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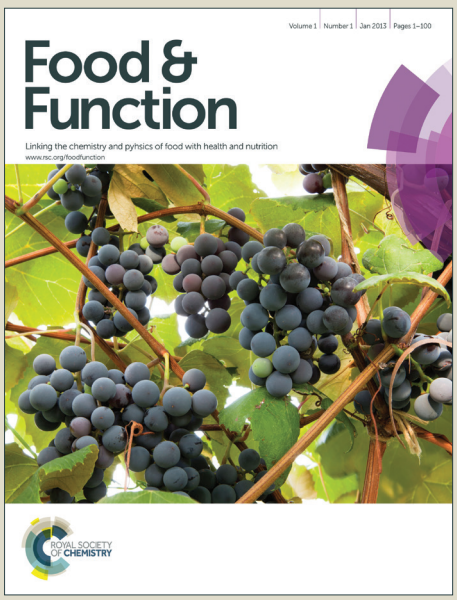

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Bioactive compounds of *Eriocaulon sieboldianum* blocking proliferation and inducing apoptosis of HepG2 cells might involve in Aurora kinase inhibition

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Eriocaulon sieboldianum **(Sieb. & Zucc. ex Steud.) is an edible and medicinal plant used in traditional Chinese medicine. Often in combination with other herbs, it is processed into healthcare beverages for expelling wind-heat, protecting eyes, and reducing blood lipids. Besides, its water decoction together with other herbs has been utilized to treat cancer in China. However, the active ingredients and the precise cellular mechanisms of *E. sieboldianum* remain to be elucidated. Aurora kinase family plays critical roles in the regulation of cell division and has attracted great interest to the identification of small- molecules Aurora kinase inhibitors for the potential treatment of cancer. A molecular docking study was employed for docking of the most bioactive compounds. Hispidulin (HPDL) and quercetin-3-*O*-(6″-*O*-galloyl)-β-D -galactopyranoside (QGGP) were singled out as potent inhibitors of Aurora kinase. Their inhibitory activity towards Aurora kinase was further confirmed by the obvious decrease in autophosphorylation of Aurora-A (Thr288) and Aurora-B (Thr232). Moreover, the induction of cell cycle arrest in HepG2**

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cells and the suppressed phosphorylation of histone H3 were also consistent with inhibition of Aurora kinase. The data indicate that *E. sieboldianum* extract and its two active compounds , HPDL and QGGP, could effectively induce apoptosis via p53, MAPKs and the mitochondrial apoptotic pathways. These findings could improve the understanding and enhance the development of drugs based on *E. sieboldianum* and raise its application value in anticancer therapy or prevention. In addition, our results indicated Aurora kinase might be a novel target of HPDL and QGGP.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide[1]. Chemotherapy is a common therapeutic method for the treatment of unresectable HCC. However, the majority of patients with HCC have poor prognosis and die after months of chemotherapy, mainly due to the high level of intrinsic and acquired chemo-resistances[2, 3, 4]. Thus, there is an urgent need to develop new chemotherapy agents for treatment of HCC.

Aurora kinase is a family of serine / threonine kinases in mammalian and plays a pivotal role in the tumorigenesis and development of many human tumors, including breast, colorectal and hepatocellular carcinoma[5, 6]. In human, Aurora family comprises of Aurora-A, Aurora-B, and Aurora-C. Aurora-A regulates chromosome maturation and mitotic spindle formation; Aurora-B one of the chromosomal passenger proteins, controls chromosomal segregation and cytokinesis[5, 7, 8]; whereas Aurora-C is involved in meiosis[9]. Overexpression of aurora kinases has been reported to be strongly associated with poor prognosis and shortened overall survival [8, 10, 11, 12]. It is reported that Aurora-A phosphorylates p53 and Aurora-A loss leads to upregulation of p53 signaling [13]. Aurora-B is responsible for the mitotic phosphorylation of Ser10 residue of histone H3[14] and this phosphorylation is thought to be necessary for chromosome condensation[15]. Furthermore, auto-phosphorylation of Aurora-A (Thr288) and Aurora-B (Thr232) in human is important for the full activation of Aurora kinase[16]. Previous studies proposed Aurora kinases as promising drug targets in cancer treatment [8, 17, 18] and much effort has been invested in the development of small-molecule Aurora kinase inhibitors. So far, Alisertib, the selective Aurora-A inhibitor, is in phase 3 clinical trial. Aurora-B inhibitors, such as Barasertib[19] and GSK1070916 [20] are in phase1clinical trial. The suppressed action of Aurora kinases by Aurora inhibitors, such as ZM447439 and VX680, increases p53, the ratio of Bax/Bcl-2 and cleaved caspase-3 [21, 22, 23]. Recently, Aurora-A was also found to have the abilities to increased ERK1/2 activity [24]. In HepG2 cells, Aurora kinase is over-expressed and inhibition of Aurora kinase by a chemical agent is reported to cause polyploidy and cell morphology changes followed by apoptosis in HepG2 cells in vitro and in vivo[6, 25, 26, 27]. However, considering the severe adverse events and side effects of some chemosynthesis drugs, e.g. decrease in neutrophil counts [28]. Therefore, the research of natural aurora kinases inhibitors has raised concerns. Recent studies revealed that three natural flavonoids, S39, luteolin and eupatorin, have been demonstrated to be inhibitors of aurora kinases [29, 30, 31].

Eriocaulon sieboldianum Sieb. & Zucc. ex Steud. is a genus of *Eriocaulon* in the Eriocaulaceae family under the name ‘‘Sai-Gu-Jing-Cao” (Siebold Pipewort) in China. *Eriocaulon sieboldianum* is a substitution herbal of *Ericaulon buergerianum*. In fact, they are exactly alike in medicinal uses in many parts of China. Both have been used in traditional Chinese medicine as an anti-inflammatory, ophthalmic and antimicrobial medicine [32, 33, 34]. They are also processed into healthcare beverages often in combination with other herbs for expelling wind-heat, protecting eyes, and reducing blood fat[35]. *E. sieboldianum* has long been used to treat cancer, alone or in conjunction with other herbs and achieved remarkable curative effect[36, 37, 38, 39]. However, the anticancer active ingredients and mechanisms of *E. sieboldianum* remain to be determined.

In order to determine the precise cellular mechanisms of the action of *E. sieboldianum*, molecular docking studies were performed to investigate the binding affinities and interaction modes for the active compounds that we have isolated from *E. sieboldianum* extracts. They were docked against Aurora-A and Aurora-B by 3D molecular docking. The results of virtual screening showed that two flavonoids of *E. sieboldianum* can perfectly bind with the amino acid residues in the active site of Aurora kinase. Therefore, the effects of *E. sieboldianum* extract and two active compounds on Aurora kinase and apoptosis were further investigated. In short, the active ingredients and the mechanism of the anticancer activity of the *E. sieboldianum* against HepG2 cells were examined. We expected to elucidate the anti-cancer mechanism of *E. sieboldianum*, and also hoped to exploit a low toxicity Aurora kinase inhibitor based on the structure of the two active compounds.

Materials and methods

Chemicals and reagents

Silica gel (200-300 mesh) for column chromatography was obtained from Qingdao Marine chemical company (Qingdao, P. R. China). Sephadex LH-20 was a product of Amersham Co. RP-18 (15-30 um) silica gel was purchased from Merk chemical Ltd. Preparative HPLC was carried out on a JASCO PU-2087 apparatus with an ODS column (YMC-Pack ODS-A, 150×10 mm). HPLC grade methanol (MeOH) (HiPure Chem., Elmsford, NY); analytic grade MeOH, petroleum ether (b.p. 60-90℃), CH_2Cl_2 and EtOAc (Tianjin Fuyu Fine Chemical Industry Co., Tianjin, China).

RPMI1640 medium was purchased from Hyclone (Logan, UT) and fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY). Antibodies for Bcl-2 (#sc-492), Bax (#sc-493), cytochrome C (#sc-13156), Caspase-3 (#sc-7148) and cleaved-Caspase-3(#sc-22171-R), anti-Histone H3 (#sc-10809), anti-p-Histone H3 (#sc-8656-R) (Ser10), anti-GAPDH (#sc-20357) and anti-β-actin (#sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p53 (#2527), PARP(#9542), p38(MAPK) (#8690), phospho-p38MAPK

(Thr180/Tyr182) (p-p38) (#4511), p44/42 MAPK (Erk1/2) (#4695), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (p-ERK1/2) (#4870), SAPK/JNK (#9252) and Phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK) (#4668), anti-aurora kinases A (#14475) /B (#3094)and anti-Phospho-Aurora A (Thr288) / Aurora-B (Thr232) / Aurora C (Thr198) (#13464) were were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell lines

Human hepatoblastoma cell line (HepG2 cells) was donated by professor He Zhong-gui from Shenyang Pharmaceutical University, Shenyang, China. HepG2 cells were cultured in a Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L of glucose (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Lonza, Walkersville, MD) at 37℃ with 5% CO_2 in a humidified atmosphere.

Herb authentication

E. sieboldianum was purchased from Jiangxi province of China. It was identified and authenticated by Prof. Jincai Lu, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China. A voucher specimen (No. 20130301) was deposited at the Department of Pharmacy, General Hospital of Shenyang Military Area Command, Shenyang, China.

Extraction and isolation

Chopped and air-dried whole plants of *E. sieboldianum* (10.0 kg) were extracted with 60% EtOH for 3 times (2 h each time) and concentrated under reduced pressure to give the crude extract (195.18g). The crude extract was dissolved in 8L H_2O, filtered and then fractionated with petroleum ether, EtOAc, and n-BuOH (each, 8L×3), successively. The EtOAc extract (55.2g) was subjected to silica gel column chromatography (CC) using increasing MeOH in CH_2Cl_2 (0%, 20%, 70% and 100%, v/v) to give four fractions (E1-E4). Then, most of pigments and impurities were removed. Afterward, fraction E3 (70% MeOH, hereinafter referred to as E3) was evaporated to dryness under reduced pressure. E3 (31.2 g) was purified using a silica gel column, eluted with a CH_2Cl_2-MeOH gradient (20:1-1:1) to give five fractions (E3-1-E3-5). Fraction E3-1 (625mg) was subjected to Sephadex LH-20 column chromatography (ϕ 2×80 cm) with MeOH/H2O (1:1, v/v) and then purified by RP-HPLC to yield compound 1 (14.71mg) and compound 2 (14.28mg). Fraction E3-2 (1.19g) was chromatographed on MPLC on ODS (ϕ 2×20 cm) with MeOH/H_2O (from 0:100 to 100:0, v/v) to yield six fractions (E3-2-1-E3-2-6). Fraction E3-2-1 was subjected to Sephadex LH-20 with CH_2Cl_2-MeOH (1:1, v/v) and then purified by RP-HPLC with MeOH-H_2O (22:78) to yield compound 3(28.7mg). Like E3-2-1, E3-2-2, E3-2-3, E3-2-4 and E3-2-5 were purified by Sephadex LH-20 with CH_2Cl_2 - MeOH (2:3, v/v) and RP-HPLC with MeOH-H_2O in a proportion 25:75, 28:72, 32:68 and 35:65, respectively. Compound 4(65.8mg), compound 5 (102mg), compound 6 (32.7mg) and compound 7 (18.9mg) were obtained. Fraction E3-3 was successively subjected to ODS CC with MeOH/H_2O (from 20:80 to 100:0, v/v) to give fractions E3-3-1-E3-3-4. E3-3-2 and E3-3-3 were purified by Sephadex LH-20 with CH_2Cl_2 - MeOH (2:3, v/v) and RP-HPLC with MeOH-H_2O (30:70, v/v) to give compound 8 (18.22mg) and compound 9 (40.13mg). E3-3-4 and E3-3-5 were purified by RP- HPLC with MeOH-H_2O (26:74, v/v) to give compounds 10 (89.57mg) and compound 11(4.86mg), respectively. The structures of the known compounds were identified by comparing ^{1}H NMR and ^{13}C NMR data with the literature as Irigenin (1)[40], Hispidulin (2) [41], Quercetin (3)[42], quercetin-3-*O*- (6''-*O*- galloyl)-β-D-galactopyranoside (4) [43], Hyperin (5) [44], hispidulin-7-*O*-β-D-glucopyranoside (6) [45], Apigenin-7-*O*-β-D -glucopyranoside (7) [46], Acacetin-7-*O*-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside(8) [47], Gallic acid (9) [48], protocatechuic acid (10) [49],Corilagin (11) [50] (Fig. 1). E3 and the compounds were dissolved in DMSO and diluted with RPMI 1640 (Logan, UT, USA) to serial concentrations when they were used.

Evaluation of growth inhibitory potential

The cytotoxicity of E3 and compounds 1-11 on HepG2 cells was measured by a standard MTT assay. HepG2 cells were seeded at a density of about 5× 10^3 cells per well in a 96-well microtiter plate. After culture for 12 h, cells were treated with DMSO or various concentrations of E3(0-100μg/mL) and compounds 1-11 (0-50μM), and then incubated for 72 h. 20μL of MTT (Amresco, Solon, USA) solution (5 mg/mL) was added to each well. After the incubation for 4 h, the culture mediums were removed and the formazan crystals were dissolved with 150μL DMSO. The optical density (OD) of each well was measured using a microtiter plate reader at a wavelength of 492 nm after being shaken for 5 min. Cell viability was calculated as follows: The inhibition rate (%) = [OD_{492} (control)-OD_{492} (compounds) / OD492 (control)] × 100%.

Molecular modeling

The Autodock 4.2 was used to perform docking calculations. The crystal structures of aurora-A (pdb: 4UYN) and Aurora-B (pdb: 4C2V) [51, 52] in complex with INCENP and VX-680 were used as the receptors for docking. Its active site was used as the center of the grid box for docking, and the size of the grid box was 60 × 60 × 60 Å with a grid spacing of 0.375 Å. The structures of Aurora kinase for docking were treated with the Autodock Tools program suite (http: //mgltools. scripps. edu). The ligand structures were built with Sybyl 6.9.1 software package (Tripos Associates: St. Louis, MO, 2003), assigned charges using the Gasteiger-Hückel method and minimized with the Powell method (Tripos force field) to an energy gradient of 0.05 kcal/ (mol Å). Flexible torsions in the ligands were assigned and all dihedral angles were allowed to rotate freely. The Lamarckian genetic algorithm (LGA) was used to find the appropriate binding positions, orientations, and conformations of the ligands. Besides, the evolved population terminated after 27,000 generations and a maximum of 2,500,000 energy evaluations. Other running parameters for docking were used as the default values in the

Autodock program. Molecular graphics were generated with Discovery Studio v4.0.

Morphologic evaluation of cell death

To assess the effect of E3 and Hispidulin (hereinafter referred to as HPDL) and quercetin-3-*O*-(6"-*O*-galloyl)-β-D-galacto-pyranoside (hereinafter referred to as QGGP) on cell morphology, HepG2 cells were seeded in 6-well plates. After treatment with the indicated concentrations of E3, HPDL and QGGP for 72h, the morphological changes of HepG2 cells were observed by invert microscope.

Cell cycle analysis

The effect of E3, HPDL and QGGP on the cell cycle distribution was determined using flow cytometry analysis as described previously[53]. Briefly, cells were seeded at a density of about 2.0×10^5 cells per well in a 6-well microtiter plate. After culture for 12 h, the cells were treated with the indicated concentrations of E3, HPDL and QGGP for 72h. Then, cells were fixed with 70% ethanol and incubated overnight before staining with Propidium iodide mixed with RNase. The cells were kept under dark conditions for 30 min and subject to flow cytometry analysis.

Apoptosis assay

Apoptosis was detected using Annexin V-propidium iodide staining. Cells were seeded at a density of about 4.0×10^5 cells per well in culture dish. After culture for 12 h, the cells were treated with the indicated concentrations of E3, HPDL and QGGP for 48h. The treated cells were harvested and rinsed with cold PBS twice. After centrifugation at 1500 rpm for 5 min, cells were resuspended in 500μl of 1×Annexin V binding buffer and then added 5μl of Annexin V-FITC and 5μl of Propidium iodide. After incubation for 15 min at room temperature in the dark, the samples were analyzed by flow cytometry.

Western blot

Western blotting was performed as described previously [53]. HepG2 cells (6×10^5) were plated in cell culture flasks. After 12 h, the cells were treated with various concentrations of E3, HPDL and QGGP for 24h or 48h, and then cells were harvested. After washing with ice-cold PBS, the cells were lysed and protein concentration was determined using Bradford method. The samples were subjected to 12% of SDS-PAGE and transferred to polyvinylidene fluoride membrane. Blots were blocked in blocking buffer. The membrane was probed with the indicated antibodies overnight. The secondary antibody consisting of horseradish peroxidase-conjugated IgG was used for the detection. The band was visualized by the ECL detection system.

Statistical analysis

All statistical analyses were carried out in triplicate (n=3) and calculated in SPSS17.0 software. Results are expressed as means ± standard deviations (S.D.). Statistical evaluation was determined by the one-way analysis of variance (ANOVA) followed by the post hoc Turkey's test. Differences with $P<0.05$ were considered statistically significant.

Results

Cytotoxicity of E3 and the separated compounds of E3 on HepG2 cells

To evaluate the effect of E3 and compounds1-11 on cell growth, proliferation assays were performed on HepG2 cells, using increasing drug concentrations. Cell viability was determined by the MTT assay. As shown in Table 1, E3, compounds 2 (HPDL), compounds 4 (QGGP) and compounds 11 (Corilagin) significantly reduced proliferation of HepG2 cells in a dose-dependent manner after 72 h incubation. The IC50 values were 15.29±0.69μg/mL, 17.83±0.82μM, 11.28±0.65μM and 16.67±0.52μM, respectively.

Binding affinity study through docking against aurora-A and Aurora-B

In order to detect which active compounds could interact with Aurora kinase, molecular docking studies were performed to investigate the binding affinities and interaction modes for compound2, compund4 and compound11. Fig 2A shows that HPDL (compound2) binds to the binding pocket of Aurora-A through three H-bond interactions with the residues Leu139, Lys162 and Ala213. Likewise, QGGP (compund4) also forms H-bond interactions with the residues Lys141, Phe144, Lys162, Glu260 and Ala273. In addition to the main interactions, further stabilization of the binding was mediated by the contact with hydrophobic interaction formed by the Leu139, Val147, Leu210, Tyr212, Leu216, Thr217 and Leu263 amino acid side chains54. As shown in Fig. 2B, HPDL and QGGP could directly binds to ATP-binding cleft in Aurora B kinase. Residues ARG97, LEU 99, LYS 101, LYS 122, GLU141, ALA 173 and GLU177 are responsible to form hydrogen bonds with the HPDL and QGGP. In addition, HPDL and QGGP have Van der Waals interaction with LEU99, VAL107, LEU154, LEU168, PHE172, GLU177 and LEU223. The previous study suggested these hydrogen bonds and Van der Waals interaction contribute to the inhibition of Aurora-B. Both ^{Glu177}Aurora-B hydrogen-bonding and van der Waals contributions might justify the selective inhibition towards Aurora B [51]. Therefore, we hypothesize that QGGP might exhibit higher selectivity than HPDL for the ^{Glu177}Aurora-B hydrogen-bonding. This binding model suggests that HPDL and QGGP might bind to the ATP-binding site of Aurora kinase and thereby inhibit Aurora kinase activity in the competing way with ATP. Thus, the effects of E3, HPDL and QGGP on the activity of Aurora kinase were further investigated.

E3, HPDL and QGGP induced cell cycle arrest in HepG2 cells

A number of publications have demonstrated inhibition of Aurora kinases could result in G2/M cell cycle arrest and lead to polyploidy [55, 56]. In this case, obvious apoptosis features are not observed during this period. We therefore investigated the effects of E3, HPDL and QGGP on cell cycle progression at lower concentration to avoid massive apoptosis of HepG2 cells. Peak analysis quantified percentage of 2N (G1 phase), 4N (G2 phase), and polyploid cells (>4N). As shown in Fig. 3, in HepG2 cells, treatment with E3(15μg/mL), 10μM of HPDL and QGGP resulted in accumulation of cells with 4N DNA contents at 72 h and polyploid cells were observed following the increasing concentrations.

Inhibition of Aurora kinase activity

Aurora kinase is regulated at many levels. Both Aurora-A and Aurora-B have more than one way to be active. Like other kinases, Aurora kinase can self-activate, but the details of this process are not well understood. It is generally accepted that the activity of Aurora-A kinase is regulated by auto-phosphorylation in a cell cycle dependent manner. This phosphorylation occurs on a conserved residue, threonine 288, within the activation loop. Inhibition of Aurora-A could result in G2/M accumulation [57]. Likewise, Aurora-B activation depends on autophosphorylation at a threonine 232 residue in its activation loop. Aurora kinase B inhibition would lead to failed cytokinesis. The accumulation of polyploid cells implied failed cytokinesis following inhibition of Aurora B kinase activity[58]. The inhibition of Aurora B kinase can be observed by its specific cellular substrate histone H3 (Ser10)[59].

Our results indicated that E3, HPDL and QGGP induced dephosphorylation of histone H3(Ser10) in HepG2 cells in a concentration-dependent manner after drug exposure for 24 h (Fig. 4).This indicated that Aurora-B activity might be attenuated. Further investigation revealed that autophosphorylation of Aurora-A (Thr288) and Aurora-B (Thr232), indicators for Aurora kinase activity, was significantly decreased. However, no obvious variations of total Aurora-A, Aurora-B and histone H3 proteins expression were found (Fig. 4). The data suggested that aurora kinase activities were suppressed by E3, HPDL and QGGP.

E3, HPDL and QGGP promote apoptosis in HepG2 cells

Apoptosis in the cells treated with E3, HPDL and QGGP was measured using flow cytometry. We selected a larger drug concentration scope than that of cell cycle analysis to investigate the apoptosis induction effect on HepG2 cells. As shown in Fig. 5, when the cells were treated with DMSO alone, only small percentages (about 2%) of cells were underwent apoptosis. When cells were treated with E3, HPDL and QGGP at the indicated concentrations for 48h, the rate of apoptotic cells was significantly increased. The apoptotic population in 25μg/mLE3, 20μM HPDL and QGGP treated cells was 24.72%, 20.33% and 22.24%, respectively. Unlike HPDL and QGGP, which mainly caused early apoptosis, E3 is largely responsible for late apoptosis. In addition, cells were treated with the three mentioned above were found to be greatly decreased and shrinking compared to the controlled group (Fig.S1).

Effect of E3, HPDL and QGGP on MAPK signaling pathway

MAPKs, a family of serine/threonine kinas, mediate cell growth and apoptosis. Recently, Aurora-A was found to have the abilities to increased ERK1/2 activity [24]. Besides, the induction of apoptosis is also under the control of MAPK signaling pathways. On the basis of these, we examined the effects of E3, HPDL and QGGP on levels of phosphorylation forms of ERK, JNK and p38. The three mentioned above significantly restrained the phosphorylation of ERK1/2, and increased the levels of p-JNK and p-p38 in a dose-dependent manner (Fig. 6). The results indicated that E3, HPDL and QGGP could also induce apoptosis via MAPK signaling pathways.

E3, HPDL and QGGP induced apoptosis through a p53-dependant manner and via mitochondrial apoptotic pathway

P53 plays important roles in the apoptotic pathways, especially in the early onset of apoptosis induced by AZD1152-HQPA, an Aurora-B selective inhibitor [60]. It was supposed to be a linkage between polyploidization and apoptosis. P53 was reported to be up-regulated during endoreduplication and polyploidization after exposure to aurora kinases inhibitors [60, 61]. Therefore, the expression level of p53 was determined by Western blot analysis (Fig. 7). We also had investigated the effects of E3, HPDL and QGGP on the expression of apoptosis-related proteins including Bax and Bcl-2 in HepG2 cells (Fig. 7). As shown in Fig. 7, all the three fronts could up-regulate the expression of proapoptotic proteins p53 and Bax, while reduce the expression of Bcl-2. To further explore the role of the three fronts played in apoptosis of HepG2 cells, we determined the expression levels of mitochondrial related proteins, such as cytochrome c, activated PARP and cleaved caspase-3. The data indicated that E3, HPDL and QGGP could also significantly increase the levels of Cyt C, cleaved caspase-3 and cleaved PARP (Fig. 8). Taken together, the data indicated that E3, HPDL and QGGP could induce cell apoptosis and decreased survival of HepG2 cells in a p53-dependent manner and via mitochondrial apoptotic pathway.

Discussion and conclusion

E. sieboldianum, an edible and medicinal plant used in traditional Chinese medicine, has been used to treat cancer together with other herbs and achieved favorable results. But its active ingredients and the precise cellular mechanisms remain to be illustrated. Therefore, it is necessary to investigate the active compounds and their action mechanism. Herein, we investigate the anti-tumor active components (Fig. 1)and reporte the effects of *E. sieboldianum* extract and its two active compounds, HPDL and QGGP, on the growth of HepG2 cells for the first time (Table 1).

Due to the continuously deepening research on aurora kinases recently, the key functions of aurora kinases in cell mitosis and tumorigenesis have been gradually revealed. Aurora kinases have become new promising targets of anti-tumor therapy [8, 18, 64]. Previous research has indicated that aurora kinases were over-expressed in HepG2 cells and inhibition of Aurora-B by a chemical inhibitor is reported to cause polyploidy and cell morphology changes followed by apoptosis in HepG2 cells in

vitro and in vivo[6, 25, 26, 27]. However, Aurora inhibitors exert more toxic effects to the treated tumors compared with normal tissues, in previous cases [65]. HPDL was reported to suppress the angiogenesis and growth of human pancreatic cancer by targeting vascular endothelial growth factor receptor 2-mediated PI3K/Akt/mTOR signaling pathway and have fewer side effects on normal cells [66]. While our results revealed the activity of aurora kinase inhibition, which is another new important anticancer mechanism of HPDL (Fig. 4). In this study,

flow cytometry of DNA content indicated that E3(15μg/mL), HPDL (10μM) and QGGP (10μM) resulted in accumulation of cells with 4N DNA contents at 72 h and polyploid cells were observed following the increasing concentrations (Fig. 3). Losing of Aurora-A activity could lead to G2-M arrest, many spindle defects, the appearance of tetraploid cells and apoptosis [5, 67]. Besides, cells with Aurora-B activity being inhibited do not divide and become tetraploid instead. In this case, obvious apoptosis features are not observed during this period [68]. Thus, we selected a lower drug concentration to avoid too much apoptosis when performed cell cycle analysis.While a larger drug concentration scope was used to investigate the apoptosis induction effect (Fig. 5). Given that many Aurora-B inhibitors could also inhibit Aurora-A and block cell cycle in G2/M phase [69], we speculated that E3, HPDL and QGGP were Aurora-A inhibitors either. In the molecular docking study, we found QGGP could directly bind to ATP-binding cleft in Aurora B kinase with Glu177 via hydrogen-bonding and van der Waals interactions (Fig.2). Both ^{Glu177}Aurora-B hydrogen-bonding and van der Waals forces are reported to contribute to the selective inhibition towards Aurora B [51]. Therefore, we hypothesize that QGGP might exhibit higher selectivity than HPDL for the ^{Glu177}Aurora-B hydrogen-bonding. The data obtained from western blot shown that the autophosphorylation at threonine 288 of Aurora-A and threonine 232 of Aurora-B in the activation loop were markedly decreased. In regard to this, further studies on the selectivity of HPDL and QGGP against other kinases are also in our planning.

To our suprise, E3, HPDL and QGGP could decrease the MAPKs pathway activation (Fig.6). Recently, Aurora-A was found to have the abilities to promote ERK1/2 activity [24]. Thus, we speculated that inhibition of Aurora-A could also decrease the activation of MAPK signaling pathways.

In addition,our results suggested that E3, HPDL and QGGP could inhibit Aurora kinase and induce apoptosis in a p53-mediated manner (Fig. 7). Consistent with our results, p53 was reported to be required for cellular senescence induced by alteration of genes involving mitosis and chromosome segregation, including Aurora kinase overexpression [21, 70]. P53 can stimulate the expression of Bax and decrease the protein level of Bcl-2 [71, 72]. P53 can also exert a direct pro-apoptotic function in the mitochondria, thereby activating the mitochondrial apoptotic pathway. Moreover, overexpression of Bcl-2 has been reported to confer drug resistance, whereas high Bax expression is associated with a favorable prognosis. Aurora kinase inhibitory VX-680 could induce apoptosis via increase Bax/Bcl-2 ratio and cleaved caspase-3 [23]. Likewise, we demonstrated all the three fronts could induce apoptosis via down-regulating Bcl-2 and up-regulating Bax (Fig. 7). They also increased the levels of cytochrome *c*, cleaved caspase-3 and cleaved PARP (Fig. 8). Future study will be needed to address whether there is other hypothesis account for the increased Bax/Bcl-2 ratio and cleaved caspase-3.

In conclusion, the results obtained in this study showed, for the first time, that *E. sieboldianum* extract (E3) and its active ingredients, HPDL and QGGP, suppress cell growth of HepG2 cells in vitro. The three mentioned above could effectively inhibit the activity of Aurora kinase and induce apoptosis of HepG2 cells through mediating the expression of apoptosis associated proteins such as p53, Bcl-2, Bax, caspase-3 and PARP. All three could also decrease the MAPKs pathway activation. These could improve the development of *E. sieboldianum* and raise its application value in anticancer. Also, our results implied that targeting Aurora kinase by natural products may be a feasible strategy to elaborate low-toxic anticancer agents. Furthermore, structure modification would help improve potency and selectivity of HPDL and QGGP.

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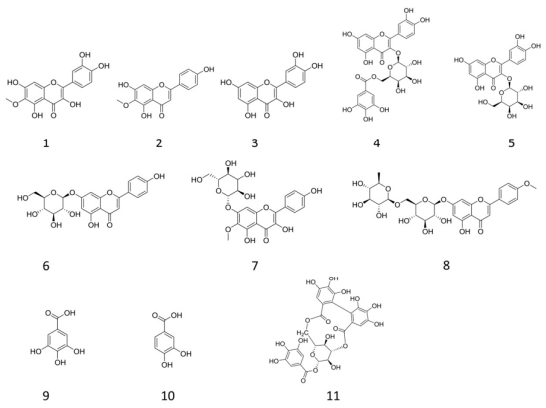


Fig.1. Structures of compounds 1-11.

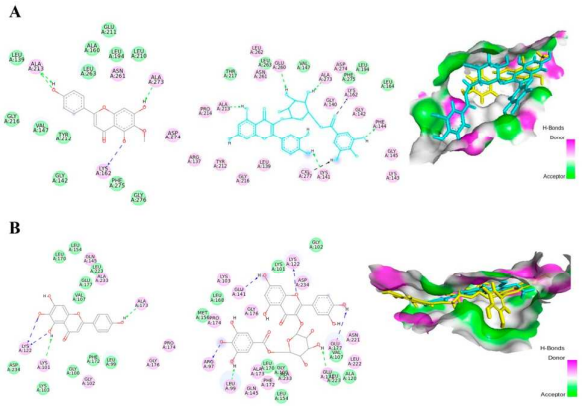


Fig.2. Docked binding modes of compounds in the ATP binding site of Aurora A and B. (A) Binding of HPDL and QGGP to the ATP-binding pocket of Aurora A. The ligands are shown in stick model, while the proteins are shown in surface model for better visualization in the three dimensional combination models. HPDL was colored yellow and QGGP was marked in blue. (B) Binding of HPDL and QGGP to the ATP-binding pocket of Aurora B. The ligands are shown in stick model, while the proteins are shown in surface model for better visualization in the three dimensional combination models. HPDL was coloured blue and QGGP was marked in yellow.

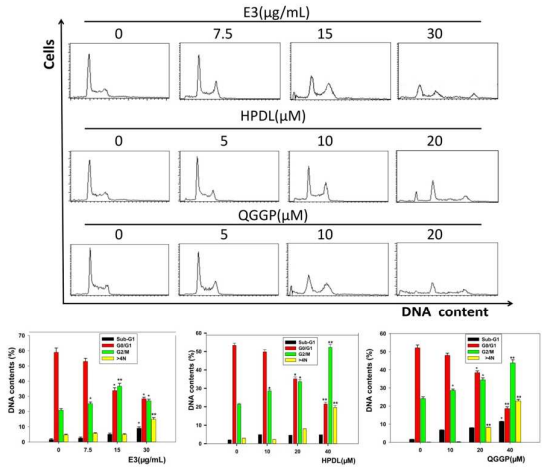


Fig.3. Effect of E3, HPDL and QGGP on cell cycle distribution. HepG2 cells were treated with the indicated drugs for 72h and then stained with propidium iodide. DNA contents were analyzed by flow cytometry. Data shown are representative of three independent experiments. Columns: means; bars: SD (n = 3). * $p < 0.05$; ** $p < 0.01$ (compared to untreated controls). Differences with p values 0.05 are considered significant.

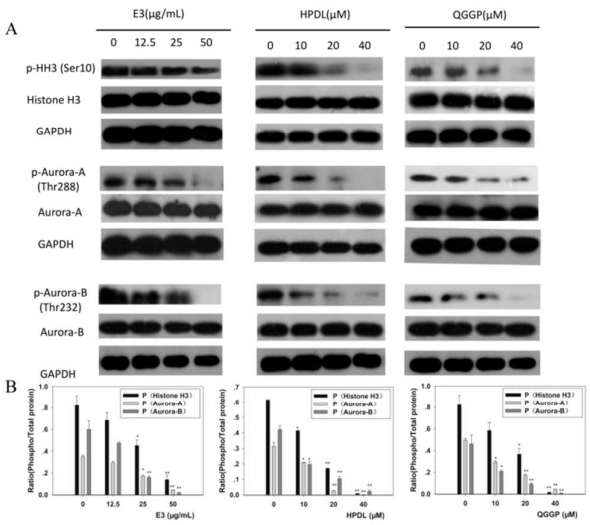


Fig.4. Aurora kinases are suppressed by E3, HPDL and QGGP. (A) E3, HPDL and QGGP inhibit histone H3 phosphorylation and phosphorylated protein expression of Aurora-A and Aurora-B in HepG2 cells in vitro. HepG2 cells were treated with E3, HPDL and QGGP for 24h. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. The expression of GAPDH is shown as an internal control. (B) The quantitative analysis of western blotting for the phosphorylation of Histone H3 (Ser10), Aurora-A (Thr288) and Aurora-B (Thr232). The data are representative examples for triplicate independent tests. Columns: means; bars: SD (n = 3). * $p < 0.05$; ** $p < 0.01$ (compared to untreated controls). Differences with p values 0.05 are considered significant.

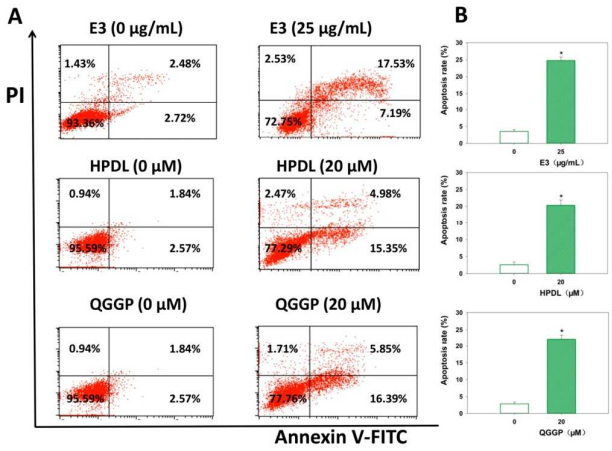


Fig.5. E3, HPDL and QGGP promote apoptosis of HepG2 cells in a concentration-dependent manner. HepG2 cells treated with DMSO or E3, HPDL and QGGP at the indicated concentrations for 48 h. The treated cells were stained with FITC-Annexin V (FL1-H, x-axis) and propidium iodide (FL2-H, y-axis) and analyzed by flow cytometry. The data are representative examples for triplicate independent tests. Columns: means; bars: SD (n = 3). * $P < 0.05$; ** $p < 0.01$ (compared to untreated controls). Differences with p values 0.05 are considered significant.

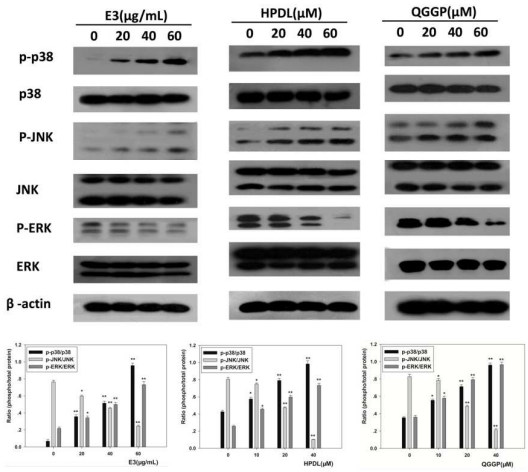


Fig.6. Effect of E3, HPDL and QGGP on MAPKs pathway. E3, HPDL and QGGP Cells were treated with the indicated concentrations of E3, HPDL and QGGP for 24h. Cell lysates were subjected to SDS-PAGE and analyzed by Western blotting. The data are representative examples for triplicate independent tests. Columns: means; bars: SD (n = 3). * P < 0.05; ** p < 0.01 (compared to untreated controls). Differences with p values 0.05 are considered significant.

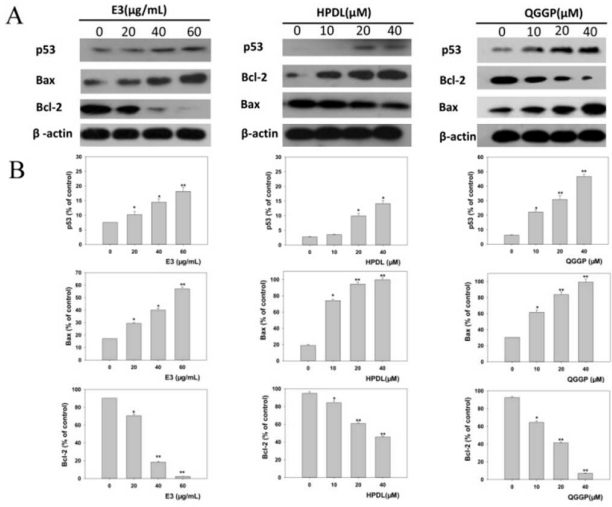


Fig.7. Effect of E3, HPDL and QGGP on apoptotic-related proteins (p53, Bax and Bcl-2). (A) E3, HPDL and QGGP Cells were treated with the indicated concentrations of E3, HPDL and QGGP for 48h. Cell lysates were subjected to SDS-PAGE and analyzed by Western blotting. (B) The quantitative analysis of western blotting for p53, Bax and Bcl-2. The data are representative examples for triplicate independent tests. Columns: means; bars: SD (n = 3). * P < 0.05; ** p < 0.01 (compared to untreated controls). Differences with p values 0.05 are considered significant.

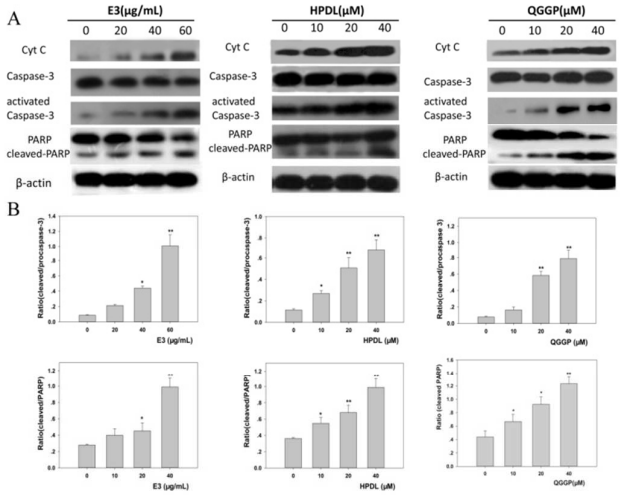


Fig.8. Effect of E3, HPDL and QGGP on Cytochrome C, caspase-3 and PARP. (A) Cells were treated with the indicated concentrations of E3, HPDL and QGGP for 48h. Cell lysates were subjected to SDS-PAGE and analyzed by Western blotting. (B)The quantitative analysis of western blotting for cleaved caspase-3 vs. procaspase-3 and cleaved PARP vs. PARP. The data are representative examples for triplicate independent tests. Columns: means; bars: SD (n = 3). * P < 0.05; ** p < 0.01 (compared to untreated controls). Differences with p values 0.05 are considered significant.

Table 1-Effect of *Eriocaulon sieboldianum* extract and its compounds on proliferation of HepG2 cells.

Extract/ Compounds	IC50 μg/mL or μM
Extract	15.29±0.69
1	>50
2	17.83±0.82
3	>50
4	11.28±0.65
5	>50
6	>50
7	>50
8	>50
9	>50
10	>50
11	16.67±0.52

The concentration range of extract was 0-60μg/mL. The concentration range of compounds 1-11 was 0-60μM.

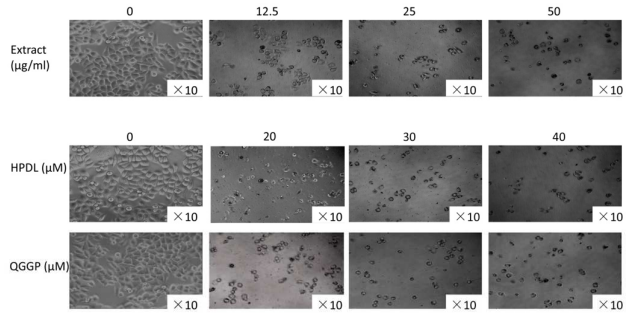
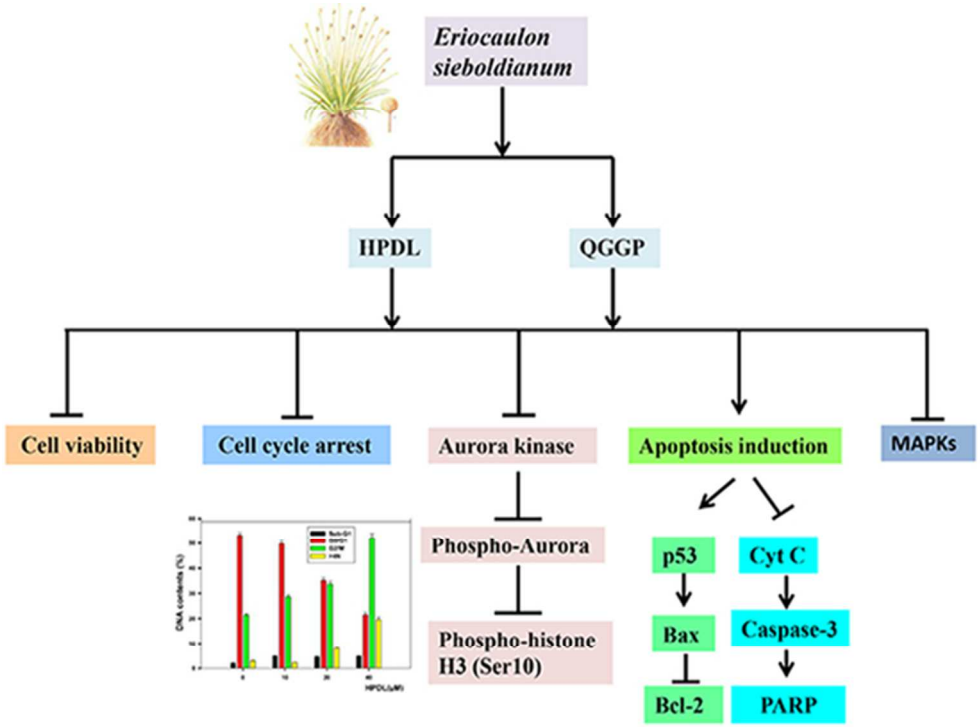


Fig.S1. Cell morphologic alteration in HepG2 cells. HepG2 cells were seeded in 6-well plates. After treatment with the indicated concentrations of E3, HPDL and QGGP for 72h, the morphological changes were observed by the inverted microscope.



We found that E. sieboldianum extract and its two active compounds , HPDL and QGGP, could effectively inhibit aurora kinase and induce apoptosis via p53, MAPKs and mitochondrial apoptotic pathways.
59x44mm (300 x 300 DPI)