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Potential effects of hesperidin isolated from *Zanthoxylum rhetsa* against NTERA-2 cancer stem cells

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Recent studies have suggested that cancer stem cells (CSCs) may represent novel targets for cancer therapies. Besides, flavonoids derived from plants have attracted interest because of their promising bioactivities and medicinal potential. Hesperidin (HES) isolated from *Zanthoxylum rhetsa* has been demonstrated to have potent cytotoxic effects against a category of cancer cell lines. This study firstly proposes to evaluate the bioactivity of hesperidin against NTERA-2 cancer stem cells. Hesperidin has been examined for the suppression capacities on proliferative ability and stemness properties of NTERA-2 cells. The compound was investigated potential activities *via* 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay in 2D, 3D culture, flow cytometry, caspase assay and western blot analysis. The results showed that hesperidin exerted anti-proliferative activity, with a half-maximal inhibitory concentration (IC_{50}) of 57.80 ± 3.55 µM against NTERA-2 cells, but showed much less toxicity against HEK-293A human embryonic kidney cells (IC_{50}> 150 µM). The anti-CSC effects of HES on NTERA-2 cells might involve in the p53 pathway, inducing apoptosis by increasing early and late apoptotic rates and activating caspase-3, -8, and -9, increasing expression of Bax, p53 proteins, and significant cell cycle arrest at the G2/M phase. Moreover, hesperidin was observed remarkably down-regulating the stemness characteristics of NTERA-2 cell by inhibiting cell proliferation in tumorsphere and suppressing the expression of Oct4, Nanog proteins. Together, these results suggest the potential of hesperidin in CSC-targeted treatment for more efficient clinical therapy.

Keywords: Apoptosis, Caspase 3/8/9, Nanog, Oct4, p53 pathway, Stemness

Treatment of cancer faces many challenges because of the uncontrolled ubiquitously growth of cancer cells in the body[1]. So far, surgery, chemotherapy, and radiation have been known as standard therapies that have been used for many decades. However, surgical removal of a solid tumor does not mean that it prevents the tumor from recurring[2].

There is a hypothesis that cancer stem cells (CSCs), a specific subgroup of cancer cells, are accountable for directly resulting in tumor formation and driving tumor spread[3]. CSCs have been shown to be elevated in different types of cancers, contributing to the limitation of therapy such as chemical and radial therapies[4]. A large CSC population indicated a rapid proliferation rate of tumor cells, emphasizing the aggressive nature of the cancer[4]. The inability of tumor cells to differentiate results in genetic instability and prevents the generation of differentiated progeny, making them more likely to survive and thrive despite the cancer treatments[5]. Autologous CSCs have mechanisms to evade programmed cell death, such as apoptosis and autophagy, allowing them to self-renew and maintain their population across various types of cancers[3]. The exact mechanisms behind CSC drug resistance are not fully understood, but it may involve the increased production of cell-survival proteins or drug-processing proteins that expel the drugs from the cells[6].

There were several drugs applied in CSCs treatment, but their effects can lead to several negative events, including loose stools, queasiness, lightheadedness, emesis, nerve damage affecting movement, and increased liver enzyme levels[7]. Therefore, it is essential to discover active compounds of natural origin that exhibit anti-CSC effects while minimizing adverse side effects.

Zanthoxylum rhetsa, often referred to as Indian ivy-rue, was a woody plant of moderate size, which is characterized by numerous conical spines that extend from the base to the apex and found across India, Thailand, Myanmar, Laos, and Vietnam[8]. This plant, belonging to the Rutaceae family, included about two

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thousand species, many of which have yet to be characterized. The plant contained alkaloids and flavonoids with potential bioactivity[9]. Hesperidin (HES) from methanol extraction of *Z. rhetsa* bark exhibited cytotoxicity on cancer cells while less toxicity on non-cancerous cells[10].

HES, a glycoside flavonoid, exhibited several types of biological activity, including anticancer, hepatoprotective, cardioprotective, antidiabetic, anti-inflammatory, antimicrobial, and skin protective effects[11-13]. For anticancer activities, various researchers have demonstrated significant inhibitory effects of HES across different cancer cell lines. It suppressed the spread and the growth of mammary carcinoma cells by triggering apoptosis and cell cycle arrest[14,15]. In colon cancer cells, HES exhibited antiproliferative activity by modulating signaling pathways involved in cell growth and survival[16]. For HeLa cells derived from cervical cancer, HES triggers apoptosis and impedes cell migration *via* the endoplasmic reticulum stress pathway[17]. Additionally, HES suppressed the proliferation of the HEp-2 laryngeal carcinoma cell line by decreasing anti-apoptotic Bcl-2 expression and increasing tumor suppressor gene p53 expression[18]. Similarly, in liver cancer cells, HES's anticancer effects were evident through the suppression of cell growth and promotion of programmed cell death *via* the mitochondrial pathway and death receptor pathway[19]. Pulmonary carcinoma cells, such as non-small cell lung cancer types, were also sensitive to HES, which reduced their viability and metastatic potential through downregulation of β-catenin/c-myc, mitochondrial apoptotic pathway, and induced G0/G1 arrest, inhibiting the interaction between p53 and a p53-interacting protein[20,21]. Additionally, HES exerted strong cytotoxic effects on ovarian cancer cells by activating endoplasmic reticulum stress pathways, further underscoring its broad-spectrum anticancer properties[22]. These multifaceted actions made HES a promising candidate for the development of novel anticancer therapies. Moreover, utilizing bioinformatics, HES significantly induced apoptosis and inhibited the proliferation and self-renewal capabilities of BCSCs[23]. This inhibition was accomplished by suppressing p53 signaling pathways, Wnt signaling pathway, and inducing cell cycle arrest[23]. However, despite these promising findings, there are still very few reports on the effects of HES on CSCs. Therefore, there is a need to expand research on the anticancer effects of HES in other human cancer stem cell lines to fully understand its potential and broaden its application in cancer therapy.

This study initially investigated whether HES isolated from *Z. rhetsa* exerts an inhibitory effect on the proliferation and stemness properties of NTERA-2 cancer stem cells.

Materials and Methods

Materials

The stem bark of *Zanthoxylum rhetsa* was collected in Que Phong district, NgheAn province, Vietnam. HES was isolated from the stem bark of *Z. rhetsa*, and its purity was verified with NMR spectrum by following our previously reported procedure[24]. Chemicals were from Merck (Darmstadt, Germany), Duksan (Ansan, Korea), and YMC (Kyoto, Japan). The NTERA-2 cell line was kindly provided by Prof. P. Wongtrakoongate, Mahidol University, Thailand. HEK-293A cell line was from ATCC (Cat. CRL-1573, Manassas, USA). The culture reagents were from Thermo Fisher Scientific (Waltham, USA).

Cell culture

Cancer stem cell line NTERA-2 and non-cancerous cell line HEK-293A were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic, 1% MEM Non-Essential Amino Acids, 1% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 1M. Cells were incubated at 37°C with 5% CO_2 and subcultured every three days at a ratio of 1:3.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay

The MTT assay was conducted as previously reported[25]. In brief, NTERA-2 cells and HEK-293A cells were seeded into 96-well plates (1×10^4 cells/well) for 24 h, then treated with HES (from 4 to 500 µM) and DMSO 0.5% as negative control (NC) for a further 48 h. Thereafter, the cell was incubated with 20 µL of MTT solution (5 mg/mL). The formed formazan crystals were dissolved with 100 µL/well of dimethyl sulfoxide (DMSO 100%). The optical density (OD) was measured at 540 nm using a microplate reader (BioTek, ELx800).

Tumor sphere assay

In each 96-well ultra-low attachment plate well, 5000 NTERA-2 cells were cultured to form tumor spheroids over 48 h. Following this, the cells were exposed to NC and varying concentrations of HES for

an additional 48 h. Cell viability was then measured as described in the previous report. The area of tumor spheroids was analyzed using Image J software (https://imagej.nih.gov/ij/). The result was expressed as a percentage, with the sphere area of the untreated control considered to be 100%. The MTT assay was to measure the cellular proliferative rate.

Cell cycle arrest

Seeding 3 x 10^5 of NTERA-2 cells/well in a 6-well plate for 24 h at 37°C in 5% CO_2 before treatment of HES and NC in another 48 h. The experiment used the accessible procedure to assess cell cycle arrest with some modification in which DNA content determined by staining with propidium iodide (PI) at concentration of 0.1 mg/mL in 1X Phosphate Buffer Saline (PBS)[26]. The cell cycle arrest was analyzed using the Novocyte system flow cytometry and NovoExpress software (ACEA Bioscience Inc.)

Detection of apoptosis using Annexin V-FITC flow cytometry analysis

Cells were cultured into a 6-well plate at 1 × 10^5 cells/mL for 24 h. Then, NTERA-2 cells were exposed to HES at different concentrations and NC in 48 h. The proportion of apoptotic cells was determined by analyzing approximately 10,000 cells. Experiments were performed based on the prior report[26] using the eBioscience™ Annexin V Apoptosis Detection Kit FITC (Thermo Fisher Scientific, ref. 88-8005-74). The ratio of apoptotic cells was identified from an approximately counted 10,000 cells using NovoCyte Flow cytometer system and NovoExpress software (ACEA Bioscience Inc.)

Caspase assay

The caspase inducible activity of HES was assessed using Caspase-3 (ab39401, Abcam), Caspase-8 (BS9718958, MyBioSource), and Caspase-9 (MBS9718959, Abcam) Colorimetric Assay Kits by sharply following the guidance from the producer. After 48 h of treatment with HES and NC on an initial 1 × 10^5 cells/mL of NTERA-2 cells, the cells were lysed, and the total protein was collected. Equal quantities of protein (50 µg) from the various treatments were mixed with 50 µL of assay buffer, 50 µL of Dithiothreitol (DTT), and 5 µL of DEVD-pNA (for caspase 3), Ac-LEHD-pNA (for caspase 9), Ac-IETD-pNA (for caspase 8) in each well of a 96-well plate, then incubated at 37°C for 2 h. The absorbance was measured using the ELx800 system at 405 nm.

Western blot analysis

The NTETA-2 cells were plated in a 6-well plate at a density of 1 x 10^5 cells/mL and cultured for 24 h. Following this, NTERA-2 cells were treated with HES at different concentration and NC for 48 h. After that, the cell pellets were harvested and washed with PBS (pH 7.2), homogenized in RIPA lysis buffer and centrifuged at 13,000 rpm for 10 min. Protein concentration was measured by Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific). Equal amounts (20 µg) of protein and 2x Laemmli Sample Buffer (1610737, Bio-Rad Laboratories) were added to the sample lysates and boiled at 95°C for 5 min. The GangNam-STAIN™ Prestained Protein Ladder (24052, iNtRON Biotechnology) served as a molecular weight marker. The protein samples were loaded into 10% SDS-PAGE gel and then transferred to polyvinylidene fluoride (PVDF) membranes (1620177, Bio-Rad Laboratories)[27]. Membranes were blocked with 5% Bovine serum albumin (BSA) in Tris-Buffered Saline with 0.1% Tween-20 (TBST) for 1 h at 4°C, and were then incubated with respective primary antibodies (Nanog, 1:1000, Abcam, Cat.No. ab109250; Oct4, 1:1000, Abcam, Cat.No. ab181557; GAPDH, 1:5000, Abcam, Cat.No. ab181602; p53, 1:1000, Abcam, Cat.No. ab32389; Bax 1:1000, ProteinTech, Cat.No. 60267-1-lg) for approximately 18 h at 4°C in blocking solution. After washing, the membrane was incubated with anti-rabbit or anti-mouse Horse Radish Peroxide (HRP)-conjugated secondary-antibodies (1:3000, Abcam, Cat.No. ab6721 and Abcam, Cat.No. ab97023, respectively) for 1 h at room temperature. Bands were visualized by Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Cat. No. 170-5060). Images were acquired using sequential exposure times (Amersham™ Imager 680). Signals were analyzed using ImageJ software (https://imagej.nih.gov/ij/).

Data analysis

Statistical analysis of the data was conducted by using Student's t-tests on GraphPad Prism 5, and the results were presented in the format of mean ± standard deviation (SD) resulting from three repeats. Statistical significance was denoted as *P< 0.05 and **P< 0.01.

Results

Cytotoxic activities

The 48 h inhibitory effects of HES against NTERA-2 cancer stem cells as a model of CSCs and

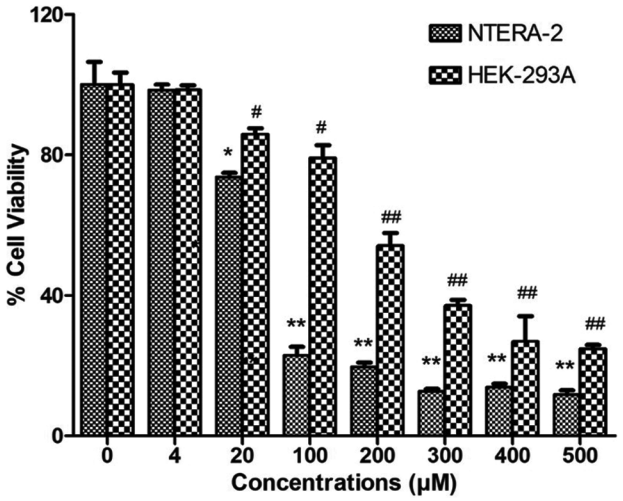

Fig. 1 — Effect of HES at different concentrations ranging from 4 μM to 500 μM on cancer stem cell line NTERA-2 and non-cancerous cell line HEK-293A after 48 h of incubation. Data are expressed as means ± SD (n=3). The viability of control cells is defined as 100%. */$^{\#}P$< 0.05 and **/$^{\#\#}P$< 0.01 vs. control cells

HEK-293A cells as a model of non-cancer cells were dosage depended (Fig. 1). Results are presented as percentages of survival HES-treated cells relative to the control. HES at 20 μM, 100 μM showed significant suppressions of NTERA-2 cells, with 78.79% and 21.52% cell survival, respectively. Therefore, the half-maximal inhibitory concentration (IC_{50}) was 57.80 ± 3.55 μM. Importantly, HES presents much less toxic activity on non-cancerous HEK-293A cells, with IC_{50}> 150 μM.

Cytotoxicity against tumorspheres

To further determine the specific cytotoxicity of HES against CSCs, we performed tumorspherical assays. The impact of HES on the growth of NTERA-2 cells in a 3-dimensional (3D) tumorsphere configuration was evaluated following exposure for 48 h. At a concentration of 500 μM, HES remarkably induced a reduction in the size of NTERA-2 tumor spheroids by 31.66% compared to that of controls. The survival rate of NTERA-2 cells in tumourspheres was also examined in a 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. The results showed that HES significantly repressed NTERA-2 cell growth in sphere structure at 100 μM and 500 μM with an IC_{50} of 437.90 ± 21.50 μM (Fig. 2).

Cell cycle arrest

In order to assess the effects of HES on the NTERA-2 cell cycle, the cells were incubated with HES at various concentrations for 48 h. There was a gradual volatile trend in the cell population at the G1 and S phases between the three HES concentrations. In contrast, the percentage of NTERA-2 cells distributed at the G2/M phase increased gradually from 23.29% to 25.44% in a dose-dependent manner. The difference at the $2xIC_{50}$ concentration of HES was significant compared to untreated control (19.28%), indicating that HES-treated NTERA-2 cells were arrested at the G2/M phase (Fig. 3).

Induction of apoptosis

Cells treated with IC_{50} and $2xIC_{50}$ concentration of HES showed induction of early and late apoptosis, with a significant increase in the percentage of apoptotic cells from 11.05% in untreated control cultures to 22.71%, and 32.65%, respectively (Fig. 4).

The apoptosis-inducing effect of HES was further examined by monitoring fold changes of apoptotic proteins, including caspase 3, caspase 8, caspase 9, Bax, and P53 (Fig. 5). Through an ELISA assay, NTERA-2 cells incubated with HES for 48 h showed a dose-dependent increase in the expression of caspase 3, caspase 8, and caspase 9 (Fig. 5A-C). Treatment with HES at concentrations of IC_{50} and $2xIC_{50}$ significantly elevated caspase 3 levels from 1.55 to 2.0-fold change compared to the negative control (Fig. 5A). Caspase 8 expression also notably increased with HES treatment at different doses, with levels ranging from 1.5 to 2.5 times higher than the control (Fig. 5B). Caspase 9 expression was elevated by approximately 1.66-fold at concentrations of IC_{50} and $2xIC_{50}$ compared to controls (Fig 5C). Western blotting analysis of NTERA-2 whole-cell protein lysates with antibodies to Bax and p53 showed significant induction of the expression of both proteins after HES treatment at a concentration of $2xIC_{50}$ for 48 h (Fig. 5D & E). Specifically, treatment for 48h with HES at concentrations of $2xIC_{50}$ resulted in 1.47 ± 0.20-fold increase in Bax expression, compared to untreated control. Similarly, p53 showed 1.38 ± 0.12-fold changes compared with controls.

HES was investigated for its potential to inhibit the activity of Oct4 and Nanog expression in NTERA-2 cancer stem cells. Figure 6 illustrated that at a concentration of two times the IC_{50}, HES had a significantly down-regulating impact on the expression of both Oct4 and Nanog with fold changes of 0.63 ± 0.05-fold and 0.34 ± 0.15-fold, respectively.

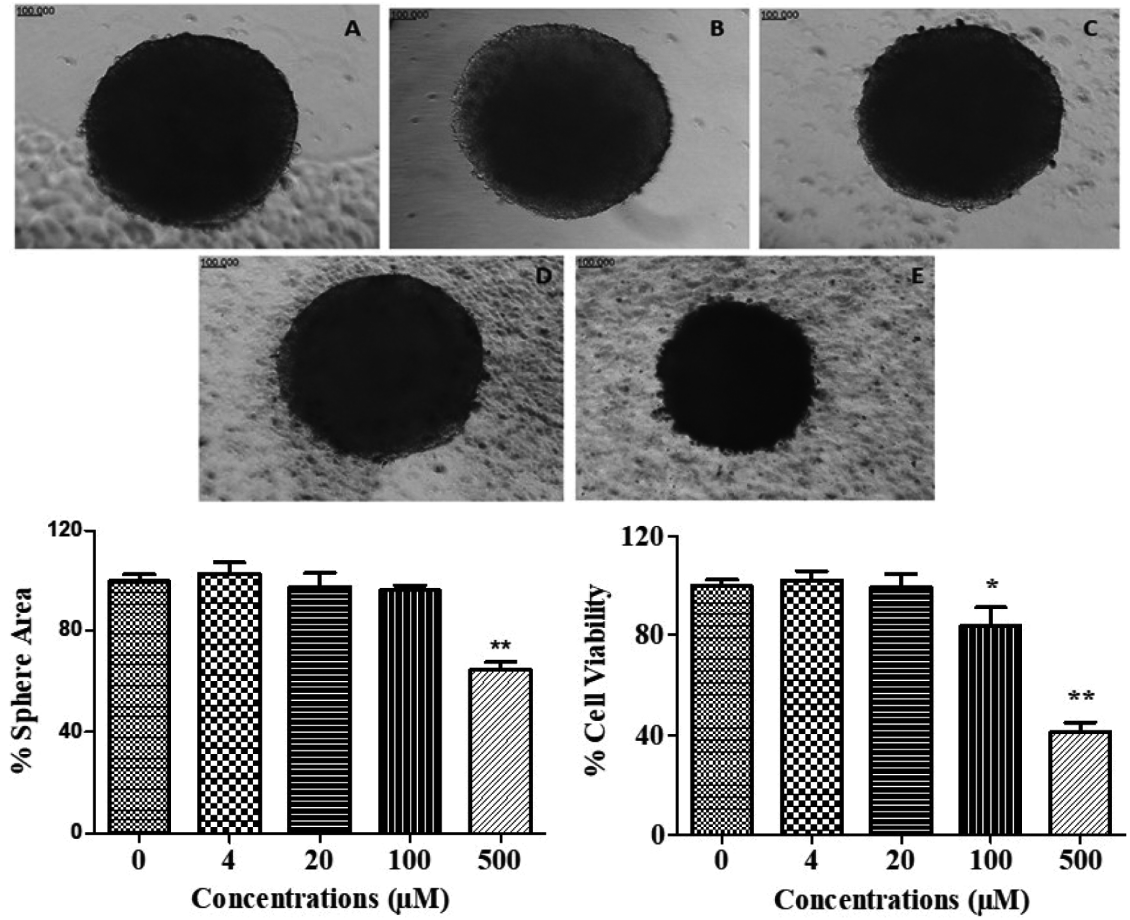

Fig. 2 — Inhibitive effects of HES on the proliferation of NTERA-2 cells in 3-dimensional cell culture model after 48 h of incubation; (A) DMSO 0.5% as negative control; (B, C, D, E) NTERA-2 tumor spheroids under treatment with HES at 4, 20, 100, 500 µM, respectively. Data was expressed as means ± SD (n=3). *P< 0.05 and **P< 0.01 vs. control cells

Discussion

In the previous reports, for non-stem cancer cells, HES had IC_{50} values of 150.43 ± 12.32 µM for HepG2 cells, 93.4 µM for HEp-2 cells, 48 µM for HeLa cells, and 78.6 µM for human osteosarcoma MG-63 cells[19,28,29]. Besides, HES isolated from *Z. rhetsa* detected the presence possessing significantly selective cytotoxic effects on lung cancer cells (LU-1) and KB cancer cells with IC_{50} values of 66.48 and 67.41 µg/mL, respectively[24]. In this study, as a result of the above experiments, HES showed potential cytotoxic effects against NTERA-2 cells as a model of CSCs with IC_{50} about 58 µM. Interestingly, the results showed that HES had little cytotoxicity against HEK-293A cells (IC_{50}>150 µM). These results were consistent with a previous study that showed little effect of HES on a non-tumor epithelial cell line such as vero and MCF10A[24,30]. Besides, HES could induce G2/M-phase arrest in NTERA-2 cancer stem cells in a dose-dependent manner, consistent with previous reports that HES influenced the cell cycle in various cancer cell lines. Notably, in gall bladder carcinoma, HES-induced cell cycle arrest at the G2/M phase, with a peak effect of > 17%, was observed after 24 h of treatment at a concentration of 200 µM[31]. Furthermore, after 48h, HES at 40 µM to 160 µM impacted the G0/G1 phase in HeLa cells[17]. In another study, the proportion of human osteosarcoma MG-63 cells suspended at the G2/M phase was elevated compared to controls following treatment with HES, with increases ranging from 5.9% at 5 µM to 43.2% at 150 µM[28]. In A549 cells, treatment with HES for 72 h at concentrations of 50, 75, 100, and 125 µg/mL induced arrest at the G0/G1 phase[21].Hes increased about 21.67% of apoptotic cells in the treated group compared with the untreated control. Previous studies have demonstrated apoptosis in human osteosarcoma MG-63 cells treated with HES at

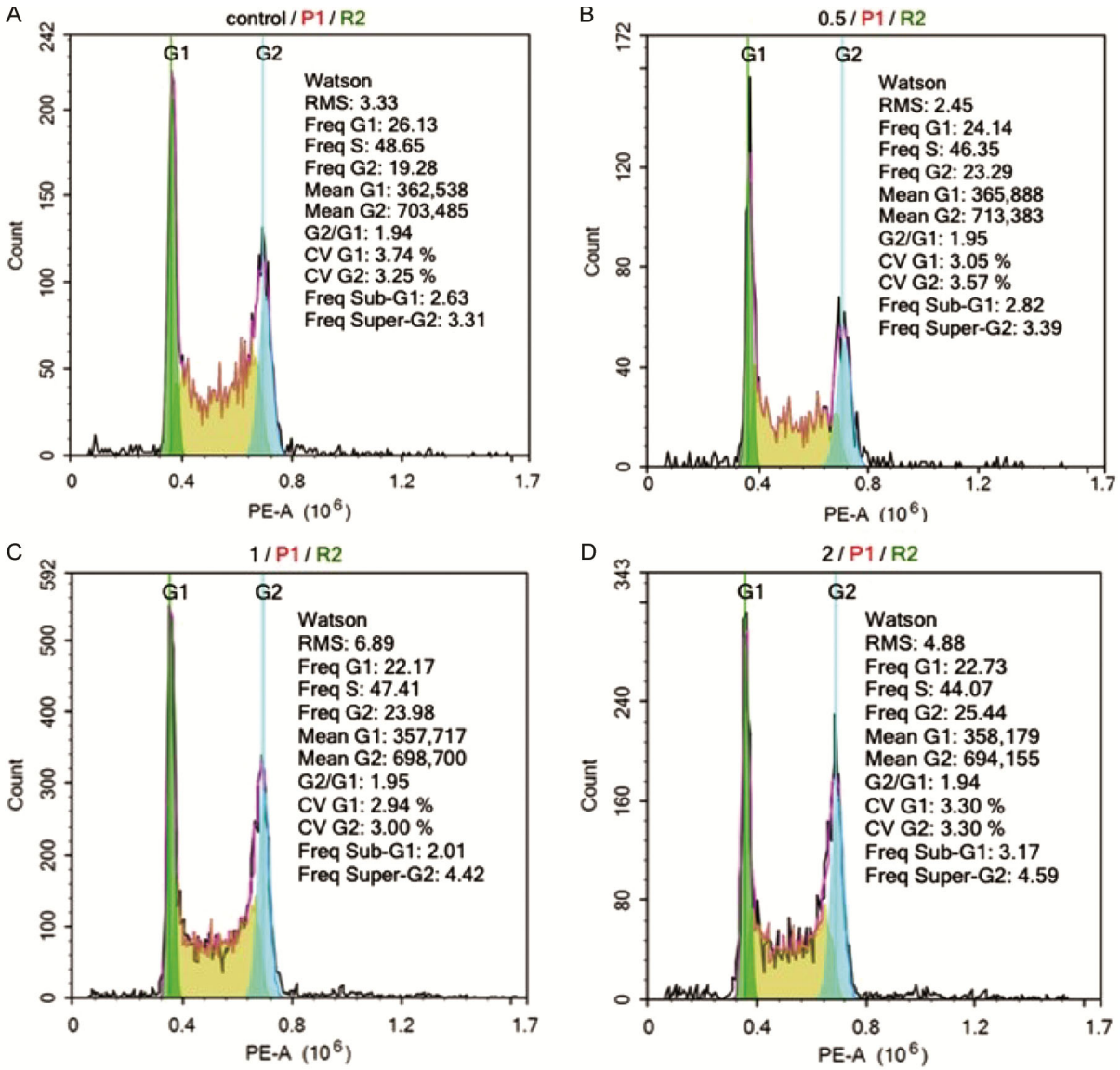

Fig. 3 — Effects of different concentrations of HES on NTERA-2 cell cycle after 48 h of incubation. (A) negative control; (B, C, D) 0.5 x IC_{50}, IC_{50}, and 2xIC_{50} of HES

150 µM, with apoptosis rates increasing from approximately 5% in the negative control to 68.3% in HES treatment[28]. Furthermore, as a result of this study, HES induced significant increases in the expression levels of caspase 3, caspase 9, caspase 8, Bax, and p53 in NTERA-2 cells in a dose-dependent manner, indicating substantial induction of apoptosis. The initiator caspases, caspase 9 and caspase 8, are positively correlated with the rapid cleavage of caspase-3 expression, facilitating the induction of apoptosis in cancer cells *via* intrinsic and extrinsic pathways, respectively[32]. The intrinsic pathway was also demonstrated by the significant increase in the tumor suppressor protein p53, which plays a vital role in responding to DNA damage by promoting the expression of various apoptotic genes such as Bax and caspase expression. In addition, the elevation of p53 was also well-linked to cell cycle regulation by establishing a role in regulating the G2/M checkpoint[33]. Therefore, HES might activate the p53 pathway to induce apoptosis on NTERA-2 cancer stem cells. This suggestion was also consistent with a prior study that also demonstrated the apoptosis-inducing HES-impacted p53 pathway in breast cancer stem cells[23]. In addition, Hesperidin significantly suppressed CSCs through inhibitory effects against

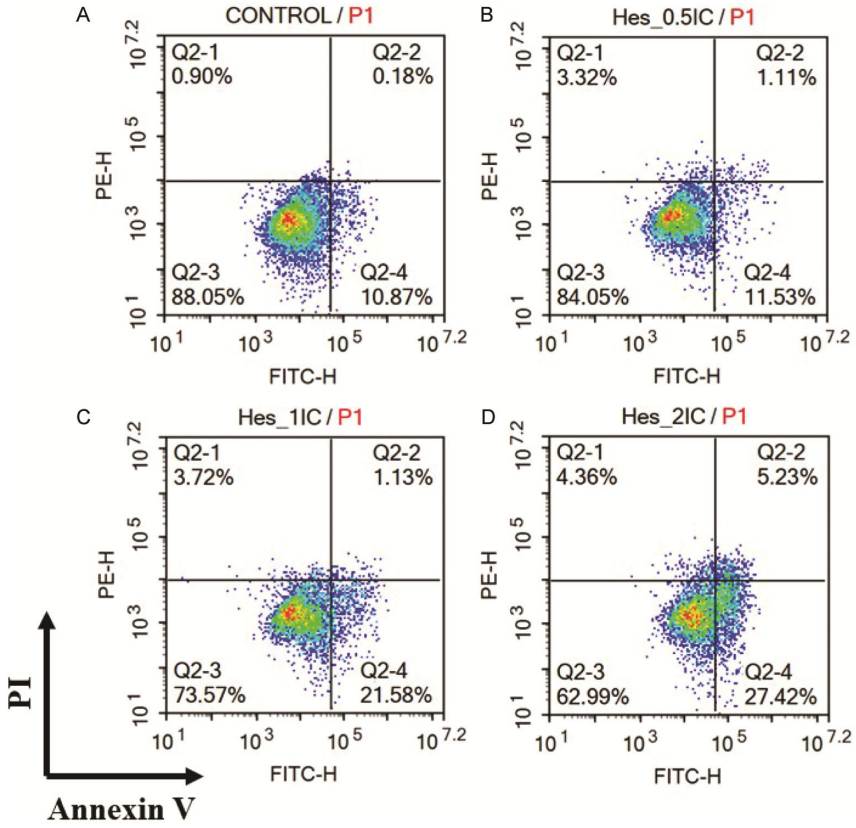

Fig. 4 — Enhanced apoptotic inducible activities of HES in NTERA-2 cancer stem cells after 48 h incubation. Configuration diagrams using double staining with Annexin-V -FITC and PI presented the percentage of apoptotic cells following the exposure to three concentrations of HES 0.5xIC_{50}, IC_{50} and 2xIC_{50} (B, C, D). DMSO 0.5% as NC (A)

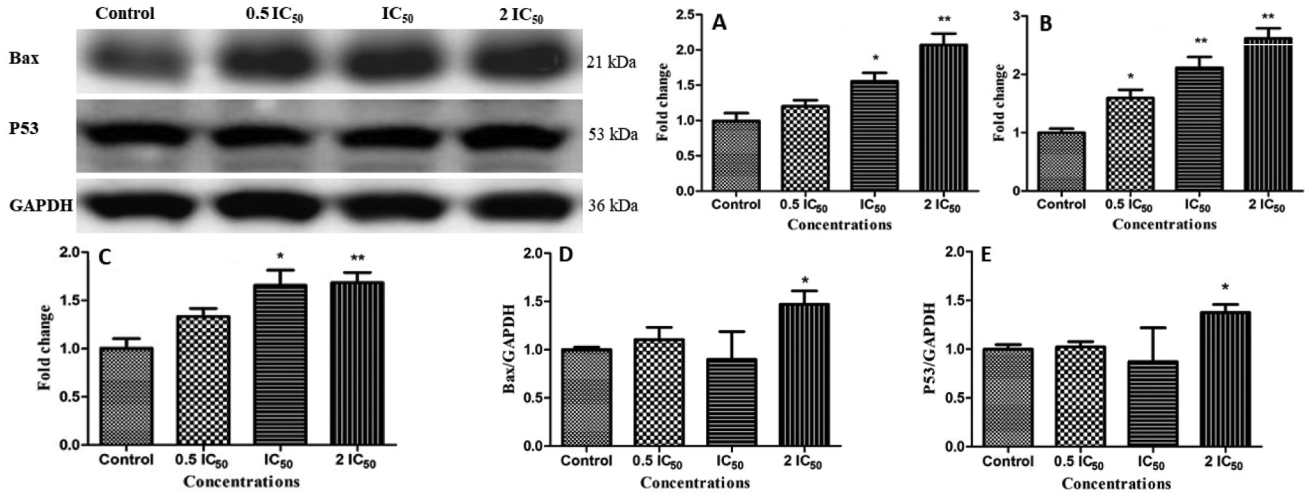

Fig. 5 — HES represented intrinsic and extrinsic apoptotic inducible activity by elevating Caspase 3 (A), Caspase 8 (B), Caspase 9 (C), Bax (D) and P53 (E) expressed protein levels in NTERA-2cells at 48 h of assessment. HES concentration was examined at 0.5xIC_{50}, IC_{50} and 2xIC_{50}. Data was expressed as means ± SD (n=3). *P< 0.05 and **P< 0.01 *vs.* control cells

tumorspheres and the expression of Oct4 and Nanog, which function to preserve the stemness, self-renewal, and tumorigenic potential characteristics[34]. A previous study also showed decreased mammosphere formation from MCF7 cells incubated with HES for 120 h at a concentration of 200 μM[23]. However, there were still few reports on this activity. Nanog and Oct4 serve as biomarkers for CSCs, notably elevated tumorigenic

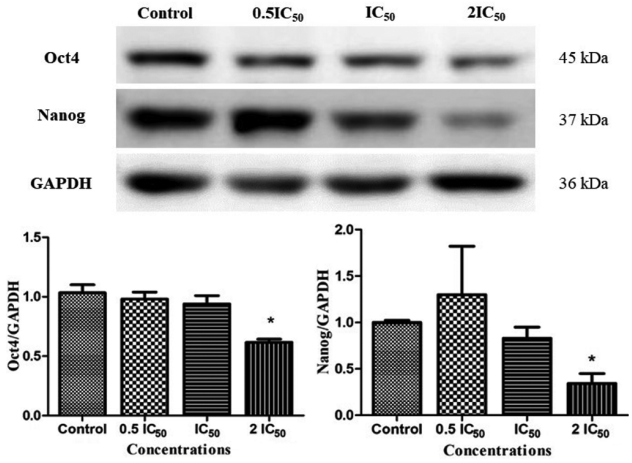

Fig. 6 — HES potentially down-regulated the expression of Oct4 and Nanog, the typical cancer stemness markers, of NTERA-2 cells. The cells were exposed with HES at $0.5xIC_{50}$, IC_{50} and $2xIC_{50}$ for 48 h. Data was expressed as means ± SD (n=3). *P< 0.05

potential and drug resistance. Together, they regulated cancer advancement by enhancing cell regeneration and proliferation[35]. Nevertheless, their activity could be suppressed by increasing p53 expression[36]. There were no reports about the expression of Nanog and Oct4 by treating HES. This study represents the initial documentation of HES's impact on the expression of Nanog and Oct4 in cancer stem cells. Hesperidin exhibited potent anticancer effects against NTERA-2 cancer stem cells by targeting their proliferation, and key stemness markers. Its multifaceted anticancer properties, coupled with its favorable safety profile with healthy cells, position it as a promising candidate for further research on cancer treatment.

Conclusions

In conclusion, HES isolated from *Z. rhetsa* might effectively activate the p53 pathway on NTERA-2 cancer stem cells by inducing apoptosis, G2/M arrest, and suppressing specific markers like Nanog and Oct4. Therefore, hesperidin represents a promising candidate for future anti-cancer drug development. Its potential role in regulating the p53 signaling pathway needs to be ensured by further investigation into downstream signaling, especially through the analysis of p53-related protein expression in NTERA-2 cancer stem cells. Furthermore, studies examining p53-knockdown both *in vitro* and *in vivo* will be critical to elucidate underlying working mechanism of hesperidin in terms of anticancer effects and its impact within this pathway.

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Conflict of interest

All authors declare no conflict of interest.

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