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Anticancer activity of Hebesu extracts in cancer cell lines

Eun-Jung Kim[1], Sohyun Kim[1], Nayeon Bak[1], Senthil Kumar Baskaran[1], Suyeon Lee[1], Junsu Kim[1], Masahito Uchiyama[2], Jong-Min Lee[1], Han-Sung Jung[1]

[1]Division in Anatomy and Developmental Biology, Department of Oral Biology, Taste Research Center, Oral Science Research Center, BK21 FOUR Project, Yonsei University College of Dentistry, Seoul, Korea, [2]Uchiyama Group, Hyuga, Japan

Abstract: Hebesu, a small citrus fruit from Miyazaki, Japan, has the potential to advance anticancer activity due to its high content of bioactive flavonoids, including narirutin, naringin, hesperidin, and neohesperidin. This study explored the anticancer effects of Hebesu extract (HBS) in various human cancer cell lines: MDA-MB-231 (breast cancer), HepG2 (liver cancer), and A549 (lung cancer). The results showed that treatment with HBS led to a substantial reduction in cell viability in a dose-dependent manner and induced morphological changes, accompanied by increased expression of apoptosis-related markers. TdTomato fluorescence imaging demonstrated that cytotoxicity in breast cancer cells was dependent on both the concentration and duration of exposure. Furthermore, HBS reduced *Ki-67* expression, increased the levels of *Caspase-3* and *NRF2*, and downregulated the expression of the oncogenic genes *SMO*, *KRAS*, and *BRAF*. These findings suggest that HBS has a multi-targeted anticancer effect by inhibiting cell proliferation, promoting apoptosis, and activating oxidative stress responses, while also suppressing oncogenic signaling pathways. This study emphasizes the potential use of HBS and its flavonoid components as natural cancer suppressive agents. Further investigation using *in vivo* models and clinical studies are essential to fully assess their efficacy, bioavailability, and safety.

Key words: Hebesu extracts, Breast neoplasms, Liver neoplasms, Lung neoplasms, Cell line

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Introduction

Cancer, characterized by uncontrolled cell proliferation resulting from loss of sensitivity to regulatory growth signals, remains a significant challenge in oncology [1]. Breast, lung, and liver cancers are among the most common malignancies and account for a substantial proportion of global cancer mortality. Despite advances in current therapies, significant side effects, limited selectivity, and drug resistance remain major challenges, underscoring the need for multi-targeted therapeutic strategies [2, 3].

Natural products have contributed to the development of anticancer therapies and their clinical applications [4]. Among them, flavonoids—polyphenolic compounds widely distributed in fruits and vegetables—exhibit potent biological activities, including antioxidant, anti-inflammatory, and antitumor effects [5]. Citrus fruits are rich in flavonoids such as hesperidin, naringenin, and narirutin, which exert anticancer effects by inducing apoptosis, suppressing proliferation, and targeting oncogenic signaling, oxidative stress, and tumor-promoting inflammation [5, 6]. These flavonoids

Corresponding author:
Eun-Jung Kim
Division in Anatomy and Developmental Biology, Department of Oral Biology, Taste Research Center, Oral Science Research Center, BK21 FOUR Project, Yonsei University College of Dentistry, Seoul 03722, Korea
E-mail: blueleah@yuhs.ac
Han-Sung Jung
Division in Anatomy and Developmental Biology, Department of Oral Biology, Taste Research Center, Oral Science Research Center, BK21 FOUR Project, Yonsei University College of Dentistry, Seoul 03722, Korea
E-mail: hsjung@yuhs.ac

have attracted considerable attention due to their strong inhibitory effects on tumor cell growth and their therapeutic potential [7].

Citrus Hebesu (Hebesu extract [HBS]) is a small Japanese citrus, which is rich in bioactive flavonoids [8]. Although its pharmacological properties remain underexplored, recent studies suggest that HBS may exert antioxidant and anti-inflammatory effects, offering potential advantages over other citrus fruits [8]. Citrus extracts exhibit anticancer activity against various cancer cell lines, including HepG2 cells, by inducing apoptosis through mitochondrial and death receptor pathways via bioactive flavonoids such as hesperidin, nobiletin, tangeretin, and narirutin [9-12]. However, the anticancer potential of HBS, a lesser-known citrus variety, remains largely unexplored, and its identification may help expand natural product–based therapeutic options for cancer treatment.

In this study, we investigated the cytotoxic activity of HBS against cancer cells, specifically focusing on its anticancer effects in three human cancer cell lines: MDA-MB-231 (breast cancer), A549 (lung cancer), and HepG2 (hepatocellular carcinoma). We evaluated its effects on cell viability, morphology, and apoptosis to assess its potential as a natural cancer suppressive agent. Our findings demonstrated that HBS exerted significant cytotoxic effects on these cancer cell lines, accompanied by distinct changes and induction of apoptosis. These results highlight its potential as a natural anticancer agent and suggest its relevance for future therapeutic development.

Materials and Methods

Ethical approval

This study did not involve human participants or animal experiments and therefore did not require ethical approval. All experiments were performed using established human cancer cell lines.

Cell culture

MDA-MB-231 and HepG2 cells were cultured in high-glucose DMEM (4.5 g/L glucose) supplemented with 10% FBS and 1% penicillin–streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin), while A549 cells were maintained in F-12K medium with the same supplements. All cell lines were incubated at 37°C with 5% CO_2, passaged at 70%–80% confluence using 0.25% trypsin-ethylenediaminetetraacetic acid.

Preparation of HBS

Fresh citrus Hebesu fruits were washed, sliced, and freeze-dried. The dried samples were ground into a fine powder and extracted with 70% ethanol at room temperature for 48 h with gentle agitation. The extract was filtered through Whatman No. 1 filter paper, and the solvent was removed under reduced pressure using a rotary evaporator. The concentrated extract was subsequently freeze-dried to obtain a powder form of HBS, which was stored at –20°C until use. For cell experiments, the HBS powder was dissolved and adjusted to pH 7.0 using 1 M NaOH or 1 M HCl, followed by sterile filtration through a 0.45 μm filter. Aliquots were stored at –20°C and diluted in culture medium to final concentrations of 0–6.4% (v/v) prior to experiments, and the pH of the medium was confirmed to be neutral.

Comparative flavonoid composition

The comparative flavonoid composition presented in Fig. 1B was adapted from reports by the National Agriculture and Food Research Organization (NARO). These data were included to provide phytochemical context for HBS and were not derived from direct compositional analysis of the experimental extract batch used in this study.

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

Cell viability was measured using the MTT-based EZ-CYTOX assay to evaluate anti-proliferative effects of HBS on MDA-MB-231, HepG2, and A549 cells. Cells were seeded in 96-well plates (5,000 cells/well), treated with HBS (0%–5%, v/v) for 48 h, and incubated with EZ-CYTOX solution for 4 h. Absorbance was measured at 450 nm, and viability was expressed relative to controls. IC_{50} values were calculated using non-linear regression with GraphPad Prism.

RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and analyzed by RT-qPCR using a Thermal Cycler Dice™ Real-Time System with SYBR Premix EX Taq™ (Takara). The reaction was initiated at 95°C for 1 min, followed by 40 cycles of 95°C for 5 s, 55°C–60°C for 10 s, and 72°C for 10 s. Gene expression was normalized to β-2-microglobulin, and primers are listed in Table 1.

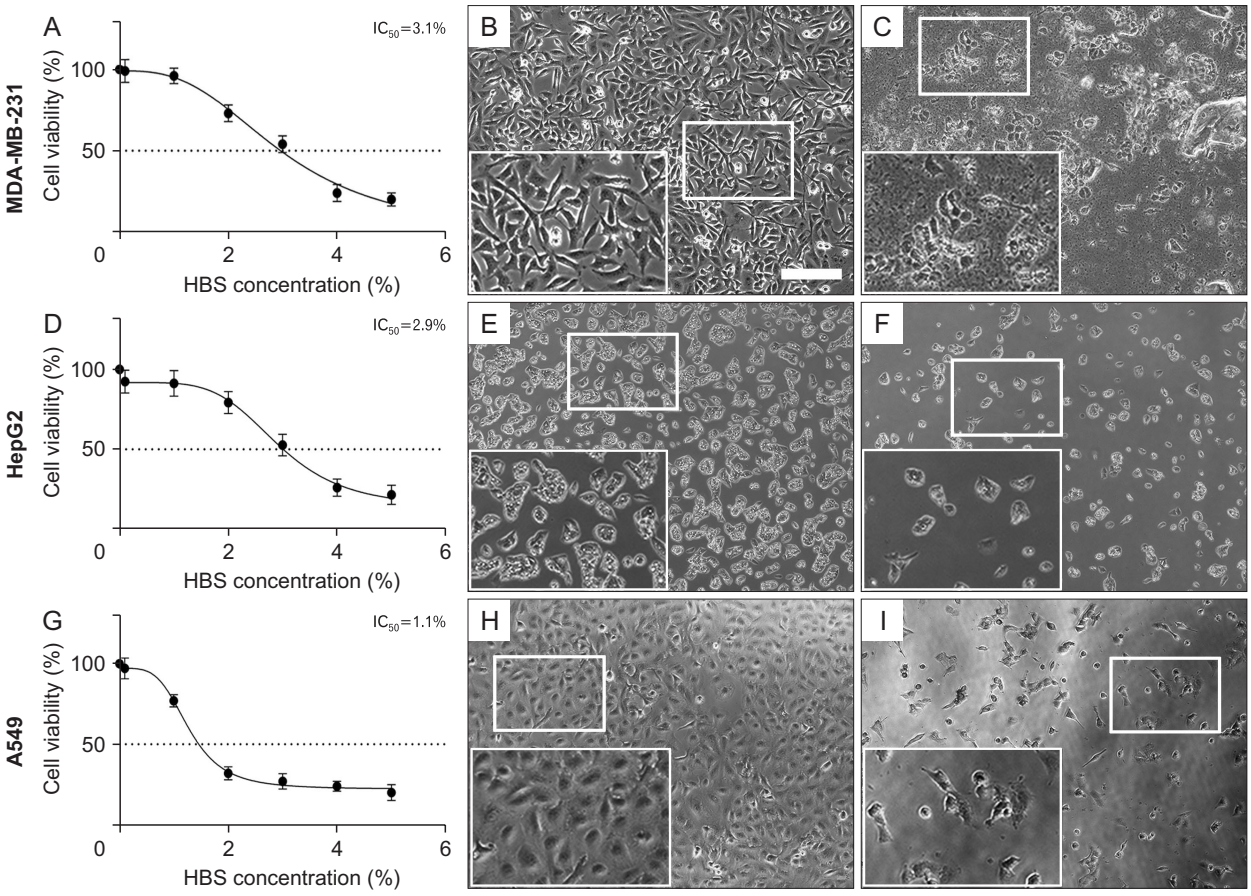

Fig. 1. Effects of Hebesu extract (HBS) on the morphology of cancer cell lines. The effects of HBS on different cancer cell lines, (A–C) MDA-MB-231, (D–F) HepG2 cells, (G–I) A549 cells. (C, F, I) Cell viability was assessed using the MTT assay after treatment with increasing concentrations of HBS (0%, 1%, 2%, 3%, 4%, and 5%) for 48 h, with untreated cells (0%) serving as controls. The IC_{50} values for each cell line were determined and expressed as the percentage of viable cells relative to controls. (B, E, H) Morphological changes in each cell line cultured for 48 h without HBS treatment. (C, F, I) Morphology of each cell line after treatment with the IC_{50} concentration of HBS for 48 h, which showed reduced cell viability. Scale bar=100 μm.

Table 1. List of primers

	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	GACGCTGGGGCTGGCATTG	GCTGGTGGTCCAGGGGTC
Ki-67	TCAAGAGGGGAGGTCGCAAA	CATGATGACCACGGGTTCGG
Cleaved Caspase-3	TGTGTGCTTCTGAGCCATGGT	ACCACGGCAGGGCTCAATAA
NRF-2	TTCTCCCAATTCAGCCAGCC	AACGTAGCCGAAACCTCA
SMO	GGCAAGAGTGCCTTCACG	CCTCTTCCTCCGCTTTTTCT
KRAS	GGCAAGAGTGCCTTGACG	CACAAAGAAAGCCCTCCCCA
BRAF	ATCGGTCTCGTTGCCCAAAT	AGAGGCGTCCTTACGAGAGA

All primer sequences were designed for RT-qPCR and are listed in the 5′-3′ orientation.

Transfection

MDA-MB-231 cells were transduced with a TdTomato-expressing lentivirus produced in HEK293T cells using psPAX2 and pMD2.G. Transduction was performed with polybrene (8 μg/ml; Sigma-Aldrich), and TdTomato-positive cells were expanded. Cells were seeded in 24-well plates and treated with HBS (0%–6.4%, v/v) for up to 48 h. Fluorescence images were acquired using a CQ1 high-content imaging system (Yokogawa Electric Corporation) and analyzed with ImageJ software (National Institutes of Health).

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Fig. 2. A representative image of Hebesu.

Statistical analysis

Data are presented as mean±SD from at least five independent experiments. Statistical significance was assessed using Student's *t*-test with GraphPad Prism 9, with **P*<0.05, ***P*<0.01, and ****P*<0.001.

Results

Composition of HBS and its inhibitory effects on cancer cell viability

HBS, a small green citrus fruit native to the Miyazaki Prefecture in Japan (Fig. 2), belongs to the citrus family. It is closely related to other citrus species, such as sudachi (*Citrus sudachi*) and kabosu (*Citrus sphaerocarpa*), but remains relatively unknown outside Miyazaki. Comparative analysis indicates that HBS is characterized by a distinct flavonoid composition compared with other citrus species, including Kabosu, Shinko, and Yuzu (Table 2) [13]. HBS is relatively enriched in narirutin with substantial levels of hesperidin and moderate levels of neohesperidin. The flavonoid composition presented in Fig. 1B is provided for phytochemical reference to contextualize the biological effects observed in this study, rather than as a direct quantitative analysis of the experimental extract batch. The anti-proliferative effects of HBS were evaluated in MDA-MB-231, HepG2, and A549 cells, showing a dose-dependent reduction in cell viability by MTT assay. The IC_{50} values were 3.1% for MDA-MB-231, 2.9% for HepG2, and 1.1% for A549 cells (Fig. 1A, D, G). Consistently, HBS treatment markedly reduced cell density and induced morphological changes in MDA-MB-231 (Fig. 1B, C), HepG2 (Fig. 1E, F), and A549 cells (Fig. 1H, I).

Table 2. Comparative flavonoid composition of HBS [13]

Flavonoids	Fruits (μg/100 g)			
	HBS	Kabosu	Shinko	Yuzu
Narirutin	380	100	160	130
Naringin	450	80	120	180
Hesperidin	380	300	-	270
Neohesperidin	210	130	240	180

Comparative flavonoid composition of selected citrus fruits, including HBS, Kabosu, Shinko, and Yuzu.

Anticancer activities of HBS in breast, liver, and lung cancer cell lines

To elucidate the molecular mechanisms underlying the anticancer effects of HBS, we analyzed the expression of genes associated with cell proliferation, apoptosis, oxidative stress, and oncogenic signaling. HBS treatment significantly reduced *Ki-67* expression, indicating suppressed cell proliferation, while markedly increasing *Caspase-3* expression, suggesting enhanced apoptosis (Fig. 3A, B). These effects were more pronounced in MDA-MB-231 cells than in the other cancer cell lines. In addition, HBS upregulated *NRF2* expression, a key regulator of oxidative stress responses, and significantly downregulated oncogenic genes including *SMO*, *KRAS*, and *BRAF* (Fig. 3C–F). To further evaluate cytotoxic responses, TdTomato-labeled MDA-MB-231 cells were analyzed by fluorescence imaging at 3.2% (approximately IC_{50}) and 6.4% HBS. Treatment with HBS resulted in a dose- and time-dependent reduction in fluorescence intensity and cell density, with more pronounced effects observed at 6.4%. Quantitative analysis confirmed that HBS suppressed cell growth in a concentration- and time-dependent manner (Fig. 3G, H).

Discussion

Recently, many studies have suggested that the antitumor efficacy of citrus-derived flavonoids [11, 12]. These compounds act as antioxidants and influence various cancer-related mechanisms, including cell cycle regulation, apoptosis, anti-inflammatory effects, angiogenesis inhibition, and metastasis prevention [5, 7]. Narirutin and hesperidin, major flavonoids in HBS, induce apoptosis and cell cycle arrest and suppress proliferation in various cancer cells, including A549 and HepG2, through miR-34a–PD-L1–NF-κB signaling and mitochondrial- and death receptor–mediated apoptotic pathways [10, 12]. In this study, we found the many kinds of flavonoid compound, including nariruitin, hesperidin, neohesperidin in HBS. Furthermore, the viability of breast,

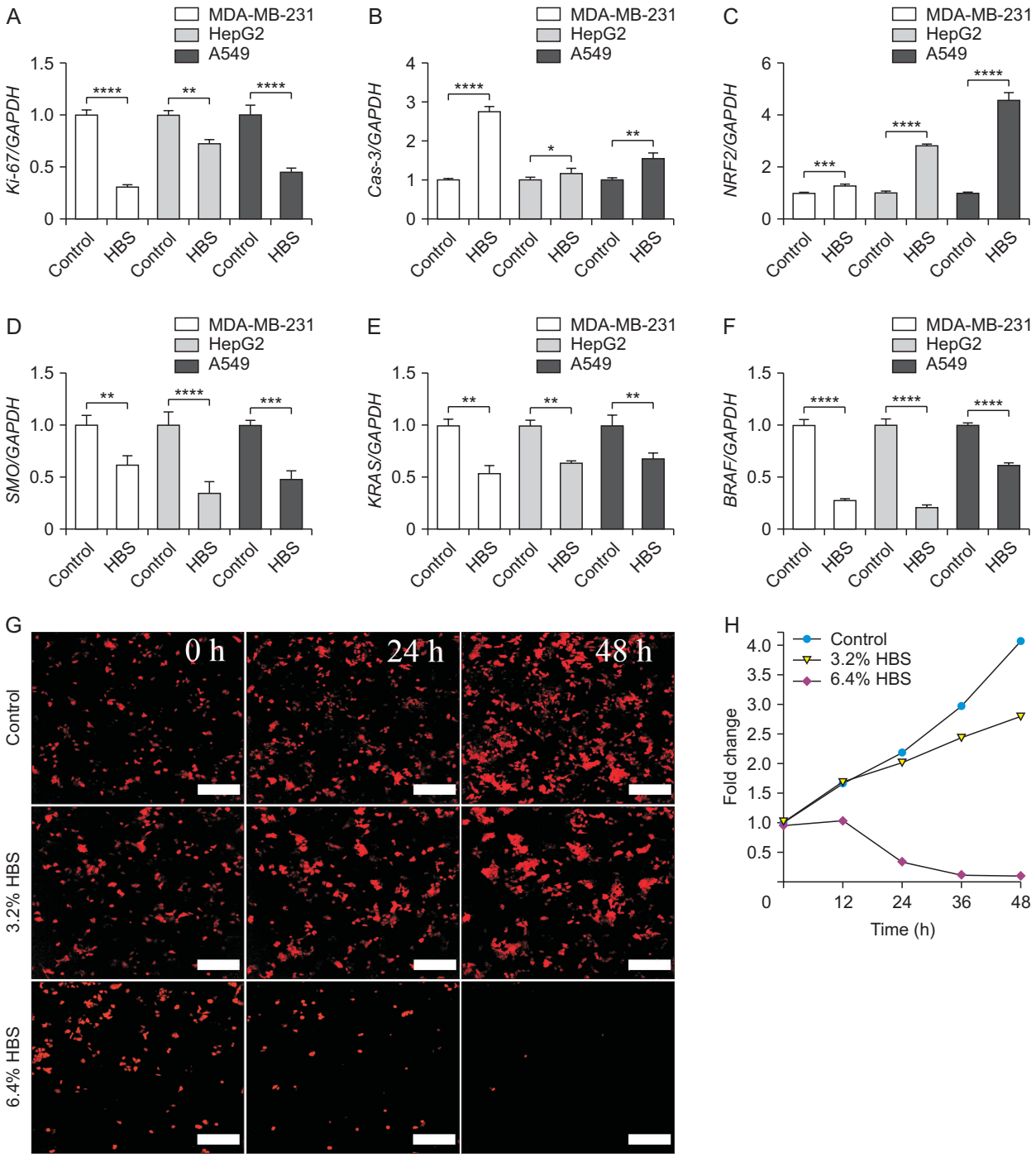

Fig. 3. Effects of Hebesu extract (HBS) on cell proliferation, apoptosis, and oncogenic signaling in cancer cell lines. (A) The expression of *Ki-67*, a marker of cell proliferation, was decreased in MDA-MB-231, HepG2, and A549 cells treated with the IC_{50} concentration of HBS. (B) The expression of *Caspase-3*, an indicator of apoptosis, increased in the same cells following HBS treatment. (C) The upregulation of *NRF2*, a key regulator of the antioxidant response, was observed in all three cancer cell lines treated with HBS. The expression of oncogenic signaling molecules, including *SMO* (D), *KRAS* (E), and *BRAF* (F), was downregulated in response to HBS treatment. (G) Fluorescence imaging of TdTomato-expressing MDA-MB-231 cells treated with HBS (0%, 3.2%, and 6.4%) showed dose- and time-dependent reductions in fluorescence intensity and cell density. The 3.2% concentration approximates the IC_{50} value, while 6.4% was used to visualize enhanced cytotoxic effects. The decrease in fluorescence at higher concentrations reflects cytotoxic responses. (H) Quantification of the fluorescence intensity from imaging analysis using ImageJ revealed a dose- and time-dependent decrease in TdTomato fluorescence with HBS treatment, consistent with the observed cytotoxic effects. Scale bars=100 μm. Statistical significance is indicated as follows: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Data are presented as means±SD.

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liver, and lung cancer cell significantly decreased with HBS treatment. Therefore, various flavonoids abundantly present in HBS modulate cancer cell fate.

Many studies have demonstrated that citrus-derived flavonoids suppress tumor growth [6, 9, 14]. Flavonoids can cease cell cycle progression, inhibit tumor cell proliferation, and induce cancer cell death by targeting MAPK signaling [10, 12, 15]. The anticancer activity of citrus flavonoids, particularly hesperidin, is mediated by suppression of the AKT/mTOR survival pathway, a key regulator of cancer cell growth and survival that promotes apoptotic cell death [15]. Furthermore, citrus carotenoid extracts and flavonoids exert anticancer effects by modulating oxidative stress and disrupting redox homeostasis, leading to mitochondrial-dependent apoptosis in breast cancer cells and attenuation of tumor growth through interference with oncogenic signaling pathways involving *SMO*, *BRAF*, and *KRAS* [11, 12, 15]. In breast cancer cells, particularly the MDA-MB-231 cells, the flavonoid hesperidin in citrus has been shown to induce apoptosis and trigger cell cycle arrest, thereby disrupting malignant cell survival [16]. Notably, natsudaidain, a flavonoid uniquely detected in HBS, may also contribute to its biological activity. Previous studies have shown that natsudaidain regulates inflammatory and apoptotic signaling pathways, including suppression of TNF-α and cyclooxygenase-2 expression, and modulation of PI3K/Akt and p53-related apoptosis pathways. Although its specific role was not directly evaluated in this study, the presence of natsudaidain as a characteristic component of HBS suggests a potential contribution to the multi-target anticancer effects of HBS [17, 18]. In this study, flavonoid-rich HBS regulated proliferation and apoptosis in MDA-MB-231, HepG2, and A549 cells in a dose-dependent manner, with particularly strong effects in MDA-MB-231 cells. These findings suggest that flavonoids in HBS play a crucial role in its anti-proliferative and pro-apoptotic activities by modulating oxidative stress responses and interfering with oncogenic signaling pathways in breast, liver, and lung cancer cells.

In conclusion, HBS effectively inhibited cell viability and proliferation and increased the expression of apoptosis-related markers, while modulating oncogenic signaling and oxidative stress responses. Fluorescence imaging of TdTomato-labeled MDA-MB-231 cells confirmed dose- and time-dependent cytotoxic effects of HBS. These findings indicate that HBS exerts promising multi-targeted anticancer activity. Notably, although individual flavonoids such as naringenin and hesperidin have documented anticancer properties, HBS represents a complex phytochemical mixture with a distinct compositional profile; therefore, the observed biological effects are more likely mediated by synergistic multi-component interactions rather than a single bioactive compound. However, future studies are required to delineate the specific contributions of individual flavonoids, particularly hesperidin and the unique natsudaidain. Rigorous *in vivo* validation, including xenograft models, pharmacokinetic analyses, and mechanistic pathway studies, will be essential to support the translational development of HBS as a natural anticancer therapeutic.

ORCID

Eun-Jung Kim: https://orcid.org/0000-0002-9515-7590
Sohyun Kim: https://orcid.org/0009-0009-1510-018X
Nayeon Bak: https://orcid.org/0009-0005-3820-8290
Senthil Kumar Baskaran: https://orcid.org/0009-0008-8158-6731
Suyeon Lee: https://orcid.org/0009-0005-6313-8554
Junsu Kim: https://orcid.org/0009-0001-2268-5157
Masahito Uchiyama: https://orcid.org/0009-0006-8109-4046
Jong-Min Lee: https://orcid.org/0000-0002-9466-7644
Han-Sung Jung: https://orcid.org/0000-0003-2795-531X

Author Contributions

Conceptualization: MU, HSJ. Data acquisition: SK, NB, SKB, SL, JK. Data analysis or interpretation: EJK, HSJ. Funding acquisition: HSJ. Drafting of the manuscript: EJK, HSJ. Critical revision of the manuscript: MU, JML. Approval of the final version of the manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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