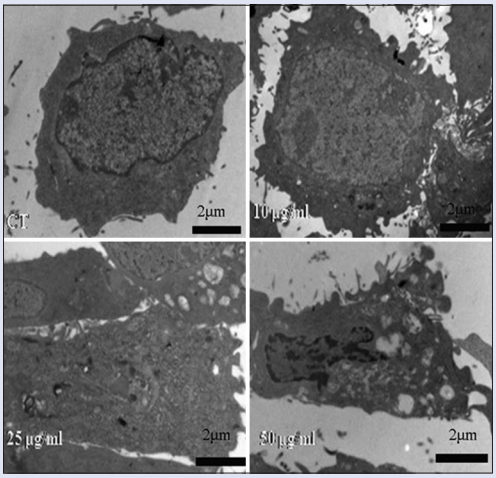

Figure 7: The cells were treated with Gallic acid (0, 10, 25, 50 μg/ml) then observed by transmission electron microscopy. Dimethyl sulfoxide treatment was used as a control. Scale bar: 2 μm. Arrows: The autophagic vacuoles

CONCLUSION

The active ingredients in traditional Chinese medicine and its extracts were usually determined by HPLC. Interestingly, HPLC was performed for simultaneously quantitative analyses of three compounds of EH in this study.

EH exerted autophagy-associated cell death in NRK and HeLa cells. The results may support further researches of EH and GA in some fields of cancer and related diseases' treatment.

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Conflicts of interest

There are no conflicts of interest.

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