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Original Article

Anti-proliferative, anti-migration, and anti-invasion activity of novel hesperidin glycosides in non-small cell lung cancer A549 cells

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Abstract

Background and purpose: Several attempts have been made to synthesize and investigate modified flavonoids to improve their potential anticancer efficacy. This study aimed to determine the *in vitro* anti-viability, anti-migration, and anti-invasive effects of two novel hesperidin glycosides, hesperidin glucoside (HG_1) and hesperidin maltoside (HG_2), compared to original hesperidin and diosmin.

Experimental approach: Inhibitory effects on normal (MRC5) and cancer (A549) cell viability of hesperidin glycosides were investigated by the trypan blue and MTS assays. A scratch assay determined the suppressive effects on cancer cell migration, and inhibition of cancer cell invasion was investigated through Matrigel™. The selectivity index (SI), a marker of cell toxicity, was also determined for A549 relative to MRC5 cells.

Findings/Results: The cell viability trypan blue and MTS assays showed similar results of the inhibition of A549 cancer cells; HG_1 and HG_2 had lower IC_{50} than original hesperidin and diosmin. The SI of HG_1 and HG_2 was > 2 after 72-h culture. Investigation of cell migration showed that HG_1 and HG_2 inhibited the ability of gap closure in a time- and dose-dependent manner. The infiltration of the Matrigel™-coated filter by A549 cells was suppressed in the presence of HG_1 and HG_2. This result implied that HG_1 and HG_2 could inhibit cancer cell invasion.

Conclusion and implication: Our results suggest the inhibition of cancer cell migration and invasion in a time- and concentration-related manner with a favorable toxic profile. Moreover, HG_1 and HG_2 appeared potentially better agents than the original hesperidin for future anticancer development.

Keywords: Anticancer; Anti-invasion; Anti-migration; Anti-proliferation; Hesperidin glycosides.

INTRODUCTION

Cancer is one of the major public health problems in many countries around the world (1). Different environmental and genetic factors, such as oxidative stress, diet, radiation, smoking, etc. cause cancers in the human body. The meta-analyses showed a relationship between smoking with lung cancer risk, clearly seen for ever-smoking, current smoking, and even ex-smoking. It was stronger for squamous than adenocarcinoma and evident in both sexes (2). However, other factors such as asbestos, radon gas, air pollution exposure, and infections can participate in lung carcinogenesis (3). These factors play a crucial role in the pathophysiology of cancer (4).

There are several types of lung cancer therapies such as chemotherapy, radiation, surgery, and targeted therapy (3). The main treatment for early-stage disease is surgery which offers the best choice for long-term survival (5). Currently, various studies were established to develop new anticancer drugs. Among the new drugs, natural substances have been widely focused on (6). Flavonoids are naturally occurring phenolic compounds found in vegetables, plants, fruits, bark, and tea.

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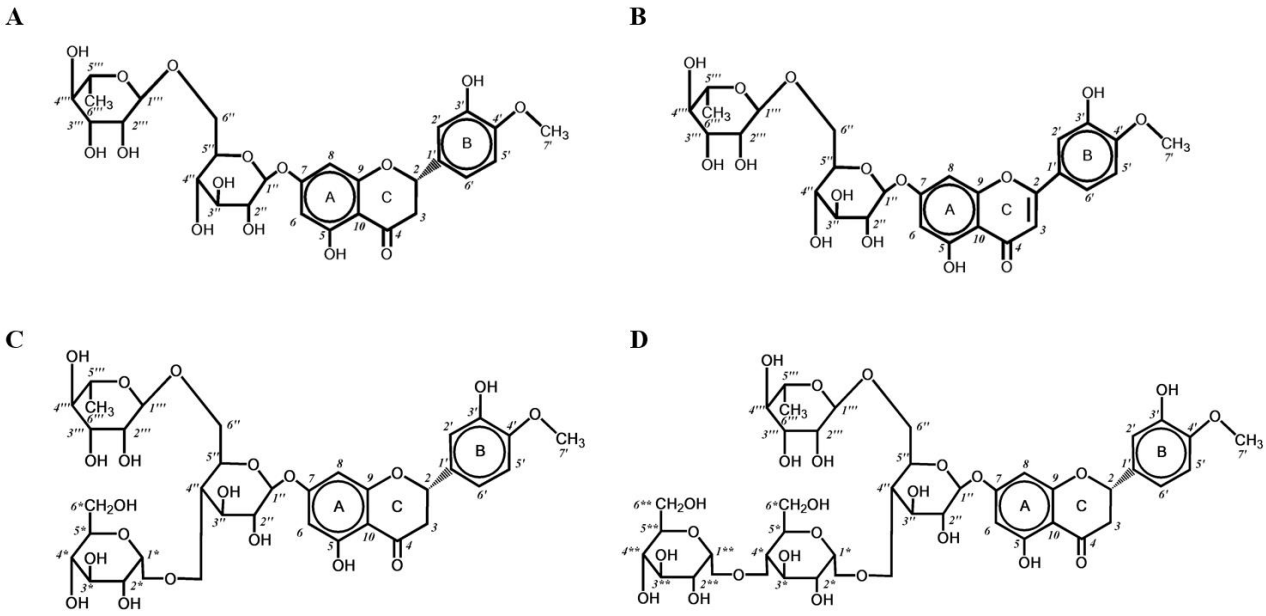

Fig. 1. Chemical structures of (A) hesperidin, (B) diosmin, (C) hesperidin glucoside, and (D) hesperidin maltoside. Modified from Chaisin *et al.* (18).

They are thought to act as an anticancer, pro-apoptotic, and anti-proliferative effect in various cancer cell types (7) and some studies have sought to re-design and re-synthesize the flavonoid to increase some of these properties. A hydroxyl group substitution at the C-3 position on ring C and methylation substitution of free hydroxyls and 4-C=S are associated with the antiproliferative properties of flavonoids (8). Several flavonoids such as apigenin, anthocyanin, and quercetin have been reported to reduce cervical cancer cell viability and suppress cell metastasis and angiogenesis (9). Moreover, luteolin, one of the most prevalent flavonoids, was able to down-regulate the AKT signaling pathway and decrease the proliferation and migration of vascular smooth muscle cells (10).

Hesperidin is a natural flavanone glycoside. The hesperidin structure includes an aglycone unit, hesperetin, and a disaccharide, rutinose. Hesperidin has been demonstrated to suppress the viability of HeLa cells in a dose- and time-related manner and apoptosis in HeLa cells could be motivated by hesperidin *via* the acceleration of nuclear condensation and DNA fragmentation (11). In lung cancer studies, hesperidin induced apoptosis and suppressed the metastasis of cancer cells (12,13).

Our previous study on acceptor specificity (14) found that, among several flavonoids, hesperidin was the best acceptor for the enzymatic synthesis of new flavonoid glycosides such as hesperidin glycosides (HGs), hesperidin glucoside (HG_1) and hesperidin maltoside (HG_2) from p19bBC recombinant cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19). The basic properties and structures of both HG_1 and HG_2 were identified together with the related-structural compounds, hesperidin (Hes) and diosmin (Fig. 1) (15-18). So, the purpose of this work is to extend the knowledge of the HGs in the disease treatment of cancer by investigating the *in vitro* anti-proliferation, anti-migration, and anti-invasion properties of HG_1 and HG_2.

MATERIALS AND METHODS

Chemicals

Hesperidin and diosmin were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). HG_1 and HG_2 were previously synthesized by cyclodextrin glycosyltransferase (CGTase, E.C 2.4.1.19) and their molecular structures were determined (18). Dimethyl sulfoxide (DMSO) and cis-diamminedichloroplatinum II (cisplatin, DDP) were obtained from Sigma-Aldrich (USA). Fetal bovine serum (FBS) was from Himedia (India); Eagle's minimum essential medium (EMEM) and Ham's F-12 (Kaighn's modification) were purchased from Cassion (USA). Basement membrane matrix Matrigel®

was from Corning Life Sciences (USA) and cellTiter 96® AQ_{ueous} one solution (MTS) was from Promega (USA). All other chemicals used were of analytical grade from Sigma-Aldrich (USA). Hesperidin, diosmin, and HGs were dissolved in DMSO.

Cell lines, culture conditions, and experimental groups

The MRC-5 (human lung fibroblast) and A549 (human lung carcinoma) cells were purchased from the American Type Culture Collection (ATCC, USA). The MRC-5 cells were cultured in EMEM supplemented with 1% penicillin/streptomycin and 10% FBS at 37 ℃ in 5% CO_2. The A549 cells were cultured in F-12K medium supplemented with 1% penicillin/streptomycin and 10% FBS at 37 ℃ in 5% CO_2.

In the present study, 6-main experimental groups were established as follows: control group containing 0.5% DMSO (the solvent of compounds); hesperidin groups (50, 100, and 150 µg/mL hesperidin); diosmin groups (50, 100, and 150 µg/mL diosmin), representing semi-synthetic hesperidin, and also diosmin has been reported to be a potential role in human diseases but only in a few lung cancer studies (19); HG_1 and HG_2 groups (50, 100, and 150 µg/mL). A chemotherapy medication DDP group (0.5, 1, and 2 µg/mL) was considered the positive control. The half maximal inhibitory concentration (IC_{50}) values of hesperidin (13) serve as a guide to decide the range of the concentration to be used in trypan blue and MTS assay for determining cell viability.

Determination of cell viability

Trypan blue assay

The MRC-5 and A549 cells were seeded at 5,000 cells/well on 96-well plates for 24 h and then treated with various treatments as mentioned before. The cells were cultured at 37 ℃ and 5% CO_2 for 24-72 h and cell growth was investigated at each time point. Cell viability was investigated by trypan blue dye exclusion assay. After trypsinization, quadruplicate wells of viable cells for each experimental group were counted on a hemocytometer. The growth curves were plotted, and the experiments were repeated at least three times. The concentration at which cell proliferation was inhibited by 50% (IC_{50} value) was determined (GraphPad Prism 5.0, GraphPad Software, Inc., San Diego, CA, USA). In addition, the selectivity index (SI), indicating the safety of HGs for anticancer therapy was evaluated by obtaining the ratio of IC_{50} for the non-cancer cell line to IC_{50} for the cancer cell line (20).

MTS assay

The MRC-5 and A549 cells were seeded at 5,000 cells/well on 96-well plates for 24 h and then treated with various treatments as mentioned before. The cells were cultured at 37 ℃ and 5% CO_2 for 24, 48, and 72 h. After reaching each time point, MTS was added to each well. Then, the 96-well plates were incubated in a dark place at 37 ℃ and 5% CO_2 for 1 h and the absorbance was measured as the optical density at 490 nm using a microplate reader (Thermo Scientific, Multiskan GO, USA). The experiment was repeated three times. The cell viability was calculated using equation (1). Then, the IC_{50} value and SI were calculated as described previously.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \qquad (1)$$

Determination of cell migration

The effects of hesperidin, diosmin, and HG_1 and HG_2 on cell migration were studied using the scratch assay. This method determines the movement of cells to close the gap between the scratch wound. The A549 cells were cultured in a 96-well plate to reach 90-100% confluence within 24 h. Then, a scratch was made on the cultured monolayer cells with a pipette tip and the scraped cells were cleaned with phosphate-buffered saline (PBS). The gap size of the wound was measured under the microscope (magnification ×400) as the width at the beginning of the scratch. Thereafter, the cells were further cultured in serum-free media containing 0-150 µg/mL of hesperidin, HGs, diosmin, and 1 µg/mL of DDP. The cells were incubated for 24-72 h at 37 ℃ in the 5% CO_2 incubator. The migration was observed using a phase-contrast microscope (magnification ×400) that also measured the width of the gaps at each time point. The experiment was repeated three times. The cell migration was determined by calculating % of wound closure from equation (2).

$$\text{Gap closure (\%)} = \frac{\text{Width at the beginning - width at a certain time}}{\text{Width at the beginning}} \times 100 \quad (2)$$

Determination of cell invasion

The Matrigel invasion assay was applied to determine the invasive capacity of the cells through Matrigel™, which acts as an extracellular matrix. Briefly, Matrigel™ was thawed, liquefied on ice, and then diluted with cold serum-free media. Then, Matrigel™ was added to a 96-Transwell® upper chamber and remained in a 37 ℃ incubator overnight to form a thin-layered gel. The A549 cells were suspended in serum-free media containing 0-150 µg/mL of hesperidin, diosmin, HG_1, and HG_2 and 1 µg/mL of DDP that was added to the Transwell® upper chamber. After that, 10% FBS (chemoattractant) was filled to the bottom of the lower chamber of the Transwell® plate. The cells were incubated at 37 ℃ in CO_2 for 24 and 48 h. After incubation, the media and remaining cells in the Transwell® upper chamber were cautiously removed and the Transwell® upper chamber was washed twice with PBS. The invasive cells attached to the Transwell® upper chamber were fixed on 3.7% paraformaldehyde and absolute methanol for 30 min, respectively. Then, the cells were stained with 50 µL of 1% crystal violet solution (Yd Diagnostics, Korea) for 15 min at room temperature and washed with PBS four times to eliminate the excess crystal violet dye. After that, the blue invasive cells on the Transwell® upper chamber were dried and counted under a microscope (magnification ×400) to enumerate the number of stained cells in five fields (21). The experiment was repeated three times. The cell invasion was determined by calculating % of invasion from equation (3).

$$\text{Invasion (\%)} = \frac{A_{NU} - A_{NM}}{A_{NU}} \times 100 \quad (3)$$

Where A_{NM} is the mean number of cells invading through the Matrigel™ matrix-coated permeable support membrane and A_{NU} is the mean number of cells migrating through the uncoated permeable support membrane.

Statistical analyses

Data were shown as mean ± SD from three independent experiments. Statistical analysis was accomplished using a one-way analysis of variance (ANOVA) test followed by a post hoc Tukey test with the IBM SPSS Statistic version 26.0 (SPSS Corporation, Chicago, IL, USA). *P*-values ≤ 0.05 were considered significantly different.

RESULTS

The viability of MRC-5 and A549 cells

Inhibitory effects investigated by trypan blue exclusion assay

The cell survivability and suppression ratio in the 0.5% DMSO-treated cells were not markedly different from non-DMSO-treated cells (data were not shown). This implied that 0.5% DMSO, as the solvent of hesperidin, diosmin, HG_1, and HG_2, did not affect the viability of MRC-5 and A549 cells. Thus, the 0.5% DMSO-treated group was used as a control throughout the study. Hesperidin, diosmin, and HG_1 inhibited MRC-5 cells with an IC_{50} value of > 150 µg/mL for 72 h, while HG_2 inhibited MRC-5 cells with an IC_{50} value of 139.67 ± 3.18 µg/mL for 72 h. In contrast, the positive control (DDP) showed a good cell survival rate in MRC-5 cells at all concentrations. The IC_{50} values at 72 h of hesperidin, diosmin, HG_1, HG_2, and DDP treatment of A549 cells were 92.90 ± 4.53, 97.66 ± 4.23, 88.85 ± 5.48, 87.35 ± 5.73, and 0.42 ± 0.04 µg/mL, respectively. It was evident that HG_1 and HG_2 had higher inhibitory activity than the original hesperidin. In addition, it was demonstrated that the cell proliferation following treatment with 150 µg/mL of hesperidin, diosmin, HG_1, and HG_2 at 24, 48, and 72 h was significantly reduced compared to the control in a time-dependent manner. In addition, the decreased cell proliferations were obtained with treatments at concentrations of 50, 100 and 150 µg/mL (Tables 1 and 2). So, it concluded that MRC-5 and A549 cell proliferation was reduced compared to the control cells in concentration-proportional manner. Furthermore, although the cell number increased with the treatments at different intervals, 24, 48, and 72 h, the MRC-5 and A549 cell proliferation rates were slowed down from time to time compared with those of the control. Moreover, the HG_2 treatment showed a selectivity index > 1.6, whereas the selectivity index of DDP at 72-h treatment was > 4. This implies that HGs have greater cytotoxic effects on normal cells compared to DDP.

Table 1. The effect of hesperidin, diosmin, HG_1, HG_2, and DDP on the number of MRC-5 cells. The data are expressed as the mean ± SD, n ≥ 3.

Sample	Concentration (μg/mL)	Number of cells			P_{period}	$P_{concentration}$
		24 h	48 h	72 h		
Control	0	14,166 ± 466	16,666 ± 365	22,916 ± 678		
Hesperidin	50	12,916 ± 498[a]	14,583 ± 472[b]	20,833 ± 404[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	10,833 ± 473[a,b]	13,333 ± 498	19,166 ± 523[a,b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	6,666 ± 662[a,b]	11,250 ± 514[a,b]	17,916 ± 508[a,b]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} \leq 0.001$
Diosmin	50	11,666 ± 387[a,b]	14,583 ± 489[b]	19,583 ± 405[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	9,583 ± 463[a,b]	12,083 ± 723[a]	17,500 ± 456[a,b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	6,666 ± 709[a,b]	10,000 ± 630[a,b]	15,833 ± 544[a,b]	$P_{c150,\ t24,48,72} = 0.002$	$P_{72} \leq 0.001$
HG_1	50	10,416 ± 598[a,b]	13,333 ± 450	16,250 ± 602[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	8,333 ± 523[a,b]	10,000 ± 668[a,b]	14,583 ± 582[b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	6,250 ± 846[a,b]	8,333 ± 712[a,b]	13,333 ± 608	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} = 0.003$
HG_2	50	9,583 ± 633[a,b]	12,083 ± 604[a]	15,000 ± 582[b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	7,500 ± 690[a,b]	9,583 ± 757[a,b]	13,333 ± 814	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	5,000 ± 852[a,b]	7,083 ± 809[a,b]	10,833 ± 826[a]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} \leq 0.001$
DDP	0.5	13,900 ± 302[b]	15,666 ± 418[a,b]	21,100 ± 488[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} = 0.051$
	1	13,516 ± 414	15,400 ± 347[a,b]	20,500 ± 465[a,b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} = 0.110$
	2	12,933 ± 396[a]	14,933 ± 292[b]	19,666 ± 389[a,b]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} = 0.022$

HG, Hesperidin glucoside; DDP, diamminedichloroplatinum; [a]$P < 0.05$ indicates significant differences compared with the data of control after 24-h treatment; [b]$P < 0.05$ versus 50 μg/mL hesperidin after 24-h treatment.

Table 2. The effect of hesperidin, diosmin, HG_1, HG_2, and DDP on the number of A549 cells. The data are expressed as the mean ± SD, n ≥ 3.

Sample	Concentration (μg/mL)	Number of cells			P_{period}	$P_{concentration}$
		24 h	48 h	72 h		
Control	0	17,500 ± 465	22,500 ± 532	38,333 ± 616		
Hesperidin	50	15,000 ± 505[a]	18,333 ± 522[b]	30,416 ± 706[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	10,833 ± 438[a,b]	13,333 ± 630[a,b]	17,500 ± 618[b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	7,500 ± 366[a,b]	9,166 ± 414[a,b]	12,083 ± 409[a,b]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} \leq 0.001$
Diosmin	50	13,333 ± 444[a,b]	18,333 ± 510[b]	26,250 ± 392[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	10,833 ± 487[a,b]	13,750 ± 538[a,b]	18,750 ± 426[a,b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	6,666 ± 539[a,b]	8,750 ± 572[a,b]	11,250 ± 559[a,b]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} \leq 0.001$
HG_1	50	13,333 ± 389[a,b]	17,083 ± 394[b]	27,500 ± 412[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	10,833 ± 493[a,b]	13,333 ± 468[a,b]	17,083 ± 334[b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	6,250 ± 711[a,b]	7,916 ± 678[a,b]	10,416 ± 702[a,b]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} \leq 0.001$
HG_2	50	13,750 ± 495[a,b]	16,666 ± 503[b]	27,916 ± 659[a,b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	100	10,416 ± 589[a,b]	11,666 ± 414[a,b]	16,250 ± 712	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	150	4,583 ± 792[a,b]	6,250 ± 805[a,b]	6,666 ± 780[a,b]	$P_{c150,\ t24,48,72} \leq 0.001$	$P_{72} \leq 0.001$
DDP	0.5	14,000 ± 310[a,b]	14,050 ± 523[a,b]	16,666 ± 420[b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} \leq 0.001$
	1	11,250 ± 545[a,b]	11,616 ± 590[a,b]	12,833 ± 564[a,b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} \leq 0.001$
	2	8,583 ± 589[a,b]	9,833 ± 650[a,b]	8,983 ± 310[a,b]	$P_{c150,\ t24,48,72} = 0.036$	$P_{72} \leq 0.001$

HG, Hesperidin glucoside; DDP, diamminedichloroplatinum; [a]$P < 0.05$ indicates significant differences compared with the data of control after 24-h treatment [b]$P < 0.05$ versus 50 μg/mL hesperidin after 24-h treatment.

Inhibitory effects investigated by MTS assay

The results at 72-h showed that hesperidin, diosmin, HG_1, and HG_2 have the potential to inhibit cell MRC-5 with IC_{50} values of 137.58 ± 5.39, 149.69 ± 6.32, 149.44 ± 5.48, and 143.32 ± 5.53 μg/mL, respectively, compared to DDP of > 2 μg/mL. Expectedly, the DPP positive control showed a good cell survival rate in MRC-5 cells at every tested concentration and every time point of treatment. On the other hand, the IC_{50} values of hesperidin, diosmin, HG_1, HG_2, and DDP treatment of A549 cells were 58.66 ± 3.02, 106.31 ± 3.52, 54.57 ± 7.08, 49.44 ± 6.28, and 0.63 ± 0.03μg/mL, respectively. It was evident that HG_1 had higher anti-proliferative properties than the original hesperidin, especially at 24- and 48-h treatment. This MTS result was in concordance with the trypan blue exclusion assay. Although the lowest inhibitory effect was changed from HG_2 in the trypan blue assay to HG_1 in the MTS assay, it was not a significant difference between HG_1 and HG_2 at every time point. Moreover, HG_2 treatment

showed a selectivity index of 2.90 toward this cell line relative to the MRC-5 cell line, whereas the selectivity index of DDP at 72-h was > 3. In addition, it was shown that the cell proliferation in the treatment of hesperidin, diosmin, HG_1, and HG_2 at 24, 48, and 72 h was a significant reduction in a concentration-related manner (Tables 3 and 4).

Inhibitory effects on migration of lung cancer cells

In the scratch assay, hesperidin, diosmin, HG_1, and HG_2 showed anti-migration activity of A549 cells as shown in the relatively wider wound gaps than that of the control in Fig. 2A and B. The control group exhibited signs of cell migration resulting in a greater percentage of gap closure in control cells *vs*. the hesperidin-treated cells, as shown in Table 5. The highest percentage of gap closure at 24 h was shown in control at 13.23% while the lowest percentage of gap closure was shown in 150 µg/mL-treated HG_2 at 2.44% So, 24 h after scratching, the 150 µg/mL HG_2-treated cells showed slower migration than control cells.

Table 3. The effect of hesperidin, diosmin, HG_1, HG_2, and DDP on the viability of MRC-5 cells. The data are expressed as the mean ± SD, n ≥ 3.

Sample	Concentration (µg/mL)	Cell viability (%)			P_{period}	$P_{concentration}$
		24 h	48 h	72 h		
Control	0	100 ± 0.00	110.46 ± 1.21	103.29 ± 0.93		
Hesperidin	50	88.70 ± 5.30[a]	85.83 ± 2.69 [a]	89.38 ± 6.40[a,]	$P_{c50, t24,48,72}$ = 0.229	$P_{24} \leq 0.001$
	100	54.82 ± 9.82[a,b]	62.20 ± 4.84[a,b]	67.65 ± 3.36[a,b]	$P_{c100, t24,48,72}$ = 0.081	$P_{48} \leq 0.001$
	150	40.70 ± 8.00[a,b]	43.36 ± 3.36[a,b]	44.26 ± 6.40[a,b]	$P_{c150, t24,48,72}$ = 0.307	$P_{72} \leq 0.001$
Diosmin	50	80.87 ± 8.46[a]	77.99 ± 9.30[a]	86.74 ± 1.38[a]	$P_{c50, t24,48,72}$ = 0.218	P_{24} = 0.002
	100	54.73 ± 7.45[a,b]	53.87 ± 9.19[a,b]	70.97 ± 9.54[a,b]	$P_{c100, t24,48,72}$ = 0.004	P_{48} = 0.006
	150	44.91 ± 5.58[a,b]	47.45 ± 2.05[a,b]	49.86 ± 8.02[a,b]	$P_{c150, t24,48,72}$ = 0.289	P_{72} = 0.002
HG_1	50	75.64 ± 9.45[a]	77.13 ± 1.74[a,b]	72.09 ± 5.39[a,b]	$P_{c50, t24,48,72}$ = 0.365	P_{24} = 0.003
	100	55.22 ± 7.69[a,b]	55.44 ± 2.76[a,b]	62.23 ± 9.78[a,b]	$P_{c100, t24,48,72}$ = 0.196	P_{48} = 0.001
	150	35.51 ± 7.60[a,b]	48.35 ± 8.56[a,b]	49.84 ± 1.27[a,b]	$P_{c150, t24,48,72}$ = 0.075	P_{72} = 0.016
HG_2	50	68.05 ± 9.20[a,b]	68.39 ± 7.87[a,b]	68.78 ± 4.63[a,b]	$P_{c50, t24,48,72}$ = 0.813	P_{24} = 0.013
	100	50.98 ± 6.11[a,b]	51.57 ± 3.03[a,b]	63.19 ± 4.81[a,b]	$P_{c100, t24,48,72}$ = 0.016	P_{48} = 0.002
	150	43.27 ± 4.97[a,b]	43.05 ± 2.47[a,b]	47.29 ± 7.14[a,b]	$P_{c150, t24,48,72}$ = 0.568	P_{72} = 0.008
DDP	0.5	98.57 ± 0.77[a,b]	95.23 ± 1.52[a]	93.06 ± 0.91[a]	$P_{c50, t24,48,72}$ = 0.007	P_{24} = 0.002
	1	96.34 ± 0.81[a]	92.63 ± 0.83[a]	91.83 ± 1.22[a]	$P_{c100, t24,48,72}$ = 0.003	P_{48} = 0.005
	2	93.76 ± 1.07[a]	90.59 ± 0.47[a]	89.79 ± 1.07[a]	$P_{c150, t24,48,72}$ = 0.009	P_{72} = 0.026

HG, Hesperidin glucoside; DDP, diamminedichloroplatinum; [a]$P < 0.05$ indicates significant differences compared with the data of control after 24-h treatment; [b]$P < 0.05$ versus 50 µg/mL hesperidin after 24-h treatment.

Table 4. The effect of hesperidin, diosmin, HG_1, HG_2, and DDP on the viability of A549 cells. The data are expressed as the mean ± SD, n ≥ 3.

Sample	Concentration (µg/mL)	Cell viability (%)			P_{period}	$P_{concentration}$
		24 h	48 h	72 h		
Control	0	100 ± 0.00	124.87 ± 6.72	136.64 ± 8.40		
Hesperidin	50	86.42 ± 3.14[a]	60.94 ± 5.53[a,b]	53.35 ± 2.14[a,b]	$P_{c50, t24,48,72}$ = 0.024	P_{24} = 0.001
	100	60.90 ± 7.39[a,b]	52.10 ± 1.19[a,b]	47.15 ± 3.07[a,b]	$P_{c100, t24,48,72}$ = 0.063	P_{48} = 0.017
	150	49.88 ± 7.83[a,b]	47.25 ± 4.31[a,b]	37.60 ± 3.85[a,b]	$P_{c150, t24,48,72}$ = 0.245	P_{72} = 0.002
Diosmin	50	72.32 ± 3.23[a,b]	71.08 ± 1.37[a,b]	54.89 ± 3.43[a,b]	$P_{c50, t24,48,72}$ = 0.025	$P_{24} \leq 0.001$
	100	61.65 ± 4.07[a,b]	57.62 ± 5.15[a,b]	50.74 ± 1.19[a,b]	$P_{c100, t24,48,72}$ = 0.106	P_{48} = 0.003
	150	45.59 ± 4.59[a,b]	47.32 ± 6.78[a,b]	35.61 ± 5.95[a,b]	$P_{c150, t24,48,72}$ = 0.258	P_{72} = 0.003
HG_1	50	63.86 ± 4.28[a,b]	50.87 ± 8.91[a,b]	52.13 ± 9.28[a,b]	$P_{c50, t24,48,72}$ = 0.249	$P_{24} \leq 0.001$
	100	47.46 ± 3.95[a,b]	42.96 ± 5.73[a,b]	44.11 ± 6.70[a,b]	$P_{c100, t24,48,72}$ = 0.101	P_{48} = 0.039
	150	31.90 ± 2.34[a,b]	31.22 ± 6.12[a,b]	32.59 ± 5.27[a,b]	$P_{c150, t24,48,72}$ = 0.823	P_{72} = 0.044
HG_2	50	74.78 ± 6.22[a,b]	57.74 ± 1.84[a,b]	49.65 ± 5.07[a,b]	$P_{c50, t24,48,72}$ = 0.062	P_{24} = 0.005
	100	58.58 ± 5.53[a,b]	49.51 ± 3.97[a,b]	37.79 ±7.11[a,b]	$P_{c100, t24,48,72}$ = 0.095	P_{48} = 0.002
	150	49.02 ± 5.84[a,b]	36.65 ± 5.80[a,b]	32.19 ± 5.62[a,b]	$P_{c150, t24,48,72}$ = 0.150	P_{72} = 0.032
DDP	0.5	83.59 ± 2.30[a]	72.06 ± 1.82[a,b]	58.74 ± 2.28[a,b]	$P_{c50, t24,48,72}$ = 0.013	$P_{24} \leq 0.001$
	1	60.93 ± 3.57[a,b]	43.73 ± 2.78[a,b]	32.21 ± 1.95[a,b]	$P_{c100, t24,48,72}$ = 0.014	$P_{48} \leq 0.001$
	2	46.86 ± 2.31[a,b]	30.31 ± 3.09[a,b]	20.61 ± 1.64[a,b]	$P_{c150, t24,48,72}$ = 0.014	$P_{72} \leq 0.001$

HG, Hesperidin glucoside; DDP, diamminedichloroplatinum; [a]$P < 0.05$ indicates significant differences compared with the data of control after 24-h treatment; [b]$P < 0.05$ versus 50 µg/mL hesperidin after 24-h treatment.

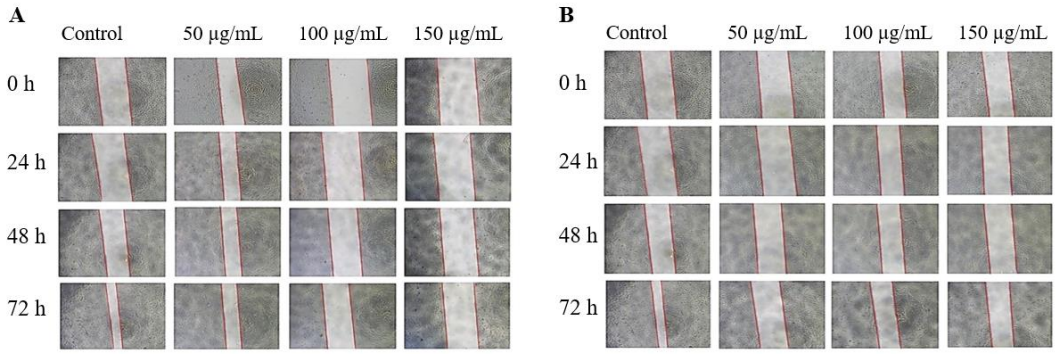

Fig.2. Representative images of the wounds at 0-72 h after treatment with 0 (control), 50, 100, and 150 µg/mL of (A) hesperidin and (B) hesperidin maltoside.

Table 5. The effect of hesperidin, diosmin, HG_1, HG_2, and DDP on the migration of A549 cells. The data are expressed as the mean ± SD, $n \geq 3$.

Sample	Concentration (µg/mL)	Gap closure (%)				P_{period}	$P_{concentration}$
		0 h	24 h	48 h	72 h		
Control	0	0	13.23 ± 2.68	23.89 ± 2.86	33.24 ± 1.75		
Hesperidin	50	0	4.64 ± 0.58[a]	8.81 ± 2.52[b]	14.03 ± 0.83[b]	$P_{c50,\ t24,48,72} = 0.016$	$P_{24} = 0.993$
	100	0	4.69 ± 0.63[a]	6.76 ± 0.82[a,b]	12.42 ± 0.48[a,b]	$P_{c100,\ t24,48,72} \leq 0.001$	$P_{48} = 0.081$
	150	0	4.65 ± 0.45[a]	5.16 ± 0.79[a]	10.11 ± 1.64[b]	$P_{c150,\ t24,48,72} = 0.013$	$P_{72} = 0.013$
Diosmin	50	0	4.88 ± 0.82[a]	7.58 ± 1.07[a,b]	10.82 ± 1.10[b]	$P_{c50,\ t24,48,72} \leq 0.001$	$P_{24} = 0.026$
	100	0	3.97 ± 0.26[a]	6.08 ± 0.87[a]	8.24 ± 0.67[a,b]	$P_{c100,\ t24,48,72} = 0.007$	$P_{48} = 0.050$
	150	0	3.32 ± 0.19[a,b]	5.32 ± 0.64[a]	6.84 ± 0.55[a,b]	$P_{c150,\ t24,48,72} = 0.006$	$P_{72} = 0.003$
HG_1	50	0	4.46 ± 0.73[a]	7.30 ± 1.57[a,b]	9.28 ± 1.49[a,b]	$P_{c50,\ t24,48,72} = 0.012$	$P_{24} = 0.124$
	100	0	4.24 ± 0.78[a]	5.25 ± 0.20[a]	6.55 ± 0.30[a,b]	$P_{c100,\ t24,48,72} = 0.023$	$P_{48} = 0.030$
	150	0	3.29 ± 0.12[a,b]	4.37 ± 0.75[a]	5.99 ± 0.26[a,b]	$P_{c150,\ t24,48,72} = 0.019$	$P_{72} = 0.008$
HG_2	50	0	2.98 ± 0.39[a,b]	4.01 ± 0.41[a]	5.37 ± 0.69[a]	$P_{c50,\ t24,48,72} = 0.006$	$P_{24} = 0.055$
	100	0	3.07 ± 0.24[a,b]	3.60 ± 0.14[a,b]	4.03 ± 0.07[a]	$P_{c100,\ t24,48,72} = 0.010$	$P_{48} = 0.011$
	150	0	2.44 ± 0.07[a,b]	3.01 ± 0.17[a,b]	3.71 ± 0.12[a]	$P_{c150,\ t24,48,72} = 0.002$	$P_{72} = 0.005$
DDP	0.5	0	3.09 ± 0.25[a,b]	4.36 ± 0.60[a]	4.77 ± 0.19[a]	$P_{c50,\ t24,48,72} = 0.021$	$P_{24} = 0.006$
	1	0	2.52 ± 0.16[a,b]	3.18 ± 0.04[a,b]	3.55 ± 0.42[a]	$P_{c100,\ t24,48,72} = 0.043$	$P_{48} = 0.005$
	2	0	2.38 ± 0.06[a,b]	2.92 ± 0.04[a,b]	3.53 ± 0.16[a,b]	$P_{c150,\ t24,48,72} = 0.004$	$P_{72} = 0.003$

HG, Hesperidin glucoside; DDP, diamminedichloroplatinum; [a]$P < 0.05$ indicates significant differences compared with the data of control after 24-h treatment; [b]$P < 0.05$ versus 50 µg/mL hesperidin after 24-h treatment.

Cell migration rate in the hesperidin, diosmin, HG_1, and HG_2 groups at 24, 48, and 72 h declined in a time-related manner as shown, especially HG_2 treatment, in the smaller relative change of % gap closure from time to time. The inhibition of cell migration also showed an increase in a concentration-dependent manner (Table 5). In addition, the anti-migration effect of HGs was substantially higher compared with both the control group and the original hesperidin. Moreover, the greatest effects on cell migration belonged to HG_2 at 150 µg/mL against the A549 cells, which was similar to that produced by DDP at 1.0 µg/mL.

Inhibitory effects on the invasion of A549 cells

The penetration of A549 cells through the Matrigel^{TM}-coated filter was suppressed in the presence of hesperidin, diosmin, HG_1, and HG_2 (Fig. 3A and B show only the results of hesperidin and HG_2). The invasion percentage results of hesperidin, diosmin, HG_1, and HG_2 treatment at 150 µg/mL at 48 h were 33.89 ± 4.82, 27.60 ± 1.62, 24.40 ± 2.38, and 20.62 ± 2.35%, respectively, compared with 92.30 ± 2.25% invasion in the control. Furthermore, it was demonstrated that the cell invasion in the cells treated with hesperidin, diosmin, HG_1, and HG_2 at 24 and 48 h considerably declined in a time- and concentration-related manner (Table 6). Moreover, HG_1 and HG_2 showed a similar inhibition rate of invasion as the DDP positive control (Table 6).

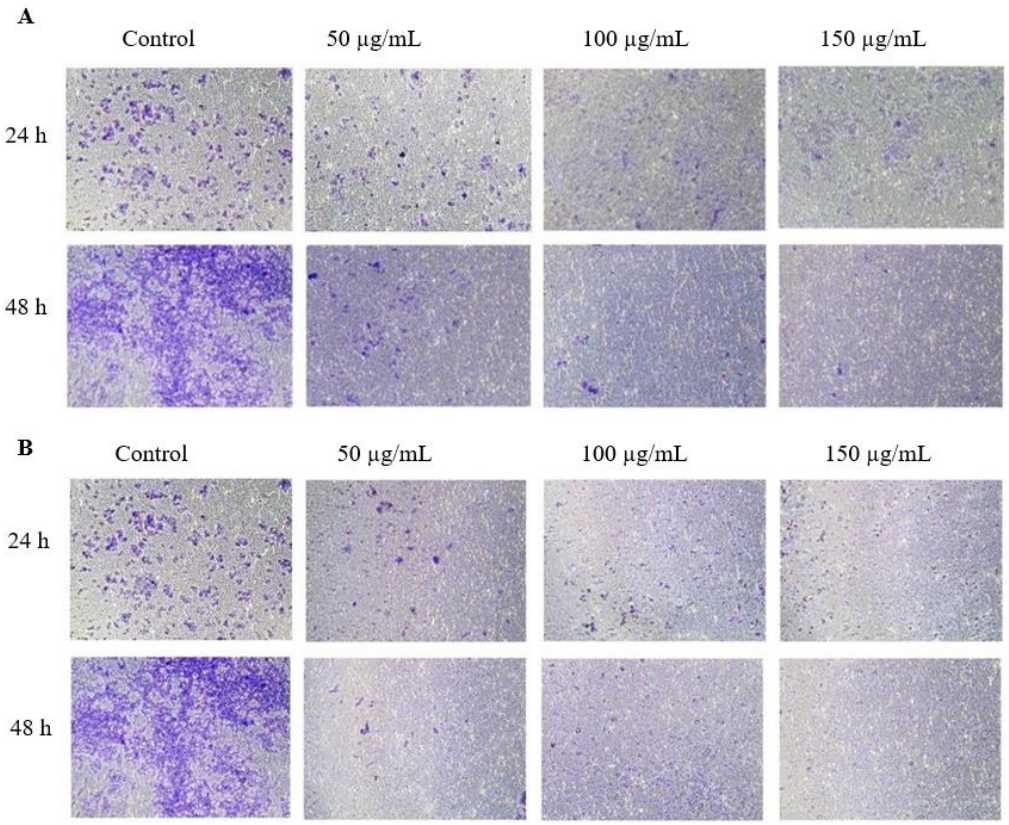

Fig. 3. Transwell® assay was performed to determine A549 cell invasion. Images captured of representative invasive cells treated with (A) hesperidin, and (B) HG_2.

Table 6. Effect of hesperidin, diosmin, HG_1, and HG_2 on the invasion of A549 cells. The data are expressed as the mean ± SD, n ≥ 3.

Sample	n	Concentration (µg/mL)	Invasion (%)		P_{period}	$P_{concentration}$
			24 h	48 h		
Control		0	87.45 ± 1.62	92.30 ± 2.25		
Hesperidin		50	52.84 ± 3.49[a]	42.78 ± 6.83[a]	$P_{c50, t24,48}$ = 0.233	P_{24} = 0.014
		100	47.11 ± 5.60[a]	37.06 ± 6.26[a,b]	$P_{c100, t24,48}$ = 0.001	P_{48} = 0.264
		150	39.03 ± 1.50[a,b]	33.89 ± 4.82[a,b]	$P_{c150, t24,48}$ = 0.115	
Diosmin		50	43.51 ± 2.39[a,b]	38.05 ± 5.14[a,b]	$P_{c50, t24,48}$ = 0.075	P_{24} = 0.039
		100	38.60 ± 3.31[a,b]	31.39 ± 2.71[a,b]	$P_{c100, t24,48}$ = 0.002	P_{48} = 0.028
		150	34.79 ± 3.59[a,b]	27.60 ± 1.62[a,b]	$P_{c150, t24,48}$ = 0.024	
HG_1		50	36.42 ± 6.96[a,b]	32.48 ± 4.76[a,b]	$P_{c50, t24,48}$ = 0.090	P_{24} = 0.148
		100	31.55 ± 5.70[a,b]	28.82 ± 4.84[a,b]	$P_{c100, t24,48}$ = 0.698	P_{48} = 0.135
		150	26.04 ± 3.13[a,b]	24.40 ± 2.38[a,b]	$P_{c150, t24,48}$ = 0.063	
HG_2		50	31.31 ± 4.51[a,b]	27.44 ± 3.03[a,b]	$P_{c50, t24,48}$ = 0.045	P_{24} = 0.049
		100	27.54 ± 2.97[a,b]	24.90 ± 1.91[a,b]	$P_{c100, t24,48}$ = 0.448	P_{48} = 0.039
		150	22.64 ± 1.97[a,b]	20.62 ± 2.35[a,b]	$P_{c150, t24,48}$ = 0.011	
DDP		0.5	29.01 ± 4.99[a,b]	18.62 ± 3.11[a,b]	$P_{c50, t24,48}$ = 0.011	P_{24} = 0.112
		1	26.12 ± 4.22[a,b]	16.13 ± 2.66[a,b]	$P_{c100, t24,48}$ = 0.008	P_{48} = 0.205
		2	20.91 ± 2.05[a,b]	14.42 ± 1.57[a,b]	$P_{c150, t24,48}$ = 0.012	

HG, Hesperidin glucoside; DDP, diamminedichloroplatinum; [a]$P < 0.05$ indicates significant differences compared with the data of control after 24-h treatment; [b]$P < 0.05$ versus 50 µg/mL hesperidin after 24-h treatment.

DISCUSSION

The anticancer properties of the flavonoid glycoside in oranges (*Citrus sinensis* L.), hesperidin, and its flavone analog, diosmin, have exhibited anti-carcinogenic activities in various studies (22). The anticancer effects of hesperidin are associated with its antioxidant and anti-inflammatory activities and its interactions with numerous cellular targets to suppress cancer cell proliferation by activating apoptosis and cell cycle arrest (23).

In the structure-activity relationship (SAR) of hesperidin, the ring B C-4′ methyl replacement of hesperidin can motivate the ring B C-3′ hydroxyl group, making hesperidin a better scavenger of free radicals (24). SAR for anticancer activity has interactions between the C2=C3 double bond (25). The substantial role of the C2=C3 double bond participates in molecular planarity and combination between rings C and A/B, which is crucial for powerful tumor suppression (26). Besides, adding glucose to the original structures of flavonoids or hesperidin, like our HG_1 and HG_2, increased their water solubility, bioavailability, and antioxidant activity (17) which could be a justification for why we found a greater anticancer activity of HG_1 and HG_2 compared to the original hesperidin regarding anti-viability, anti-migration, and anti-invasion properties. Although the anti-proliferation effect of HG was not as effective as the DDP positive control since the cytotoxic effect on normal cells of HGs was higher than DDP, its anti-proliferation properties would make a good promise. Moreover, the anti-migration and anti-invasion activities of HGs were comparable to those of DPP.

Cancer cell survival is suppressed by hesperidin over the mitochondrial apoptotic pathway and by inducting G0/G1 arrest in a time- and concentration-related manner. However, hesperidin does not have any adverse impacts on BEAS-2B normal cells (13). Xia *et al.* reported that the proliferation of A549 cancer cells was reduced by hesperidin, resulting in morphological alterations of apoptotic cells (13). They found that after treatment with various concentrations of hesperidin for 72 h, the A549 cell morphology changed and most of the cells treated with 1 µg/mL of DDP were apoptotic compared to that in the control group. Similarly, Cincin *et al.* found that hesperidin inhibits cell growth and motivates the programmed cell death pathway in two non-small cell lung cancer lines, A549 and NCI-H358, in a time- and concentration-related manner (7). They also demonstrated very low cytotoxicity of hesperidin in MRC-5 cells. Flavonoids have a dual action regarding reactive oxygen species homeostasis. They behave as antioxidants under normal cells and are strong pro-oxidants in cancer cells activating programmed cell death pathways (27). Both antioxidant and pro-oxidant activities participate in flavonoid anticancer effects (27,28).

The communication of cancer cells with the extracellular matrix is crucial for metastasis, which is the primary reason for death in cancer patients. The repressive impact of hesperidin on migration and invasion of human non-small cell lung cancer cells may be mediated by the control of the chemokine stromal-cell derived factor-1, which is involved in promoting the neo-angiogenesis of cancer (12). In addition, hesperidin can suppress programmed death ligand 1, which is overexpressed in progressive cancer, and inhibit the activation of matrix metalloproteinases such as MMP-9 and MMP-2. These properties explain why hesperidin suppresses the metastatic phenotype and cell migration (29).

A flavonoid mixture tablet of hesperidin and diosmin (daflon) is marketed as a vasoprotective venotonic agent for the treatment of venous disease. This combination may prove useful as an anticancer agent and more work is needed on HG diosmin combinations to assess their potential anti-metastatic and anti-angiogenetic effects.

So far as we know, this study is the first to inform the suppressive effects of synthetic HG_1 and HG_2 on cancer cells. However, in-depth research is necessary to elucidate the underlying mechanisms of cancer as well as on the effects of HGs on cancer behavior in a physiologic environment to provide information for innovative-drug development in the future.

CONCLUSION

Our results suggest that new the HGs, HG_1 and HG_2, have more potential to inhibit cancer cells than the original hesperidin. They were effective against A549 cell lines and had a favorable SI score of > 2.0 relative to MRC-5 normal cells, suggesting a good toxicity profile. The suppression of cell viability, cell migration, and cell invasion by HG_1 and HG_2 was time- and concentration-dependent. Taken together, our new HGs have the potential as a new

alternative anticancer agent or may be used as a combination regimen, especially against metastases. More preclinical work is needed to ascertain whether HG_1 and HG_2 should be tested in humans.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

N. Poomipark and J. Kaulpiboon conceptualized the study. T. Chaisin and J. Kaulpiboon conducted the experiments. N. Poomipark and J. Kaulpiboon contributed to the methodology. T. Chaisin prepared the raw data file for analysis. N. Poomipark and J. Kaulpiboon analyzed the data and helped in the manuscript writing. The final version of the manuscript was approved by all authors.

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