Galla chinensis exhibits anticancer activity in HPV-16 infected cervical cancer cells

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Abstract

Background

Cervical cancer induced by the human papillomavirus (HPV) is one of the most common cancers in women in developing countries. Novel treatments for cervical cancer are focused on bioactive metabolites obtained from natural sources.

Materials and Methods

The biological effects of Galla Chinensis on HPV16 + cells were analyzed by cell scratch and plate cloning, and the cycle process was evaluated by flow cytometry; PCR, WB and immunofluorescence techniques were used to explore its mechanism

Results

It was shown that compared to a blank control group, Galla chinensis is less toxic to cervical cancer cells and exhibits prominent inhibitory effects on the growth, cloning, and migration of SiHa adherent cells. The effects of Galla chinensis also included cell cycle arrest in the S phase, and our obtained results suggest that its mechanism of action may involve the downregulation of the HPV E6 oncoprotein and the reconstruction of the p53 functional protein.

Conclusion

It is proved that Galla chinensis can effectively eliminate HPV16 + cells and inhibit the development of cervical cancer. It can down regulate oncoprotein E6 and up regulate tumor suppressor protein p53.

Introduction

Cervical cancer is one of the most common malignancies in women worldwide, with an incidence of over 500,000 cases per year and a mortality rate of ~ 9%[1] wherein 75% of those who develop the disease are from developing countries.[2] Although approved human papillomavirus vaccines and drugs exist, their affordability in low-income areas is limited.[3] For patients suffering from advanced or recurrent cervical cancer, radiotherapy and chemotherapy are still commonly used; however, the treatment options for patients with metastatic or recurrent disease are very limited, and the five-year survival rate is 16.5%.[4,5] Therefore, in view of the fact that natural-product-based treatments are easier to provide than preventive and/or therapeutic vaccines due to their easy availability and low cost, they could be considered an alternative treatment for use in low-income countries.

Galla chinensis, or gallnut, is a particularly unique natural product that is formed through a parasitic interaction between plants and insects. Gallnuts are pathological excreta formed on the young branches of plants by insect colonization and egg laying.[6] This species grows in areas with a low agricultural production capacity and is widely distributed in temperate, subtropical, and tropical regions, including China, Japan, and Malaysia. Galla chinensis is usually harvested between September and October, and the gall, which is commonly employed in Chinese medicine, is dried and cleaned after removal of the larvae.[7] The main active components of Chinese gall are phenolic compounds, including gallic acid and methyl gallate, of which gallic acid accounts for 20%.[8,9] Gallic acid is known to upregulate the pro-apoptotic protein Bax in cancer cells and induce caspase cascade activity. However, gallic acid also downregulates anti-apoptotic proteins, such as Bcl-2 and Xiap, and exhibits a cell cycle arrest effect.[10,11] This is considered one important mechanism by which Galla chinensis could effectively fight cancer. Although it has been reported that Chinese Galla chinensis has preventive and therapeutic effects on various diseases and ailments, such as diarrhea, dysentery, rectal and bowel cancer, diabetes, sepsis, and oral diseases,[12] its anti-cervical cancer efficacy has yet to be examined in detail.

Thus, we herein report our experimental verification of the anti-cancer efficacy of Galla chinensis to provide a theoretical basis for the treatment of cervical cancer in humans. In addition, with the knowledge that various herbs exhibit anticancer activities,[13] we carry out a comparison with pterostilbene, which is a polyphenolic compound that is found naturally in various dietary sources, such as grapes, blueberries, red wine, peanuts, and some medicinal plants,[14] to determine the anti-cervical-cancer effect of Galla chinensis.

Methods

Gall preparation and separation of its components

The selected species is formed by aphids of the Fordinae subfamily (Pemphigidae), which parasitize the compound leaves of several trees of the genus Rhus L. of the Anacadiaceae family. Insect galls are commonly used in traditional Chinese medicines. The obtained Galla chinensis samples were crushed and passed through a 60-mesh sieve to yield 80 g of powder. This powder was then extracted three times with distilled water (240 mL) under reflux for 2 h, and the extracts were combined. Liquid chromatography-mass spectrometry (LC-MC) was used to analyze the Galla components present in the aqueous extract. This extract was then subjected to column chromatography on a polyamide resin, and gradient elution was performed using 30 column volumes of 0% ethanol, 40% ethanol, and 70% ethanol. Following the evaporation of the solvent, a concentrated eluate that contained the components of Galla chinensis was obtained. Pterostilbene was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution with a concentration of 100 mM. E6 antibody (ChinaPetides Co., Ltd.), p53 antibody, FITC-labeled goat anti-mouse IgG, β-actin, and mouse were purchased from ZSGB-BIO, China.

Cell culture

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin–amphotericin B (Lonza, Walkersville, MD, USA) and maintained at 37°C in a 5% CO_2 humidified incubator.

Cell proliferation assay

Cells were seeded into 96-well plates (Jet Bio-Filtration, China) in triplicate at a density of 1000 cells per well. The cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (KeyGEN BioTECH) according to the manufacturer's instructions. The optical density at 450 nm was measured at the indicated time points using a multifunctional microplate reader (Thermo Fisher Scientific, USA). Cell growth curves were generated using the GraphPad Prism software (GraphPad Software, USA).

Clonogenic assay

SiHa cells were seeded and cultured in a 6-well plate at a density of 1×10^3 cells/well in DMEM containing 10% FBS for 24 h. The cells were then treated with various concentrations of Galla chinensis or pterostilbene (3, 6, and 12 μg/mL). Following treatment, the cells were resuspended in DMEM containing 10% FBS and cultured in 5% CO_2 at 37°C for 15 d to allow colony formation. The plates were then washed with cold PBS. The colonies were fixed with 4% paraformaldehyde at room temperature, then dyed with 1% crystal violet for 30 min at room temperature. Colonies of more than 100 cells were counted using a microscope (Leica Microsystems, Germany). Each experiment was performed three times. The colony formation rate (CFR) was calculated as follows:

CFR = number of colonies containing at least 50 cells/number of inoculated cells) × 100

Wound healing assay

SiHa cells (2×10^5 cells/well) were seeded in a 12-well culture plate. The confluent monolayer cells were scratched using a pipette tip, and each well was washed with PBS to remove any non-adherent cells. The cells were treated with Galla chinensis or pterostilbene (3, 6, and 12 μg/mL) and then incubated for up to 48 h. The perimeter of the central cell-free zone was observed using an optical microscope (Olympus, Center Valley, PA, USA).[15]

Cell cycle analysis

The cell cycle distribution was analyzed using a Cell Cycle Detection Kit (KeyGEN BioTECH, JiangSu, China) according to the manufacturer's instructions. For this purpose, SiHa cells (4×10^5 cells/well) were seeded in a 6-well plate and treated with Galla chinensis or pterostilbene (3, 6, and 12 μg/mL) for 24 h. After this time, the cells were collected by centrifugation, washed with PBS, and fixed in ice-cold 70% ethanol at − 20°C for 2–24 h. The cells were then incubated with the Cell Cycle Detection reagent for 30–60 min at room temperature in the dark. Cells were analyzed for their DNA content by measuring the PI fluorescence using an Accuri C6 flow cytometer (BD, USA).

Real-time quantitative reverse transcription PCR (qRT-PCR) gene expression analysis

A total of 250,000 cells were cultured overnight in 6-well plates. Cells were treated with Galla chinensis or pterostilbene for 24 h. The total RNA was isolated from master cells using the Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA), and quantified using a NanoDrop spectrophotometer (Thermo Scientific). The A_{260}/A_{280} and A_{260}/A_{230} ratios displayed by all samples were determined to be ~ 2.0. iScriptTM was used to reverse transcribe RNA into cDNA using a cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). SsoAdvanced™Universal SYBR Green Supermix (Bio-Rad) was used for the qPCR reaction in a Bio-Rad CFX96 Touch. The PCR cycle was carried out as follows: 98°C for 30 s, 40°C for 98°C for 5 s, and 60–62.9°C for 30 s. Subsequently, melt curve analysis was conducted from 65 to 95°C in increments of 0.5°C for each cycle. Table S1 provides a list of the primers, nucleotide sequences (or Bio-Rad analysis ID), and annealing temperatures employed. To avoid false negatives due to the degradation of mRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Target gene mRNA expression levels relative to GAPDH were automatically calculated using the comparative Ct method with CFX Manager Software v1.6 (Bio-Rad) or Genex Software (Bio-Rad). Gene expression was assessed using the comparative Ct method (ΔΔCt method), which represents the mRNA expression level relative to the reference gene. The amount of target, normalized to an internal housekeeping gene, GAPDH, and relative to a calibrator, is given by 2 − ΔΔCt according to the following equation:

Name	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
E6	TGCTAGTGCTTATGCAGCAA	ATTTACTGCAACATTGGTAC

Western blot analysis

A total of 250,000 cells were cultured on 6-well plates. Cells were lysed using RIPA buffer (H8080, Solarbio, China) supplemented with a protease inhibitor cocktail (Solarbio, China) on ice. Protein concentrations of the extracts were determined using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of cell lysate were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore) using standard electroblotting procedures. The blots were blocked in Tris-buffered saline with Tween-20 (TBST) containing 5% skimmed milk at room temperature for 1 h and immunolabeled with primary antibodies against E6, p53 (dilution 1:1000), and β-actin (dilution 1:10000) overnight at 4°C. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (dilution 1:15000) for 2 h at room temperature. Immunolabeling was detected using an enhanced chemiluminescence (ECL) kit (Millipore, USA) according to the manufacturer's instructions. The membranes were scanned using the APHA INNOTECH Fluorochem SP imaging system. The band density was analyzed using ImageJ software (version 1.5; NIH).

Immunocytochemistry

A total of 8,000 SiHa cells were plated on 6-well chamber slides and allowed to grow for 24 h. Cells were then treated with different concentrations of Galla chinensis or pterostilbene for 24 h. After treatment, the cells were fixed in 4% paraformaldehyde at room temperature, rinsed with PBS, permeabilized, and blocked with 5% BSA and 0.5% Triton X-100 in PBS for 1 h. The cells were then incubated overnight with the primary antibodies in a blocking buffer. Following primary antibody treatment, the cells were washed and then incubated with the respective fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 2 h and washed three times with PBS, followed by incubation with a mounting medium containing the DAPI antifade reagent. The slides were then mounted with coverslips, and cell images were acquired using a Zeiss Axio Observer Z1 microscope. ImageJ software was used to measure the fluorescence intensity and for cell counting. The fluorescence intensities of the E6 and p53 antibodies were normalized to the intensity of DAPI (blue).

Statistical analyses

Statistical analyses were performed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism® 8 (GraphPad Software, Inc., La Jolla, CA, USA). Means and standard deviations were calculated for each group. One-way ANOVA with Tukey's test was used to compare three or more datasets and to determine the significance between the groups. More specifically, ANOVA is a test of variance, and the post hoc Tukey test was used to determine the significance between the groups.[16] Statistical significance was set at $P < 0.05$.

Results

Galla chinensis Inhibited the Growth of Cervical Cancer Cells

We separated the various components of Galla chinensis by chemical extraction (see Fig. 1 and Table 1). To study the cytotoxicity of Galla chinensis, we used the CCK-8 kit to determine its inhibitory effect on SiHa adherent cells at different concentrations (0–100 μg/mL). According to previous literature, pterostilbene has an inhibitory effect on cervical cancer cells; therefore, it was employed as a positive control. Overall, our results indicated that Galla chinensis and pterostilbene inhibit the growth of HPV16 + SiHa cells in a concentration-dependent manner, with IC_{50} values of 25 and 5 μg being determined for Galla chinensis and pterostilbene, respectively (Fig. 2). To avoid any issues related to cytotoxicity, we employed used a uniform sublethal concentration of Galla chinensis (i.e., 3, 6, and 12μg/mL). Subsequently, we evaluated the effects of Galla chinensis and pterostilbene on SiHa colony formation and found that Galla chinensis was more effective than pterostilbene in inhibiting the colony-forming ability of the cells (Fig. 3). These data show that Galla chinensis is effective in inhibiting the growth and clonal formation of HPV16 + adherent cervical cancer cells.

Table 1
Analytical information for each component of Galla Chinensis

Number	Composition	Mass-to-charge ratio (*m/z*)	Keep time (min)	Molecular formula
1	2,3-Di-*O*-galloyl-D-glucose	483.2170	23.50, 23.75	$C_{20}H_{19}O_{14}$
2	Digallic acid	321.1016	25.79, 26.19	$C_{14}H_{10}O_9$
3	3-*O*-Galloyl-beta-D-glucose	635.2994	26.33, 29.46, 33.07	$C_{27}H_{23}O_{18}$
4	Ethyl gallate	197.0735	35.83, 35.96, 36.31	$C_9H_{10}O_5$
5	4-O-Galloyl-beta-D-glucose	787.1998	40.56, 40.60	$C_{34}H_{27}O_{22}$
6	5-*O*-Galloyl-beta-D-glucose	939.1669	44.08	$C_{41}H_{31}O_{26}$
7	Double ethyl gallate	349.1047	52.52	$C_{16}H_{15}O_{10}$
8	6-*O*-Galloyl-beta-D-glucose	1091.8642	47.14, 55.29	$C_{48}H_{35}O_{30}$
9	7-*O*-Galloyl-beta-D-glucose	1243.3025	50.86, 55.22	$C_{55}H_{39}O_{34}$

Inhibition of the cell migration of SiHa cells treated with Galla chinensis

To determine the effect of Galla chinensis on the metastatic ability of cervical cancer cells, three different sublethal concentrations were used in a 48h scratch test (Fig. 4A), wherein a single-layer wound-healing experiment was performed to evaluate the effect on cell migration. Compared with the untreated control group, Galla chinensis significantly reduced the migration of SiHa cells at 48 h after treatment. At sublethal concentrations of 6 and 12 μg/mL, both Galla chinensis and pterostilbene significantly inhibited the migration of the SiHa cells relative to that of the untreated cells (Fig. 4B). To analyze the effects of Galla chinensis and pterostilbene on cell migration, we normalized the number of untreated cells that migrated to the scratches (wounds) to 100%. Thus, 75% and 55% of the gall-treated cells migrated for treatment concentrations of 6 and 12 μg/mL, respectively, compared with the control group (Fig. 4B). The wound-healing assay showed that the two compounds reduced the migration of SiHa cancer cells when compared to the control group.

Cell cycle arrest in the S phase of SiHa cells treated with Galla chinensis at sublethal concentrations

To determine whether the growth inhibition effect of Galla chinensis on SiHa adherent cells was caused by cell cycle arrest, flow cytometry analysis was used to quantify the effect of Galla chinensis on the cell

cycle distribution (Fig. 5). Compared with the blank control cells, the number of cells in the G2/M phase of the cells treated with either compound was found to be significantly reduced (Table 2), thereby indicating cell cycle arrest in the S phase. In addition, in the cells treated with 12 μg/mL Galla chinensis, the ratio of the S phase was nearly doubled compared with that of the blank control, which was the phase with the largest change among the four test cell cycle phases in the SiHa cells. Taken together, these results indicate that blocking cells in the S phase significantly inhibits cells from entering the G2/M phase.

Table 2
Table showing the percentage of cells in each phase of the cell cycle after treatment with different concentrations of Galla Chinensis (Gall) and pterostilbene (Pte)

Compound	Cell cycle phase (%)		
	G0	S	G2/M
Control	63.50	16.50	19.50
Gall 3 μg	60.20	21.36	18.44
Pte 3 μg	61.30	22.10	16.60
Gall 6 μg	55.74	29.62	14.64
Pte 6 μg	53.70	33.90	12.40
Gall 12 μg	57.30	31.97	10.74
Pte 12 μg	56.50	33.27	10.23

Downregulation of the E6 viral oncoprotein and upregulation of p53 in SiHa cells treated with Galla chinensis

In order to explore the mechanism of Gall inhibiting the growth and migration of SiHa cervical cancer cells, their effects on the expression of E6 gene in HPV infected cervical cancer cells were studied. Studies have shown that the pathogenesis of HPV infection involves the overexpression of viral oncoprotein, of which the most critical is the regulation level of E6 oncoprotein, which can inhibit a variety of cell proteins and affect biological processes including cell proliferation, cell cycle and apoptosis. The expression of E6 mRNA was detected by real-time fluorescence quantitative PCR (RT-qPCR). With the increase of the concentration of the two drugs, the expression decreased gradually (Fig. 6C).

Before immunostaining analysis of E6, we treated it with sublethal concentrations (3ug/mL, 6ug/mL and 12ug/mL) for 24 hours, and observed the fluorescence intensity of oncoprotein E6 in the SiHa cells after fluorescence staining (Fig. 6A). At three concentrations, Galla chinensis and pterostilbene could affect E6 activity compared with the blank control group (Fig. 6B). With the inhibition of E6, treatment at three

concentrations for 24 hours also resulted in the up regulation of p53 in cells (Fig. 7A). Compared with the blank group, gallnut treatment increased the level of p53 by 2 times (staining standardized to DAPI; Fig. 7B).

Western blotting was then used to analyze the total protein levels of E6 and p53 in the cells treated with Galla chinensis at the three different concentrations over 24 h (Fig. 8). As indicated, downregulation of the E6 oncoprotein was observed at all three concentrations; however, to further understand the molecular mechanism of the anti-cervical cancer activity of Galla chinensis, we investigated its influence on the level of the tumor suppressor protein p53. Indeed, it was found that Galla chinensis upregulates the expression of the p53 tumor suppressor protein (Fig. 8B), and the effect of Galla chinensis was more significant than that of pterostilbene. These results indicate that Galla chinensis exerts its anti-cancer activity through the downregulation of oncoprotein E6 and the upregulation of p53.

Discussion

As mentioned above, Galla chinensis has been reported to exhibit a variety of biochemical and pharmacological properties.[17,18] In addition, in the past few decades, pterostilbene has been shown to exhibit excellent anti-cancer effects with regard to lung, colon, breast, and cervical cancers.[19] However, the bioavailability of Galla chinensis is higher than that of pterostilbene, which contains two methoxy groups.[20] Importantly, this is the first paper to propose and preliminarily explore the antitumor activity of Galla chinensis against cervical cancer.

More specifically, using a wide range of sublethal concentrations to evaluate the effects of Galla chinensis (i.e., 3, 6, and 12 μg/mL) to avoid any issues related to cytotoxicity, an IC_{50} value of 25 μg/mL was determined. Our results showed that at these low concentrations, Galla chinensis can inhibit the growth of SiHa cervical cancer cells, which are known to be clonal, and our research elucidated the viability of cells treated with sublethal concentrations of Galla chinensis. Indeed, clone formation experiments demonstrated that Galla chinensis has a long-term effect on SiHa cervical cancer cells after 24 h of treatment, wherein the surviving cells were allowed to grow in a normal growth medium for 15 d afterwards. At all three concentrations examined, the cloning ability of the viable cells was significantly reduced, and the observed effect was slightly superior to that observed for pterostilbene. These results indicate that Galla chinensis has the ability to inhibit the proliferation and growth of cervical cancer cells.

Since cell migration is a key step in metastasis,[21] limiting such migration will help to better understand cancer metastasis to ultimately enable physiologically relevant drug and cell line screening.[22] Our research found that at sublethal doses, both pterostilbene and Galla chinensis exhibited anti-migration effects. These findings are supported by those of previous studies, which have shown that Galla chinensis exhibits a significant ability to prevent the proliferation of human breast cancer cells through an effective *h* LDH-A inhibitor.[23] These mechanisms may account for the inhibition of SiHa cell migration, but further studies are required to confirm this.

It has previously been demonstrated that pterostilbene promotes the arrest of the cancer cell cycle, and it has been confirmed that following treatment, cervical cancer cells remain in their S phase.[24] We, therefore, decided to study the cycle arrest effect imparted by Galla chinensis, wherein cytometry analysis showed that this treatment caused SiHa cells to stagnate in the S phase, thereby preventing cell mitosis.

E6 is a known viral oncoprotein that induces cervical cancer by inactivating the tumor suppressor protein p53.[25] The p53 gene is irreversibly mutated in most cancers; however, it has been reported that cervical cancer and cell lines retain the wild-type p53 gene, and its function is obscured by the viral E6 protein.[26] In the current study, we observed that Galla chinensis reduced the expression of E6 in SiHa cervical cancer cells. This is of importance since the downregulation of E6 may lead to restoration of the tumor suppressor function of the p53 protein. This may result in the activation of downstream signaling molecules, leading to cell cycle arrest or cell apoptosis, which will lay the foundations for further exploration of the anti-cancer activity of Chinese Galla chinensis in cervical cancer.

In recent years, an increasing number of scientists have begun to study various naturally occurring antioxidants, which seems to be a feasible method to verify the development of traditional drugs. Although modern drug design prefers to use a single chemical entity with a specific molecular target, this can result in increased drug resistance or related side effects. In addition to the necessity to find affordable cervical cancer treatments for use in developing countries, there is an urgent need to develop affordable alternative therapies. In this context, Galla chinensis and polyphenol botanicals may therefore exhibit higher bioavailabilities and lower toxicities than individual molecular drug compounds; hence, whole plant extracts may prove to be more effective. Of course, this also requires additional experimental evidence, such as further in vivo experiments on animal models of ectopic tumor implants to better verify the anti-cancer activity potential of Chinese Galla chinensis against cervical cancer.

Conclusion

The focus of this study is to initially explore the role of Galla chinensis in HPV16 + cervical cancer SiHa cells. The results show that Galla chinensis effectively inhibits the expression of oncoprotein E6 and effectively inhibits cell proliferation and migration through p53-mediated apoptosis. The cell cycle stops in S phase. We found that compared with pterostilbene, Galla chinensis may be a more promising anti-cervical cancer drug. Based on these characteristics, Galla Chinensis may become a relatively economical but promising treatment for cervical cancer in the future. Our future research will include the role of Galla chinensis in other cervical cancer cell lines and signal transduction studies using HPV-infected murine tumor models to confirm these observations in vivo.

Declarations

Ethics approval and consent to participate

Consent for publication

Availability of data and materials

Applicable

Competing interests

No conflict of interest.

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Authors' Contributions

Z.X.X and M.X.P designed and performed most of the experiments, analyzed data, and prepared the manuscript as leading authors. W.Z., Y.W and D.H.W. contributed to editing and commented on the article. H.B. and R.Y. supervised the project.

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References

1. Isaguliants, M., Nosik, M., Karlsen, A., Petrakova, N., Enaeva, M., Lebedeva, N., Podchufarova, D., Laga, V., Gromov, K., Nazarov, A., Chowdhury, S., Sinitsyn, M., Sobkin, A., Chistyakova, N., Aleshina, S., Grabarnik, A., Palefsky, J.M. Prevalence and risk factors of infection with high risk human papilloma viruses among HIV-positive women with clinical manifestations of tuberculosis in a middle-income country. *Biomedicines* **2021,** *9*, 683.
2. Arbyn, M., Weiderpass, E., Bruni, L., de Sanjosé, S., Saraiya, M., Ferlay, J., Bray, F. Estimates of incidence and mortality of cervical cancer in 2018: A worldwide analysis. *Lancet Glob. Health* **2020,** *8*, e191–e203.
3. Wigle, J., Coast, E., Watson-Jones, D. Human papillomavirus (HPV) vaccine implementation in low and middle-income countries (LMICs): Health system experiences and prospects. *Vaccine* **2013,** *31*, 3811–3817.
4. Santin, A.D., Deng, W., Frumovitz, M., Buza, N., Bellone, S., Huh, W., Khleif, S., Lankes, H.A., Ratner, E.S., O'Cearbhaill, R.E., Jazaeri, A.A., Birrer, M. Phase II evaluation of nivolumab in the treatment of persistent or recurrent cervical cancer (NCT02257528/NRG-GY002). *Gynecol. Oncol.* **2020,** *157*, 161–166.

5. Howlader, N., Noone, A.M., Krapcho, M., Miller, D., Brest, A., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D.R., Chen, H.S., Feuer, E.J., Cronin, K.A. Eds., SEER Cancer Statistics Review, 1975–2016, National Cancer Institute: Bethesda, MD, 2019.
6. Giron, D., Huguet, E., Stone, G.N., Body, M. Insect-induced effects on plants and possible effectors used by galling and leaf-mining insects to manipulate their host-plant. J. *Insect Physiol.* **2016,** *84*, 70–89.
7. Sariozlu, N.Y., Kivanc, M. Chapter 60. Galla Chinensiss (Quercus infectoria Oliv. and Rhus chinensis Mill.) and their usage in health. In Nuts and Seeds in Health and Disease Prevention, V.R. Preedy, R.R. Watson, V.B. Patel, Eds., Academic Press: San Diego, 2011, pp. 505–511.
8. Ahn, Y.J., Lee, C.O., Kweon, J.H., Park, J.H. Growth-inhibitory effects of galla rhois-derived tannins on intestinal bacteria. *J. Appl. Microbiol.* **1998,** *84*, 439–443.
9. Ahn, Y.J., Kwon, J., Chae, S.H., Park, J.-H., Yoo, J.-Y. Growth-inhibitory responses of human intestinal bacteria to extracts of oriental medicinal plants. *Microb. Ecol. Health Dis.* **1994,** *7*, 257–261.
10. Faried, A., Kurnia, D., Faried, L.S., Usman, N., Miyazaki, T. Anticancer effects of gallic acid isolated from indonesian herbal medicine, Phaleria macrocarpa (Scheff.) Boerl, on human cancer cell lines. *Int. J. Oncol.* **2007,** *30*, 605–613.
11. Hsu, J.D., Kao, S.H., Ou, T.T., Chen, Y.-J., Li, Y.-J., Wang, C.-J. Gallic acid induces G2/M Phase arrest of breast cancer cell MCF-7 through stabilization of P27(Kip1) attributed to disruption of P27(Kip1)/Skp2 complex. *J. Agric. Food Chem.* **2011,** *59*, 1996–2003.
12. Djakpo, O., Yao, W. Rhus Chinensis and Galla Chinensis - Folklore to modern evidence: Review. *Phytother. Res.* **2010,** *24*, 1739–1747.
13. Yin, S.Y., Wei, W.C., Jian, F.Y., Yang, N.-S. Therapeutic applications of herbal medicines for cancer patients. Evid. Based Complement. *Alternat. Med.* **2013**, *2013*, 302426.
14. Riche, D.M., McEwen, C.L., Riche, K.D., Sherman, J.J., Wofford, M.R., Deschamp, D., Griswold, M. Analysis of safety from a human clinical trial with pterostilbene. *J. Toxicol.* **2013**, *2013*, 463595.
15. Liang, C.C., Park, A.Y., Guan. J.L. In Vitro Scratch Assay: A convenient and inexpensive method for analysis of cell migration In Vitro. *Nat. Protoc.* **2007,** *2*, 329–333.
16. Aumailley, L., Warren, A., Garand, C., Dubois, M.J., Paquet, E.R., Le Couteur, D.G., Marette, A., Cogger, V.C., Lebel, M. Vitamin C modulates the metabolic and cytokine profiles, alleviates hepatic endoplasmic reticulum stress, and increases the life span of Gulo−/− Mice. *Aging* **2016,** *8*, 458–483.
17. Ren, Y.Y., Zhang, X.R., Li, T.N., Zeng, Y.-J., Wang, J., Huang, Q.-W. Galla Chinensis, a traditional Chinese medicine: Comprehensive Review of botany, traditional uses, chemical composition, pharmacology and toxicology. *J. Ethnopharmacol*. **2021,** *278*, 114247.
18. Gao, J., Yang, X., Yin, W., Li, M. Galla Chinensiss: A potential treasure in anticancer drug discovery. *Evid. Based Complement. Alternat. Med.* **2018**, *2018*, 4930371.
19. Liu, Y., You, Y., Lu, J., Chen, X., Yang, Z. Recent advances in synthesis, bioactivity, and pharmacokinetics of pterostilbene, an important analog of resveratrol. *Molecules* **2020,** *25*, 5166.

20. Obrador, E., Salvador-Palmer, R., Jihad-Jebbar, A., López-Blanch, R., Dellinger, T.H., Dellinger, R.W., Estrela, J.M. Pterostilbene in cancer therapy. *Antioxidants* **2021,** *10*, 492.
21. Oudin, M.J., Weaver. V.M. Physical and chemical gradients in the tumor microenvironment regulate tumor cell invasion, migration, and metastasis. *Cold Spring Harb. Symp. Quant. Biol.* **2016,** *81*, 189–205.
22. Paul, C.D., Mistriotis, P., Konstantopoulos, K. Cancer cell motility: Lessons from migration in confined spaces. *Nat. Rev. Cancer* **2017,** *17*, 131–140.
23. Deiab, S., Mazzio, E., Eyunni, S., McTier, O., Mateeva, N., Elshami, F., Soliman, K.F.A. 1,2,3,4,6-Penta-O-galloylglucose within Galla Chinensis inhibits human LDH-A and attenuates cell proliferation in MDA-MB-231 breast cancer cells. *Evid. Based Complement. Alternat. Med.* **2015**, *2015*, 276946–276946.
24. Surien, O., Ghazali, A.R., Masre, S.F. Chemopreventive effects of pterostilbene through p53 and cell cycle in mouse lung of squamous cell carcinoma model. *Sci. Rep.* **2021,** *11*, 14862.
25. Narisawa-Saito, M., Kiyono. T. Basic Mechanisms of high-risk human papillomavirus-induced carcinogenesis: Roles of E6 and E7 proteins. *Cancer Sci.* **2007,** *98*, 1505–1511.
26. Yan, L., Huang, H., Zhang, Y., Yuan, X., Yan, Z., Cao, C., Luo, X. Involvement of p53-dependent apoptosis signal in antitumor effect of colchicine on human papilloma virus (HPV)-positive human cervical cancer cells. *Biosci. Rep.* **2020,** *40*, BSR20194065.

Supplementary

Table S1 is not available with this version.

Figures

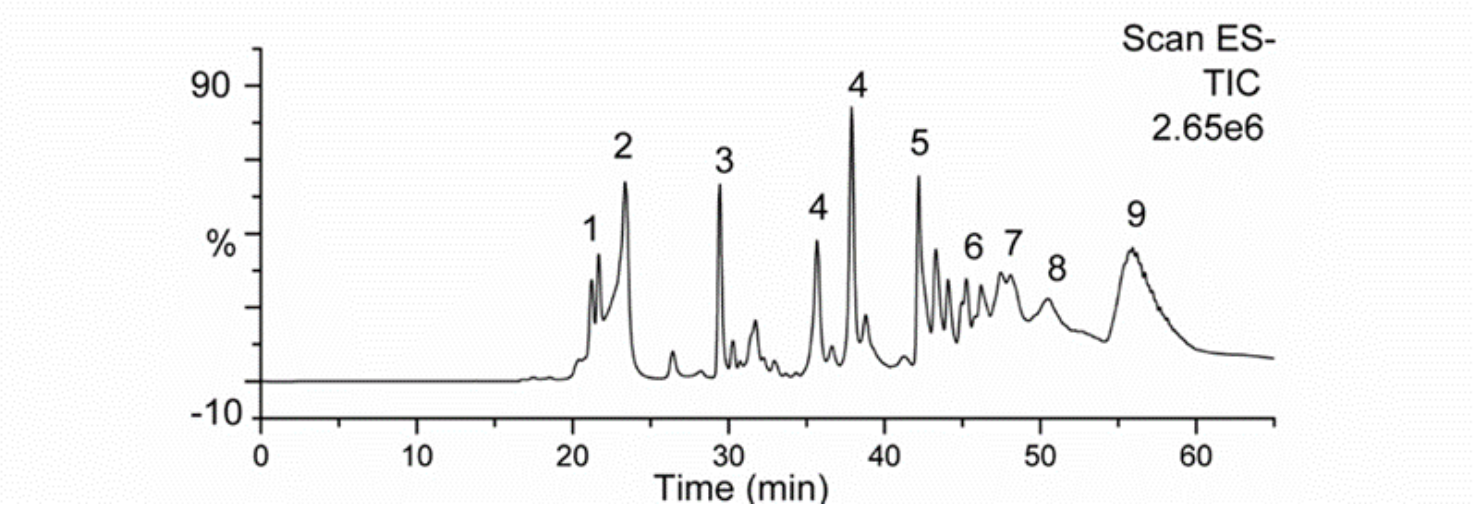

Figure 1

Liquid chromatogram of the water extract of Galla Chinensis

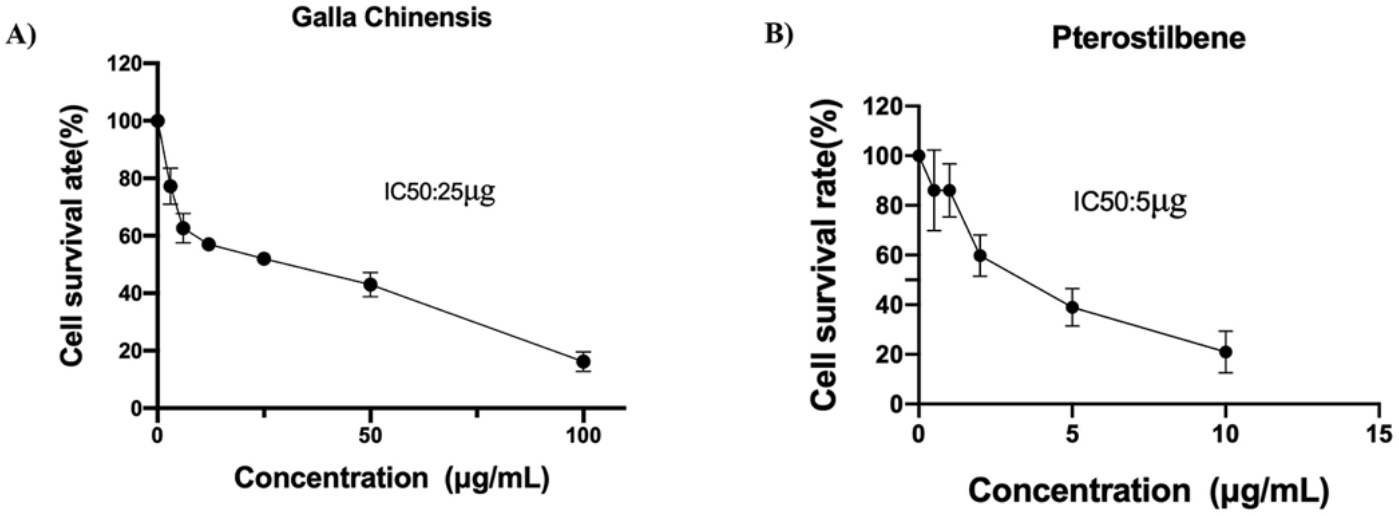

Figure 2

Analysis of IC50 values, generated by CCK-8 assay after 24 h of exposure to Galla Chinensis or pterostilbene indicates that Galla Chinensis (IC50=25μg) , pterostilbene(IC50=5μg).In order to unify the unit, we used the same concentration for subsequent experiments.The graphs represent data from three independent experiments (mean ± S.E.M. (Standard error mean)).

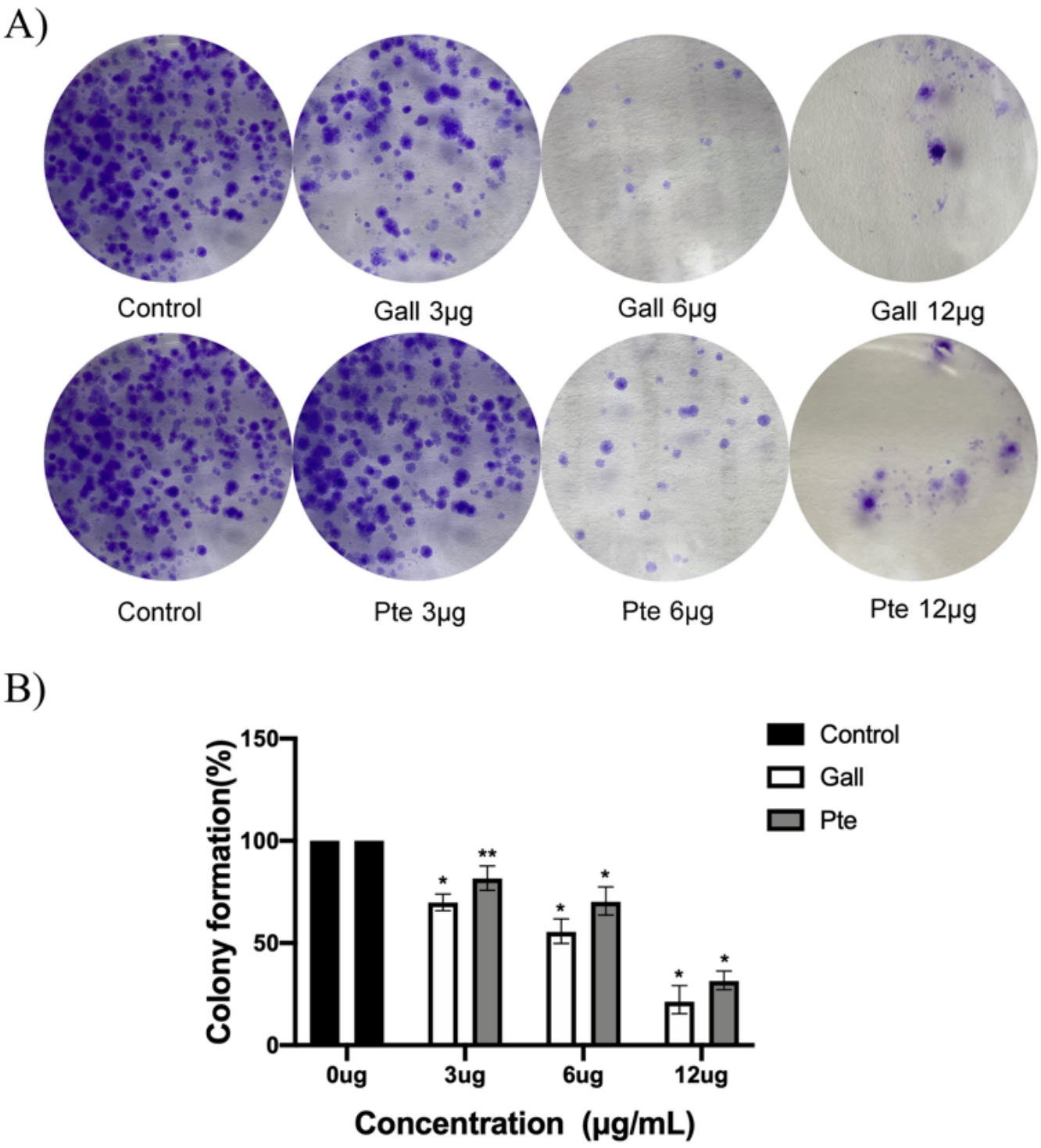

Figure 3

(A)Clonogenic assays performed to compare the relative effect of the two polyphenols on the clonogenicity of SiHa cells untreated or treated with 3μg/mL，6μg/mL，12μg/mL either Galla Chinensis or pterostilbene.(B)Results are from 15-days post-treatment and indicate that Galla Chinensis is more efficient in curbing the clonogenicity compared to Control.

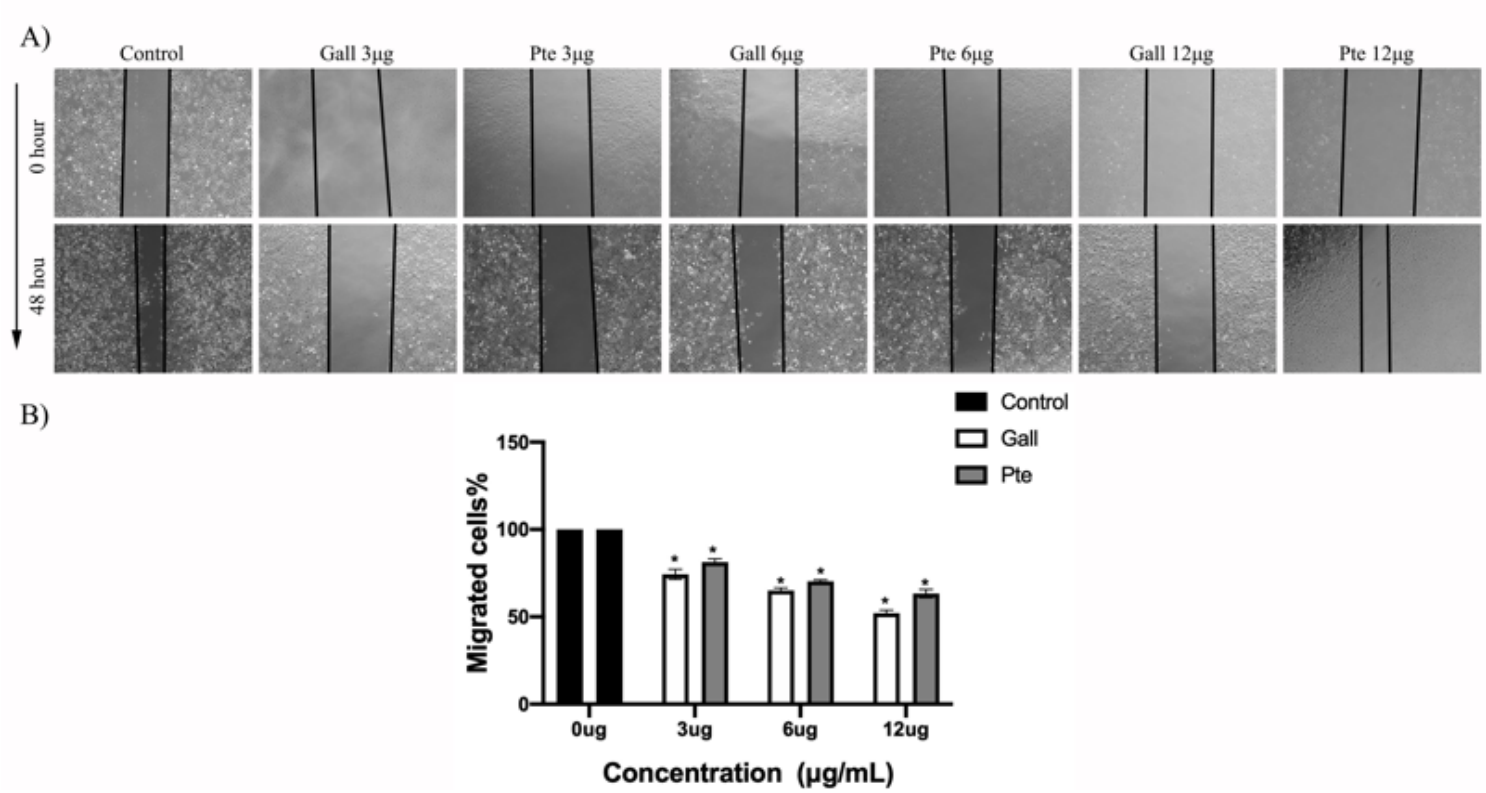

Figure 4

Galla Chinensis inhibits cell migration: (A) Scratch SiHa cells and monitor cells treated with sublethal concentrations of Galla Chinensis and Pterostilbene (3μg/mL, 6μg/mL, 12μg/mL). After 48 hours, the area where the cells migrated to the scratch was calculated, and the results showed that compared with Control, Galla Chinensis had stronger anti-migration ability.(B) The graphs represents data from triplicate sample experiments normalized to the control((mean % migrated cells in each phase ± S.E.M.*p0.05).

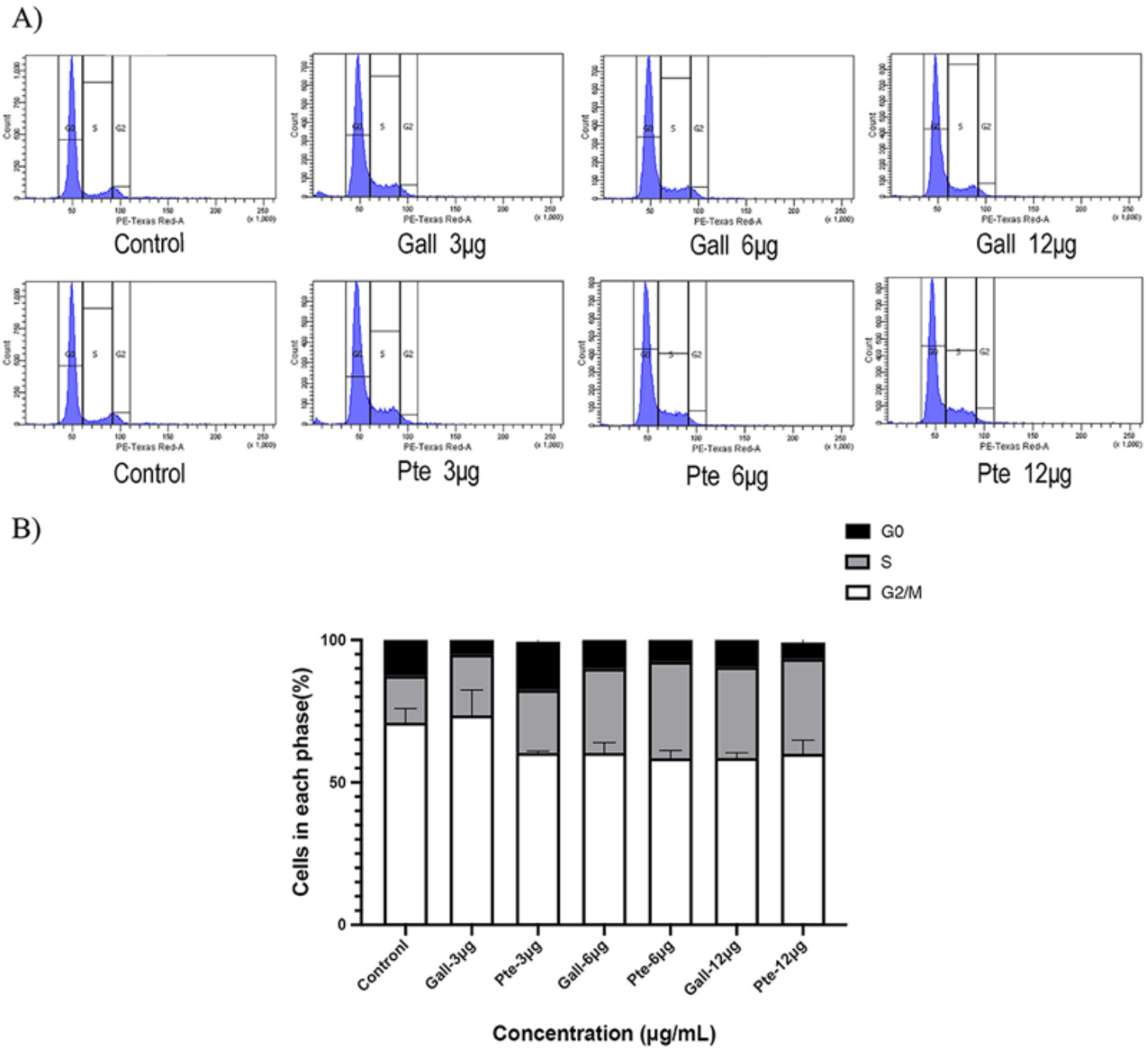

Figure 5

Effects of Galla Chinensis and pterostilbene on the cell cycle SiHa cells: (A) Flow-cytometric evaluation of SiHa cells untreated or treated with sub-lethal doses of Galla Chinensis (Gall) and pterostilbene (Pte) for 24h. Treated cells exhibited S-phase arrest and a subsequent decrease in the number of cells in G2/M. Pterostilbene was a more potent compound than resveratrol, showing a capacity to arrest cells at the S-phase at concentrations as low as 3µg/mL. (B) Graphical representation of the dose-dependent cell cycle effects induced by Galla Chinensis and pterostilbene at three different concentrations (3µg/mL, 6µg/mL, 12µg/mL). The graph represents data from triplicate sample experiments normalized to the control (mean % cells in each phase ± S.E.M.).

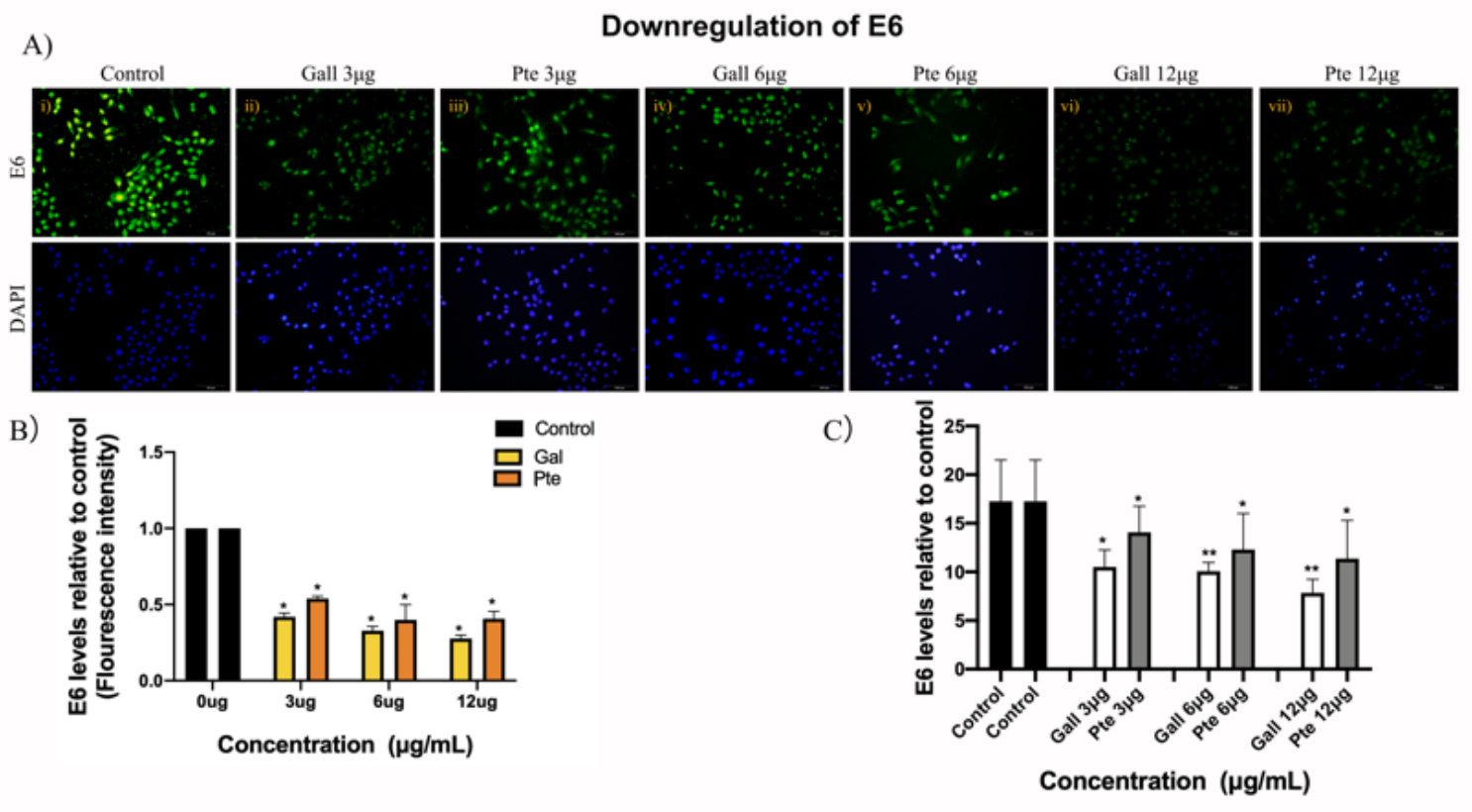

Figure 6

Downregulation of viral oncoprotein E6 in SiHa cells treated with Galla Chinensis or pterostilbene: (A) SiHa cells immunostained for E6 levels (green) and counterstained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI) (blue) after treatment with Galla Chinensis (Gall) and pterostilbene (Pte) (3µg/mL, 6µg/mL, 12µg/mL). Loss of E6 proteins are visually evident in cells treated with three concentrations of either Galla Chinensis or pterostilbene. (B) Cell image analysis of the E6 fluorescent data decrease of E6 protein levels in SiHa cells treated with Galla Chinensis both relative to the control. (C) Reverse transcription quantitative real-time PCR (RT-qPCR) analysis of oncoprotein E6 mRNA expression level:In SiHa cells treated with the two compounds for 24 hours, the oncoprotein E6 mRNA expression level decreased with the increase of the concentration(3µg/mL,6µg/mL, 12µg/mL).The graph represents data from experiments obtained from three independent experiments normalized to the control (mean % normalized to DAPI ± S.E.M., * $p < 0.05$)

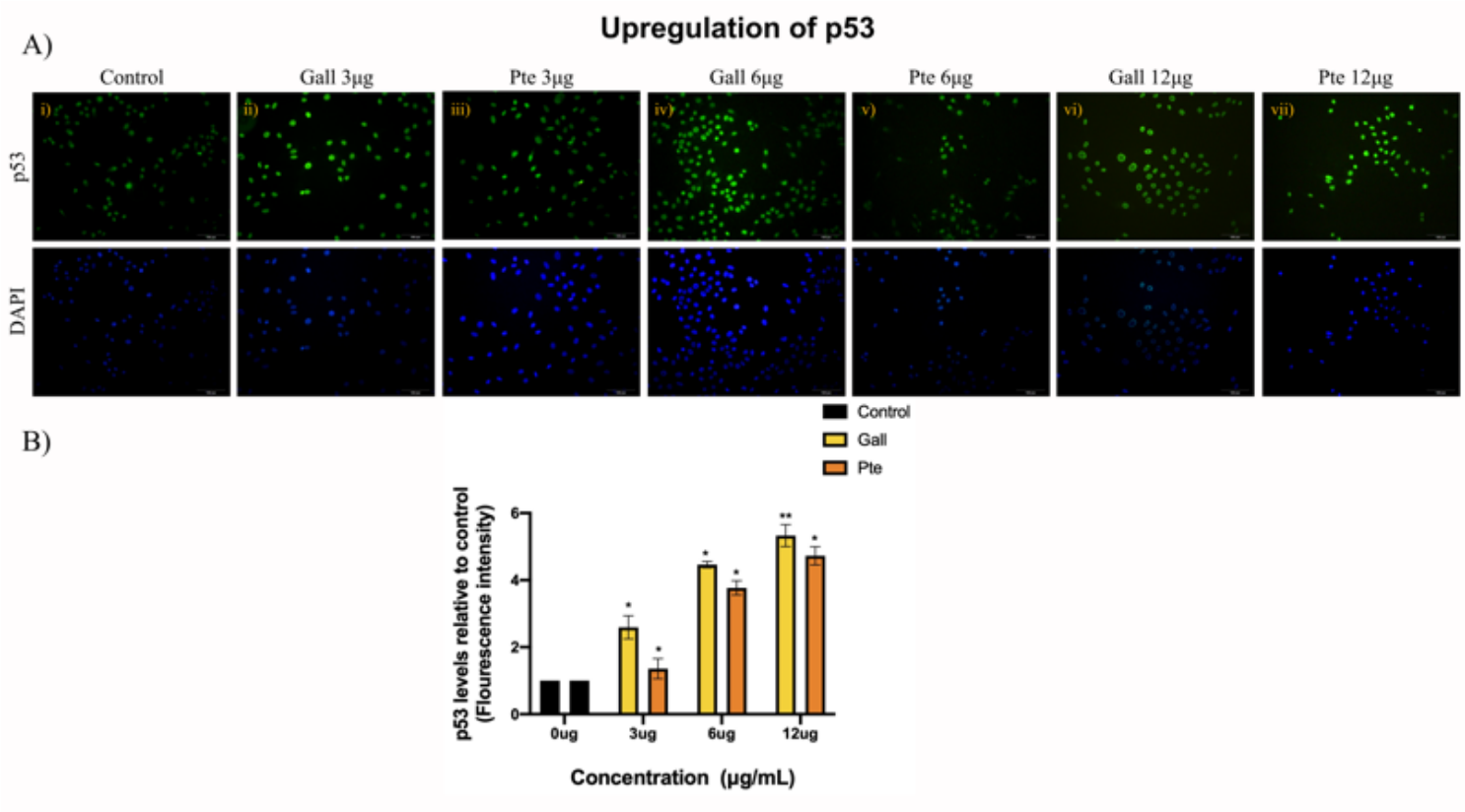

Figure 7

Upregulation of the tumor suppressor protein p53 in SiHa cells treated with Galla Chinensis and pterostilbene: (A) Immunoflourescent images of p53 protein (green) untreated or after treatment with 3µg/mL, 6µg/mL, 12µg/mL of either Galla Chinensis (Gall) or pterostilbene (Pte) for 24h. Levels of p53 are elevated in cells treated with either polyphenol. (B) Image analysis of p53 immunofluorescence indicates that Galla Chinensis (Gall) treatment at 12µg elicited a significant 5-fold increase in p53 compared with control. The graph represents data from experiments obtained from three independent experiments normalized to the control (mean % normalized to DAPI ± S.E.M. * $p < 0.05$).

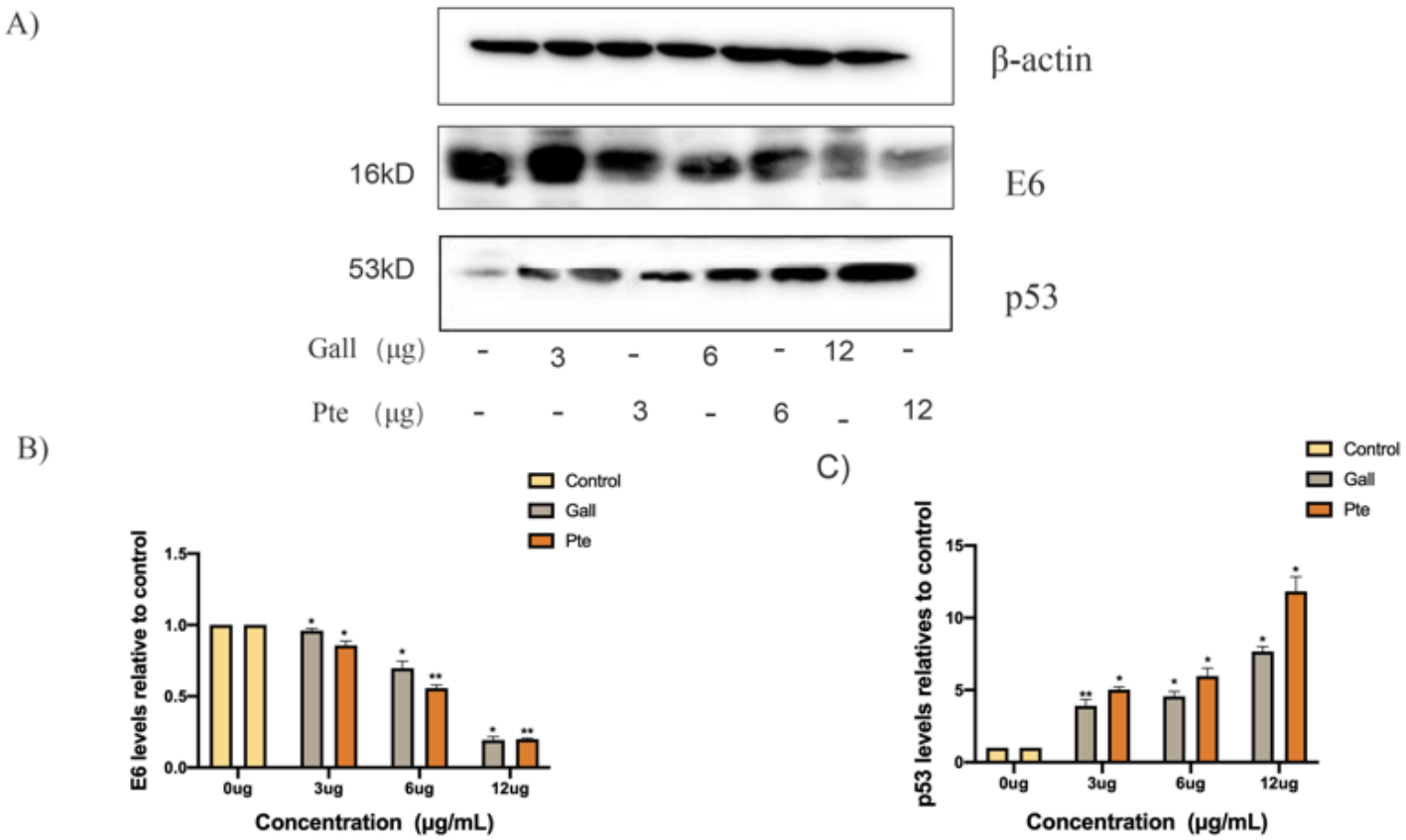

Figure 8

(A)Western blot analysis also revealed that downregulation of viral oncoprotein E6the and elevation of p53 protein levels is evident in SiHa cells treated with Galla Chinensis (Gall) or pterostilbene (Pte). (B,C) The graph represents data from experiments obtained from three independent experiments normalized to the control (mean % normalized to beta-actin ± S.E.M., *$p < 0.05$.)