α-Hederin Inhibits the Growth of Lung Cancer A549 Cells in vitro and in vivo by Increasing SIRT6 Dependent Glycolysis

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Research

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Abstract

Background: α-hederin an effective component of *Pulsatilla chinensis* (Bunge) Regel, Studies showed that α-hederin exert many pharmacological activities, However, the effect of α-hederin on metabolism is still unclear. This study aimed to illuminate the role of α-hederin in glucose metabolism in lung cancer cells and investigate the molecular mechanism of α-hederin.

Methods: CCK8 and colony formation assays were employed to assess the anti-proliferative effects induced by α-hederin. Glucose uptake, ATP generation, and reduced lactate production were measured using kits, and an A549 tumor xenograft mouse model of lung cancer was used to assess the in vivo antitumor effect of α-hederin (5, 10 mg/kg). Glycolytic-related key enzymes hexokinase 2 (HK2), glucose transporters 1 (GLUT1), pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDHA), monocarboxylate transporter (MCT4), c-Myc, Hypoxia inducible factor-1α (HIF-1α) and Sirtuin 6 (SIRT6) protein expression were detected by western blotting and immunohistochemical staining and SIRT6 inhibitors was verified in A549 cells. Results: Our results showed that cell proliferation was significantly inhibited by α-hederin in a dose-dependent manner and that α-hederin inhibited glucose uptake and ATP generation and reduced lactate production. Furthermore, α-hederin remarkably inhibited HK2, GLUT1, PKM2, LDHA, MCT4, c-Myc, HIF-1α and activated SIRT6 protein expression. Using inhibitors, we proved that α-hederin inhibits glycolysis by activating SIRT6. Moreover, a tumor xenograft mouse model of lung cancer further confirmed that α-hederin inhibits lung cancer growth via inhibiting glycolysis in vivo. Conclusions: α-hederin inhibits the growth of non-small cell lung cancer A549 cells by inhibiting glycolysis. The mechanism of glycolysis inhibition includes α-hederin activating the expression of the glycolytic related protein SIRT6.

Background

In recent years, the incidence of lung cancer has been increasing. More than one million people die of lung cancer every year worldwide, representing an important factor tumor associated death [1, 2]. Lung cancer primarily includes two types, small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC accounts for 85% of all lung cancer patients. Since early symptoms are not obvious, approximately 70% of patients with NSCLC are diagnosed with advanced lung cancer, and the 5-year survival rate is a mere 16%. Despite early lung cancer patients having a choice to receive combined chemotherapy and surgery at the same time, which is the current recommended standard treatment, chemotherapy often causes many adverse reactions, and recently, progress in treatment modalities have improved, including surgery, chemotherapy and radiation therapy [3–5]. However, despite these advances, NSCLC prognosis has not improved. Therefore, additional therapeutic strategies are needed.

Energy metabolism refers to the state in which energy is generated, released, stored and consumed by organisms or living cells during the process of metabolism. Energy metabolism is primarily divided into glucose metabolism, protein metabolism and fat metabolism. Under normal conditions, cells primarily generate energy through aerobic respiration. Only when oxygen content is insufficient do cells adopt the
method of glycolysis to generate energy. This process is called anaerobic respiration. Unlike normal cells, however, tumor cells generate energy primarily by glycolysis, even in aerobic conditions, a phenomenon known as the Warburg effect. Glycolytic capacity is characterized by rapid productivity but low efficiency. Tumor cells rapidly proliferate, which requires swift energy consumption. Meanwhile, lactic acid generated by glycolysis creates an acidic environment for tumor cells, which is conducive to their growth, leading to rapid proliferation of tumor cells [6, 7].

*Pulsatilla chinensis* (Bunge) Regel, which is used for its clearing heat as a detoxifying agent, has been reported to exert many pharmacological activities such as anti-malaria, anti-bacterial infection [8] and anti-cancer [9]. α-hederin (Fig. 1a) belong to the monosaccharide chain pentacyclic triterpenoid saponins and is one of the effective components of the Chinese traditional medicine *Pulsatilla chinensis* (Bunge) Regel, studies have found that α-hederin has many pharmacological effects, such as anti-tumor, anti-fungal, insecticidal, anti-spasmodic, analgesic, anti-fungal and hepatic protection [10–14].

This experiment, using in vitro and in vivo studies of α-Hederin, addressed NSCLC inhibition, determination of glucose in glycolytic tumor metabolism, lactate and ATP content, key enzyme in the process of glycolytic metabolism and relevant transporters. Moreover, we assessed related regulatory factors in glycolysis to clarify α-hederin’s effect on the inhibitory mechanism of non-small cell lung cancer.

**Materials And Methods**

**Chemicals and reagents**

α-hederin was provided by Cheng du PUSH BIO-TECHNOLOGY (purity 99%), cell count kit 8 (CCK8) was purchased from DOJINDO Laboratories, RPMI1640 and DMEM were purchased from Hyclone, fetal bovine serum (FBS) was obtained from Biological Industries, and SIRT6 inhibitor, OSS_128167, was purchased from MCE, c-Myc and HIF-1α antibody were purchased from Abcam.

**Cell culture**

Human non-small cell lung cancer A549 cells, human lung cancer cell NCI-H460, and human lung cancer cell NCI-H292 (lymph node metastasis) cells were provided by Stem Cell Bank, Chinese Academy of Sciences. NCI-H460 and NCI-H292 were cultured in RPMI1640 containing 10% FBS, and A549 were cultured in DMEM containing 10% FBS in a 5% CO₂ incubator at 37° C.

**Cell viability assay**

The effect of α-hederin on proliferation of A549, NCI-H292 and NCI-H460 cells was assessed by CCK8 assay. Cell suspensions were seeded into 96-well plates, 5000 cells per well. After incubation for 24 h, cells were treated with α-hederin for 48 h. Subsequently, 10 µl CCK8 was added into each well and incubated for 1 h. Absorbance was measured at 450 nm using a Microplate Reader (SpectraMax I3
Austria). The inhibition rate was calculated by the following formula: Inhibition Rate (%) = (1-Absorbance of the treated group/Absorbance of the control group) × 100%.

**Colony formation assay**

The effect of α-hederin on cell proliferation was detected by colony formation assay. 500 cells per well of A549 cells were seeded into 6-well plates and incubated for 24 h. Subsequently, cells were treated with 5, 10, or 15 µM α-hederin for 48 h. Then, cells were cultured in DMEM containing 10% FBS for 10 days followed by fixation with 4% formaldehyde and staining with 0.5% crystal violet. The number of colonies was quantified.

**Glucose uptake, lactate and ATP assays**

A549 cells were cultured in 6-well plates (2 × 10^5 cells per well). After incubation for 24 h, DMEM medium containing 10% FBS with 0, 5, 10, or 15 µM α-hederin were seeded into the 6-well plates and incubated for 24 h, and culture media was collected. Glucose, lactate generation and ATP levels were assayed using a kit (Jiancheng, Nanjing, China) following the manufacturer's instructions. Absorbance was determined by a Microplate Reader, and the amount of glucose uptake, lactate and ATP level by cells was defined per protein mass.

**Western blotting**

For western blot analysis, A549 cells (1 × 10^6 per well) in 6-cm dishes were treated with or without α-hederin for 24 h. Cells were lysed in RIPA containing protease and phosphatase inhibitors (Thermo Scientific USA). Proteins were separated by SDS-PAGE based on molecular weight and blotted onto a polyvinylidene fluoride membrane (Thermo Scientific USA). Bands were incubated with primary antibodies HK2 (1:2000), GLUT1 (1:10000), PKM2 (1:1000), MCT4 (1:1000), LDHA(1:1000), c-Myc (1:1000), p53 (1:1000), HIF-1α (1:1000), Akt (1:1000), pAkt (1:1000) (Abcam USA), SIRT6 (1:1000) (Proteintech China) overnight at 4°C, Bands were measured using Image La Software, Version 4.1 (Bio-Ra Chemi Germany).

**Animal models**

Six-week-old male BALB/c nude mice (Hunan SJA Laboratory Animal Co. Ltd, China) were used for an A549 xenograft tumor mouse model. Mice were kept in pathogen-free environment with free access with food and water at the institutional animal care facility. The animals were housed in temperature and relative humidity at 21 °C and 55%, The procedures for care and use of animals were approved by the experimental animal ethics committee of Jiangxi University of Traditional Chinese Medicine (JZLLSC2018-0053) and all applicable institutional and governmental regulations concerning the ethical use of animals were followed, Mice were subcutaneously injected with 5 × 10^6 A549 cells. After tumor reached a certain size, they were removed, stripped clean, and tumor tissue was disassociated with scissors (approximately 3 × 3 × 3 mm). A small incision was made on the lateral abdomen of recipient animals. A small piece was taken with unhooked ophthalmic tweezers and implanted into the right armpit. The blank group was injected with culture medium when tumor volume reached 100 mm^3, and
mice were randomly assigned to five groups and treated intraperitoneally with α-hederin (10 mg/kg or 5 mg/kg), 5-FU (25 mg/kg) or vehicle. Mice were euthanized after a 32-day treatment, and tumor volume (length × width² × 0.5236) and body weight were determined. Then, tumors were fixed in 4% paraformaldehyde.

**Immunohistochemical analysis**

Tumor tissues were embedded in paraffin and cut into 5 µm sections by microtome (Leica Biosystems, Germany). Following the manufacturer's instructions (CW Biotech, China), tumor sections were incubated with GLUT1 (1:400), HK2 (1:400), PKM2 (1:30), LDHA (1:400), MCT4 (1:100), HIF-1α (1:200), c-Myc(1:100) (Abcam USA), SIRT6 (1:100) (Proteintech China) antibodies overnight at 4°C. Six kidneys were taken from each group, and three sections were taken from each group. Choose 6 fields per slice, The results were calculated by mean integrated optical density. and analyzed by Image pro-plus 6.0.

**Statistical analyses**

Comparisons were made to determine significant differences between individual groups by one-way ANOVA. T-test was used for statistical analyses between two independent groups. All tests were performed using SPSS 19, and \( p < 0.05 \) was considered significant.

**Result**

**α-Hederin inhibits proliferation of human lung cancer cells**

First, we examined the inhibitory effect of α-hederin on the proliferation of human lung cancer cells. CCK8 results showed that α-hederin had significant inhibitory effects on proliferation of human lung cancer cells. The IC\(_{50}\) of α-hederin in non-small cell A549 (13.75 µM), NCI-H460 (17.57 µM) and NCI-H292 (18.04 µM) are shown in Fig. 1b. α-Hederin exhibited a superior inhibitory effect on A549 cells, and A549 cells were used for subsequent study.

Colony formation assay demonstrated that α-hederin inhibited the clonogenic effects of non-small cell lung cancer A549 cells. Compared to 0 µM, 5 µM, 10 µM and 15 µM α-hederin significantly decreased cell number (\( p < 0.05 \)) (Fig. 1c).

**α-Hederin inhibits glycolytic metabolism of human non-small cell lung cancer A549 cells**

Glycolytic metabolic levels can be assessed by glucose uptake, lactate and ATP levels, and results revealed that A549 cells treated with 10 µM and 15 µM α-hederin, glucose consumption decreased significantly (\( p < 0.01 \)). Compared to 0 µM, lactate production of A549 cells was significantly reduced in 10 µM and 15 µM treatments (\( p < 0.05 \)). Compared to 0 µM, ATP levels in A549 cells were significantly decreased after intervention with 10 µM and 15 µM α-hederin (\( p < 0.05 \)) as shown in Fig. 2.

**α-Hederin inhibits glycolytic related proteins in human non-small cell lung cancer A549 cells**
Extracellular glucose is transported into the cell by GLUT1, which then generates lactate and ATP under the catalysis of enzymes, including HK2, PKM2 and LDHA, to supply tumor cell proliferation, finally transporting intracellular lactate out of the cell via MCT4. Western Blot (WB) results showed that compared to 0 µM, 10 µM and 15 µM α-hederin significantly reduced expression of GLUT1, HK2, PKM2, LDHA, and MCT4 ($p < 0.05$) as shown in Figure. 3.

**α-Hederin inhibits glycolytic related proteins in A549 cells by inhibiting expression of c-Myc and HIF – 1α**

c-Myc, HIF-1α, Akt, and p53 have distinct effects on proteins involved in glycolysis in tumor cells. The results showed that α-hederin in each group had no significant effect on the expression of glycolysis regulators Akt and p53 protein in tumor cells, while expression of c-Myc and HIF-1α was significantly down-regulated in each group compared to 0 µM ($p < 0.05$). Expression of HIF-1α was significantly down-regulated at 10 µM and 15 µM, suggesting that α-hederin may inhibit aerobic glycolysis in tumor cells by regulating expression of c-Myc and HIF-1α. (Fig. 4).

**α-Hederin inhibits c-Myc and HIF – 1α by activating expression of SIRT6**

The results showed that expression of SIRT6 was significantly up-regulated in each group compared to control ($p < 0.05$). After addition of the SIRT6 inhibitor OSS_128167, SIRT6 protein levels were significantly down-regulated, and expression of glycolytic related proteins c-Myc, HIF-1α was up-regulated compared to control, after the combination of α-hederin and OSS_128167, c-Myc, HIF-1α, was up-regulated compared to both OSS_128167 and α-hederin alone after the combination of α-hederin and OSS_128167. (Fig. 5)

**Effect of α-Hederin combined with SIRT6 inhibitor OSS_128167 on the viability of A549 cells**

we examined the inhibitory effect of α-hederin combined with SIRT6 inhibitor OSS_128167 on the proliferation of A549 cells. CCK8 results showed that Compared to α-hederin alone, the inhibition rate of A549 cells in α-hederin combined with OSS_128167 group had significant decreased, as shown in Fig. 6.

**Inhibitory effect of α-hederin on xenograft non-small cell lung cancer A549 tumors in nude mice**

Results of tumor weight and tumor volume in xenograft nude mice showed that α-hederin had significant inhibitory effects on tumor volume and tumor weight in xenograft nude mice compared to the model group ($p < 0.05$), and the inhibitory rates of 10 mg/kg and 5 mg/kg α-hederin were 40% and 24%, respectively, as shown in Fig. 7.

**Effect of α-hederin on glycolytic related proteins in non-small cell lung cancer A549 transplanted tumors in nude mice**
Immunohistochemical results showed that compared to the model group, GLUT1 content of non-small cell lung cancer cell A549 tumors in the 10 mg/kg α-hederin dose group was significantly decreased ($p<0.01$). In addition, GLUT1 expression of the 5 mg/kg α-hederin dose group was decreased, but the difference was not statistically significant. HK2, PKM2, LDHA, MCT4, c-Myc and HIF-1α levels were all significantly decreased in the 5 mg/kg and 10 mg/kg α-hederin groups ($p<0.05$), while the SIRT6 levels were significantly increased in the 5 mg/kg and 10 mg/kg α-hederin groups ($p<0.05$) (Fig. 8).

**Discussion**

In this study, the inhibitory effect of α-hederin on tumor cells was assessed, and the effects of glucose, lactate and intracellular ATP in cell culture medium on glycolysis in A549 cells were measured. Expression of GLUT1, HK2, PKM2, LDHA, MCT4 and glycolytic regulators c-Myc, HIF-1α, p53, Akt and SIRT6 were detected by WB assay to determine the inhibitory mechanism of α-hederin on non-small cell lung cancer A549 cells. We established a non-small cell lung cancer A549 allograft transplantation tumor model to investigate the effect of α-hederin on xenograft tumors in mice by evaluating tumor volume and weight, and immunohistochemistry was used to detect the expression of related protein in glycolysis. The results showed that α-hederin inhibited non-small cell lung cancer A549 in vivo and in vitro, significantly reduced non-small cell lung cancer A549 glucose uptake, reduced generation of lactate, and reduced ATP levels in cells. The mechanism of α-hederin occurred by activating SIRT6 and regulate c-Myc and HIF-1α, which reduced key glycolytic enzymes, GLUT1, HK2, PKM2, LDHA, and MCT4.

Otto Warburg discovered in the 1920s that glycolysis is the primary source of energy for cancer cells, even when oxygen levels are sufficient [15]. Oxidative phosphorylation is a major way that normal cells produce energy; however, during the process of growth and proliferation, tumor cells need a large energy supply in a short period of time. Oxidative phosphorylation cannot provide energy for tumors very well, while the glycolysis pathway can quickly provide energy for tumors. Therefore, cancer cells primarily generate energy by glycolysis, and lactate produced by glycolysis provides an acidic environment for tumors to grow [16]. The Warburg effect primarily manifests in glucose consumption, lactate production and ATP production in cancer cells. Therefore, by measuring the glucose consumption, lactate production and ATP levels, we can determine whether the drug has an impact on the glycolytic process. The results of this study demonstrated that α-hederin significantly reduced glucose uptake, lactate production and ATP levels in A549 cells of non-small cell lung cancer, indicating that glycolysis in A549 cells was significantly inhibited in response to α-hederin treatment.

During the process of aerobic glycolysis, glucose is transferred into the cytoplasm by initial glucose transporters (GLUTs). Then, glucose is catalyzed into glucose 6 phosphate by hexokinase (HK), followed by 6-phosphate glucose transfer into pyruvate by PKM2. Pyruvate is catalyzed to lactate by lactate dehydrogenase (LDH) and finally transported out of cell by MCTs. The results of this study indicate that α-hederin significantly reduces expression of GLUT1, HK2, PKM2, LDHA, and MCT4 among the glycolytic proteins of A549 cells of NSCLC both in vitro and in vivo.
GLUT1, HK2, PKM2, LDHA, MCT4 and other relevant proteins involved in glycolysis play a role in directly affecting the glycolytic process. However, these key enzymes are also regulated by relevant regulatory factors. Akt play a very important role in cell energy metabolism, and Akt may enhance aerobic glycolysis of tumor cells [17]. p53 is a tumor suppressor gene, and p53 reprograms energy metabolism to negatively regulate cell glycolysis by promoting mitochondrial oxidative phosphorylation and inhibiting glycolysis [18, 19]. GLUTs and glycolytic related catalytic enzymes are also regulated by p53 to inhibit glycolysis of cancer cells. Expression of GLUT1 and GLUT4 can be directly inhibited and are indirectly adjusted by GLUT3 [20]. p53 also regulates the PI3K/Akt/mTOR pathway to regulate glucose metabolism [21]. Hypoxia inducible factor-1 (HIF-1α) is in a class of transcription factors that are heterodimers composed of subunits to adapt to the response of tumor cells to environmental changes in the hypoxic environment. HIF-1α can regulate glucose uptake, glycolytic enzymes, and expression of single carboxylic acid transporters, such as GLUT1, GLUT3, HK2, fructose phosphate kinase 2 (PFK2), aldolase A (ALDOA), enolization enzyme (ENO), pyruvate kinase M (PKM), and expression of LDHA and MCT 4 [22–24]. c-Myc is a member of the Myc gene family of oncogenes and is associated with a wide variety of tumor development, and c-Myc promotes glucose absorption by up-regulating GLUT1 [25–26], enhancing transcription of glycolytic enzymes HK2, PFK, and LDHA [27–28]. c-Myc also up-regulates expression of MCT and PKM2 [29–31]. In this study, we found that α-hederin significantly reduces expression of glycolytic regulators HIF-1α and c-Myc in non-small cell lung cancer A549 cells but had no significant effect on expression of p53 or Akt.

SIRT6 plays a key regulatory role in gene transcription, metabolism, maintenance of genomic stability and the integrity of telomeres, thus regulating the occurrence and development of diabetes, obesity, heart disease, cancer and other diseases, SIRT6 regulate the metabolism of fat and glucose, which is a key regulator of energy stress and closely related to the process of tumors [32], SIRT6 inhibited the activity of transcription factor HIF-1α and inhibited glucose oxidation and glycolysis through the citric acid cycle [33], SIRT6 was also found to co-inhibit the transcription activity of the central oncogene MYC of the ribosome gene [34], we found that α-hederin significantly increase expression of SIRT6, we used a SIRT6 inhibitor, OSS_128167, for verification. The results showed that expression of HIF-1α and c-Myc were decreased in OSS_128167 treatment in combination with α-hederin compared to OSS_128167 alone. Combined with the results of previous experiments, our results demonstrate that α-hederin activates expression of SIRT6 then regulate tumour glycolysis.

**Conclusion**

α-hederin inhibits the growth of non-small cell lung cancer A549 through suppressing glycolysis. The underlying mechanisms are that, α-hederin activates SIRT6 expression, then inhibits the expression of glycolytic regulatory factors HIF-1α and c-Myc, and suppresses that of glycolytic proteins. (Fig. 9)

**Abbreviations**

NSCLC
Declarations

Acknowledgements

Not applicable.

Authors' contributions

Lanying Chen designed the experiments, and was a major contributor in writing the manuscript. Cong Fang, Yahui Liu, Yingying Luo, Yaru Cui, Ni Zhang, Peng Liu, carried out the laboratory experiments. Mengjing Zhou, and Yongyan Xie analyzed the data, interpreted the results and prepared figures. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The procedures for care and use of animals were approved by the experimental animal ethics committee of Jiangxi University of Traditional Chinese Medicine (JZLLSC2018-0053) and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

Competing interests

The authors declare that they have no competing interests.

Author details
References


Figures
Figure 1
α-Hederin inhibits proliferation of human lung cancer cells. a Chemical structure of α-Hederin. b Effect of α-Hederin on the viability of human lung cancer cells. A549, NCI-H460, and NCI-H292 cells were treated with α-hederin for 48 h. CCK8 assay was performed to analyze cell viability. Result are normalized to PBS controls. c Effect of α-Hederin on the viability of non-small cell lung cancer A549 cells. Colony formation of A549 cells treated with α-hederin for 48 h, cultured by DMEM for 10 days, and stained with crystal violet. **p < 0.01 as compared to the 0 μM group. Mean ± S.E.M. n=3.

**Figure 2**

α-Hederin dramatically inhibits aerobic glycolysis in A549 cancer cells. Glucose uptake, lactate generation, and intracellular ATP levels in A549 cells in response to α-hederin treatment for 48 h. The concentration of 2-DG was 8 mM. *p < 0.05, **p < 0.01 as compared to the control group. Mean ± S.E.M. n=3.

**Figure 3**

α-Hederin inhibits glycolytic related proteins in human non-small cell lung cancer A549 cells. A549 cells were treated with α-hederin for 24 hours. Expression of GLUT1, HK2, PKM2, LDHA and MCT4 were detected by Western blot. *p < 0.05, **p < 0.01 as compared to the 0 μM group. Mean ± S.E.M. n=6.
α-Hederin reduces glycolytic levels by inhibiting c-Myc and HIF-1α in human non-small cell lung cancer A549 cells. A549 cells were treated with α-hederin for 24 hours. Expression of c-Myc, Akt, HIF-1α, and p53 was detected by western blot. *p < 0.05, **p < 0.01 as compared to the 0 μM group. Mean ± S.E.M. n=3.
α-Hederin inhibits c-Myc and HIF-1α by activating expression of SIRT6. a A549 cells were treated with α-hederin for 24 hours. Expression of SIRT6 was detected by western blot. b A549 cells were treated with α-hederin or SIRT6 inhibitor OSS_128167 for 24 hours. Expression of c-Myc and HIF-1α was detected by western blot. *p < 0.05, **p < 0.01 as compared to the 0 μM group. Mean ± S.E.M. n=3.

Figure 5
Figure 6

Effect of α-Hederin combined with SIRT6 inhibitor OSS_128167 on the viability of A549 cells. Cells were treated with α-hederin for 48 h. CCK8 assay was performed to analyze cell viability. Result are normalized to PBS controls.
Figure 7

α-Hederin inhibits orthotopic non-small cell lung cancer growth in vivo. *p < 0.05, **p < 0.01 as compared to the Normal control group. Mean ± S.E.M. n=10
Figure 8

α-Hederin suppresses expression of glycolysis-related proteins in vivo. IHC of GLUT1, HK2, PKM2, LDHA, MCT4, HIF-1α, c-Myc and SIRT6 in tumor tissues (200×). The results showed a remarkable decrease in expression of GLUT1, HK2, PKM2, LDHA, MCT4, HIF-1α, c-Myc and SIRT6 in tumors treatment with 5 and 10 mg/kg α-hederin compared to controls. *p < 0.05, **p < 0.01 as compared to the Normal control group. Mean ± S.E.M. n=6
Figure 9

α-Hederin inhibits the growth of lung cancer A549 by increasing SIRT6 dependent glycolysis.