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Original Article

The effect of Sinensetin and Imperatorin on A-549 lung cancer cell viability *in vitro*

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ABSTRACT

Introduction: Lung cancer remains the leading cause of cancer death worldwide, so research is ongoing to discover new therapeutics, such as plant-derived bioactive compounds. For example, Sinensetin, a plant-derived polymethoxylated flavonoid, and Imperatorin, a natural furanocoumarin, have anti-cancer properties. This study assessed the effects of sinensetin and imperatorin separately and in combination on A-549 lung cancer cell viability. **Method:** The A-549 lung cancer cell line was treated with sinensetin (60 µM), imperatorin (30 M), or a combination of both compounds (Sin:Imp 30:30 µM; 50:50 µM and 60:30 µM) for 48 hours. Cell viability was then assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and apoptosis was determined using fluorescein isothiocyanate (FITC) Annexin-V/Propidium iodide staining. **Results:** The combination treatment of Sin:Imp 50:50 and Sin:Imp 60:30 µM reduced cell viability more than the individual treatment of sinensetin and imperatorin, with the lowest cell viability observed for the combination treatment of Sin:Imp 50:50 µM. Likewise, the combination treatment of Sin:Imp 50:50 µM induced the most apoptosis compared to individual treatment. **Conclusion:** Sinensetin and imperatorin can decrease A-549 lung cancer cell viability and are potent apoptotic inducers, especially when they are used in combination, therefore they are potential lung cancer therapeutics.

Key words: A549, Apoptosis, Cell Viability, Imperatorin, Sinensetin..

INTRODUCTION

Lung cancer remains the leading cause of cancer death worldwide with an estimated 1.8 million deaths (18%), followed by colorectal, liver, and stomach cancers.[1] Non-small cell lung carcinoma (NSCLC) causes about 85% of all lung cancers[2] and treatment includes chemotherapy and radiotherapy.[3,4] However, research is ongoing to search for new more affordable, and effective anticancer agents targeting tumor invasion and metastasis.

Sinensetin, a plant-derived polymethoxylated flavonoid, has many pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, antitrypanosomal, anti-obesity, anti-dementia, vasorelaxant and anticancer. Many *in vivo* and *in vitro* studies have indicated that sinensetin not only shows good activity against tumor cells but also exerts minimal toxicity to normal cells, and possesses high selectivity.[5] Imperatorin, a naturally occurring furanocoumarin, also exerts apoptotic effects on a variety of cancer cell lines[6] and induces apoptosis in T98G cells[7] and SGC-7901 gastric cancer cells.[8]

A study of A549 lung cancer cells revealed the antiproliferative activities of sinensetin and that imperatorin at a concentration of less than 10 g/ml had neither cytotoxic nor proliferative effects on attached H292 and A549 cells.[9,10] Therefore, this study compared the effects of these compounds alone or in combination to investigate whether the combined treatment of sinensetin and imperatorin produces higher potency.

MATERIALS AND METHODS

Cell culture

The A549 human lung adenocarcinoma cell line was kindly provided by Professor Ristaniah Rose Effendy. The cells were grown in DMEM + Hams F12, cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), and incubated in a humidified Thermo Fischer Scientific incubator at 37˚C with 5% CO_2. When the cells reached 80 to 90% confluence, they were trypsinized to detach the cells and seeded into 96 well plates at a density of 1 x 10^4 cells/well for cytotoxicity studies.

Sinensetin and imperatorin preparation

Sinensetin (Sigma-Aldrich, St. Louis, Missouri, USA) and imperatorin [Sigma-Aldrich European Pharmacopoeia (EP) Reference Standard] were dissolved in DMSO to prepare a 5000 µM stock solution. Various combinations of sinensetin and imperatorin were prepared as follows: Sin 60 µM[9] and Imp 30 µM[11] and a combination of Sin:Imp 30:30 µM, Sin:Imp 50:50 µM, and Sin: Imp 60:30 µM.

MTT assay

A 5 mg/ml MTT (Sigma-Aldrich, Missouri, USA) stock solution was prepared by adding 50 mg of MTT to 10 mL sterile PBS (Sigma-Aldrich) in a sterile universal, stored in the dark at -20°C and pre-heated to 25-26˚C prior to use. The MTT solution was filter sterilized using a 0.2 µm syringe filter

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to remove any crystals. The A549 cells in 96 well microplates were incubated with the sinensetin and imperatorin solutions in triplicate for 48 hrs. Subsequently, the medium containing the test solutions was discarded and the cells were washed twice with pre-heated PBS before the addition of 10 µL of the MTT solution to obtain a final MTT concentration of 0.5 mg/ml. After 4 hr of incubation at 37°C, the MTT-containing medium was carefully aspirated and the resulting blue formazan crystals were solubilized by the addition of 100 µL of DMSO to each well. The plates were shaken gently and the optical density was measured at 550 nm using a microplate reader (Multiskan EX, Thermo Fischer Scientific).

Annexin V staining

Apoptosis was determined using a fluorescein isothiocyanate (FITC) Annexin V apoptosis DTEC KIT (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. First, A549 cells were incubated with the solutions of sinensetin and/or imperatorin for 48 h, then harvested and washed twice with cold PBS before resuspension in 1X Binding Buffer at a concentration of $1x10^6$ cells/ml. Subsequently, 100 µl of the suspension ($1x10^5$ cells) was transferred to a 5 ml culture tube, and 5 µl of FITC-conjugated Annexin V with 10 µl propidium iodide (PI) was added. The cells were gently vortexed and incubated for 15 min at 25˚C in the dark. Finally, 400 µl of 1X Binding Buffer was added to each tube, and apoptosis was analyzed using the FACS Calibur™ flow cytometer and CellQuest Pro software version 6.0 (BD Biosciences) within 1 hour.

Statistical analysis

The quantitative data were expressed as the mean ± standard deviation (SD) and analyzed using one-way ANOVA. A p-value < 0.05 was considered significant (*$p<0.05$, and **$p<0.01$).

RESULTS

A549 cell viability

As shown in Figure 1, after 48 hours, the cells treated with a combination of sinensetin and imperatorin exhibited decreased cell viability and increased cell death compared to untreated cells and single treatments.

The percentage viability of A549 cells is provided in Figure 2, showing that A549 cells treated with a combination of Sin:Imp 50:50 and Sin:Imp 60:30 M were less viable than cells treated with sinensetin or imperatorin alone, with the lowest cell viability observed in cells treated with Sin:Imp 50:50 µM.

Apoptosis

To determine whether the decreased viability of A549 cells following sinensetin and imperatorin treatment occurs as a result of apoptosis, the cells were stained with Annexin V-FITC/PI. As presented in Figure 3, sinensetin and imperatorin induced apoptosis in A549 cells.

All data are normally distributed and analyzed with one-way ANOVA showing that there were significant differences between groups in the necrotic and apoptotic phases (Q2 + Q3) (Figure 4). In necrotic cells (Q1) and apoptotic cells (Q2 & Q3) the highest level was in the combination treatment of Sin: Imp, 50:50 µM.

DISCUSSION

Sinensetin (SIN) is a pentamethoxyflavone, chemically known as 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-4H-1-benzopyran-4-one, and has been intensively studied for its anticancer activity.[5] Sinensetin isolated from the flavedo of *Citrus reticulata* exerted antiproliferative effects against the promyelocytic leukemia HL-60 cells and Hep G2 liver cells.[12] In another study, it also showed strong antiproliferative activity against MDA-MB-435 ER- and MCF-7 ER+, moderate activity against HT-29, DMS 114, and SK-MEL5, and the weakest activity against DU-145 cell line, with 5-desmethyl sinensetin being more active than sinensetin against all cell lines.[13] Tan *et al.* demonstrated sinensetin-induced apoptosis and autophagy in human T-cell lymphoma Jurkat cells by activating reactive oxygen species/c-Jun N-terminal kinase and blocking the Akt/mTOR signaling pathway. Apoptosis induction was related to a loss of mitochondrial membrane potential and increased caspase-3/-8/-9 and poly(ADP-ribose) polymerase (PARP) cleavage in human T-cell lymphoma Jurkat cells.[14]

Imperatorin (IMP) is a natural form of coumarin and has antioxidant, anti-cancer, anti-apoptotic, anti-inflammatory, and anti-oxidative, as well as protective effects on central nerves and the cardiovascular system.[11] Imperatorin exhibits selective antitumor effects in SGC-7901 human gastric adenocarcinoma cells by inducing apoptosis, cell cycle arrest, and targeting the PI3K/Akt/mTOR signaling pathway.[8,15] It is also a promising chemotherapeutic agent against human larynx cancer and rhabdomyosarcoma through inhibition of cell cycle progression related to specific changes in gene expression, including CDKN1A.[16] Imperatorin induces apoptosis *via* the activation of caspase-3/7, a primary and irreversible executioner pathway in HT-29 cancer cells. Apoptotic induction is a significant method to achieve significant anticancer functions and is a form of programmed cell death. It might occur naturally as well as be mediated by different stimuli such as oxidative stress, or external factors in a mitochondria-dependent manner through extrinsic signaling commonly cell death receptor-dependent manner. The results provide confirmative evidence of apoptotic signaling in HT-29 colon cancer cells.[17] In another study, imperatorin targeted MCL-1 to sensitize CD133+ lung cancer cells to γδ-T cell-mediated cytotoxicity.[18] Previously, sinensetin and imperatorin were found in similar concentrations in a root ethanol extract of *Imperata cylindrica*.[19]

Researchers have found that imperatorin combined with other drugs works better *in vitro*. The combination of quercetin (50–100 µM) and imperatorin (50–100 µM) was more effective in HeLa cells and Hep-2 cells than either drug alone. This was related to decreased Hsp27 expression and increased caspase-3 and caspase-9 activity.[20] Therefore, the mechanism of the combination of imperatorin and other drugs is complex and requires targeted research.

CONCLUSION

We have provided evidence supporting the potential effect of combined sinensetin and imperatorin as effective anticancer compounds inhibiting the proliferation and inducing apoptosis in A549 lung cancer cell lines.

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CONFLICTS OF INTEREST

No conflicts of interest to disclose.

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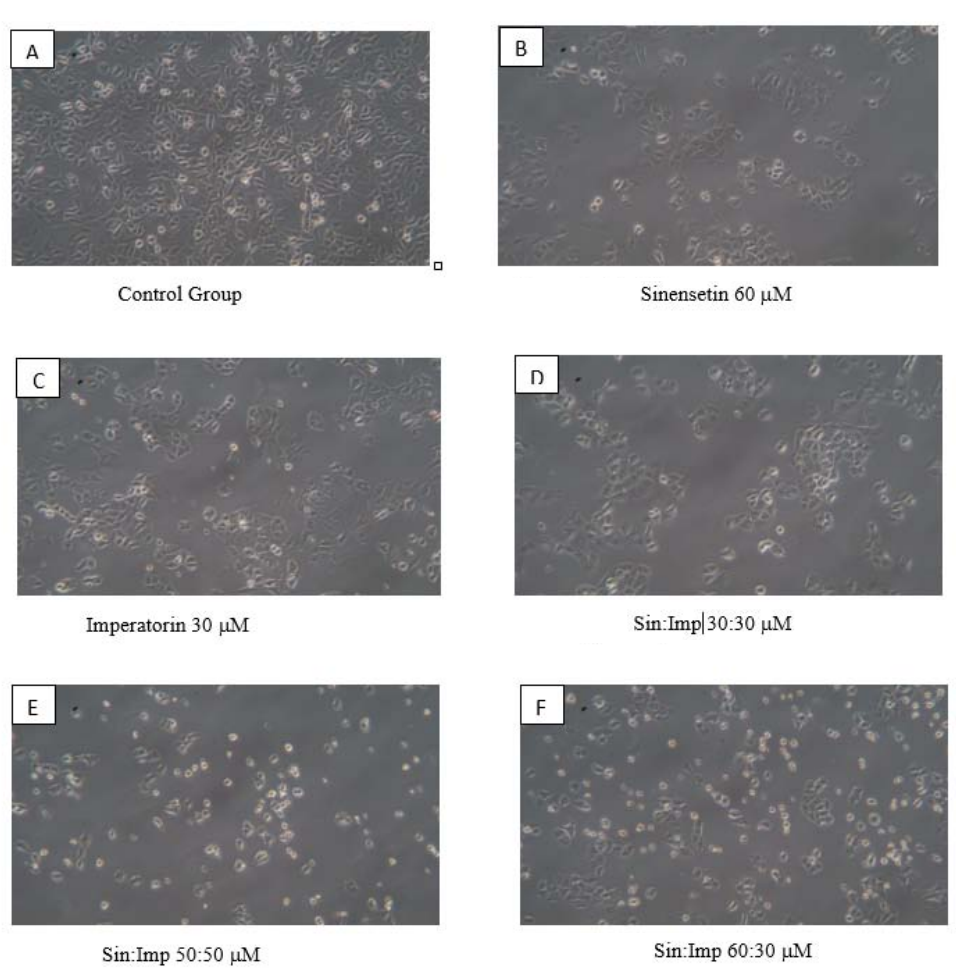

Figure 1: A-549 cells (A) were treated with 60 µM sinensetin (B), 30 µM imperatorin (C), and a combination of Sin:Imp; 30:30 µM (D), Sin:Imp; 50:50 µM (E) and Sin:Imp; 60:30 µM (F) for 48 hr and then visualized using an inverted phase contrast microscopy (100x magnification).

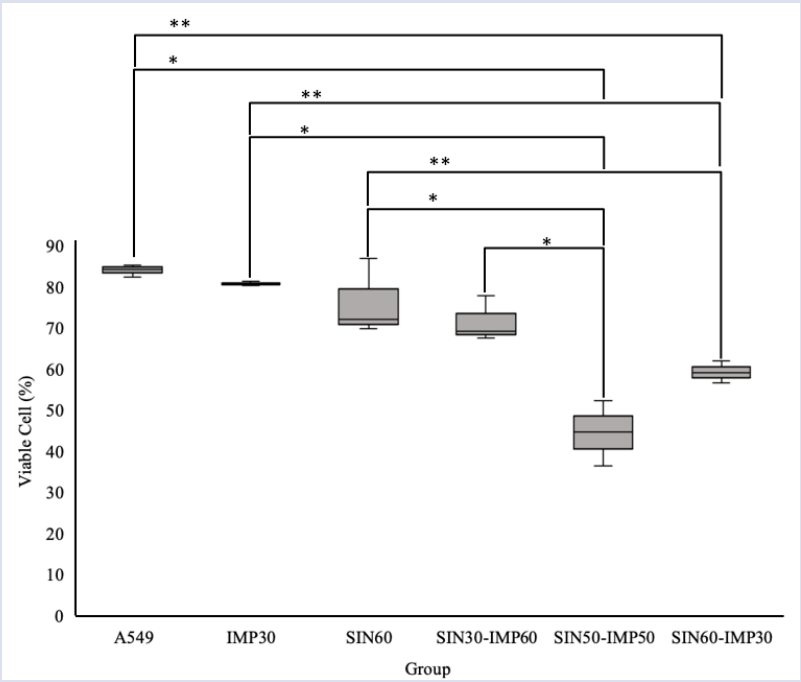

Figure 2: A549 cell viability after sinensetin, imperatorin, or combined sinensetin/imperatorin treatment. IMP: Imperatorin, SIN: Sinensetin, *p<0.05, and **p<0.01.

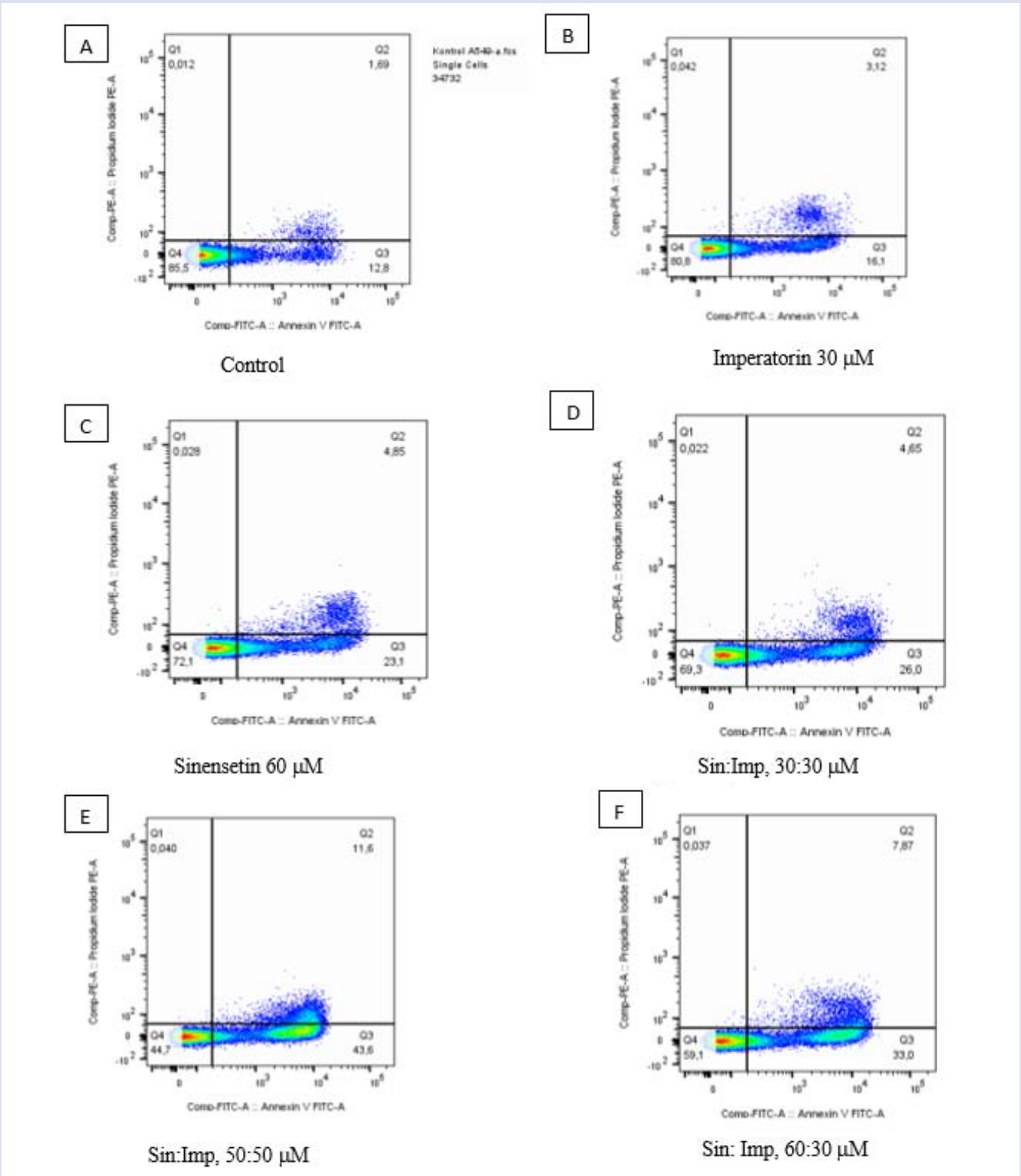

Figure 3: Effect of sinensetin and imperatorin on A549 cell apoptosis. A549 cells were treated with imperatorin 30 µM (B), sinensetin 60 µM (C), and a combination of Sin:Imp 30:30 µM (D), Sin:Imp 50:50 µM (E), and Sin:Imp 60:30 µM (F) for 48 h and apoptosis was measured by annexin V/propidium iodide staining and analyzed by flow cytometry.

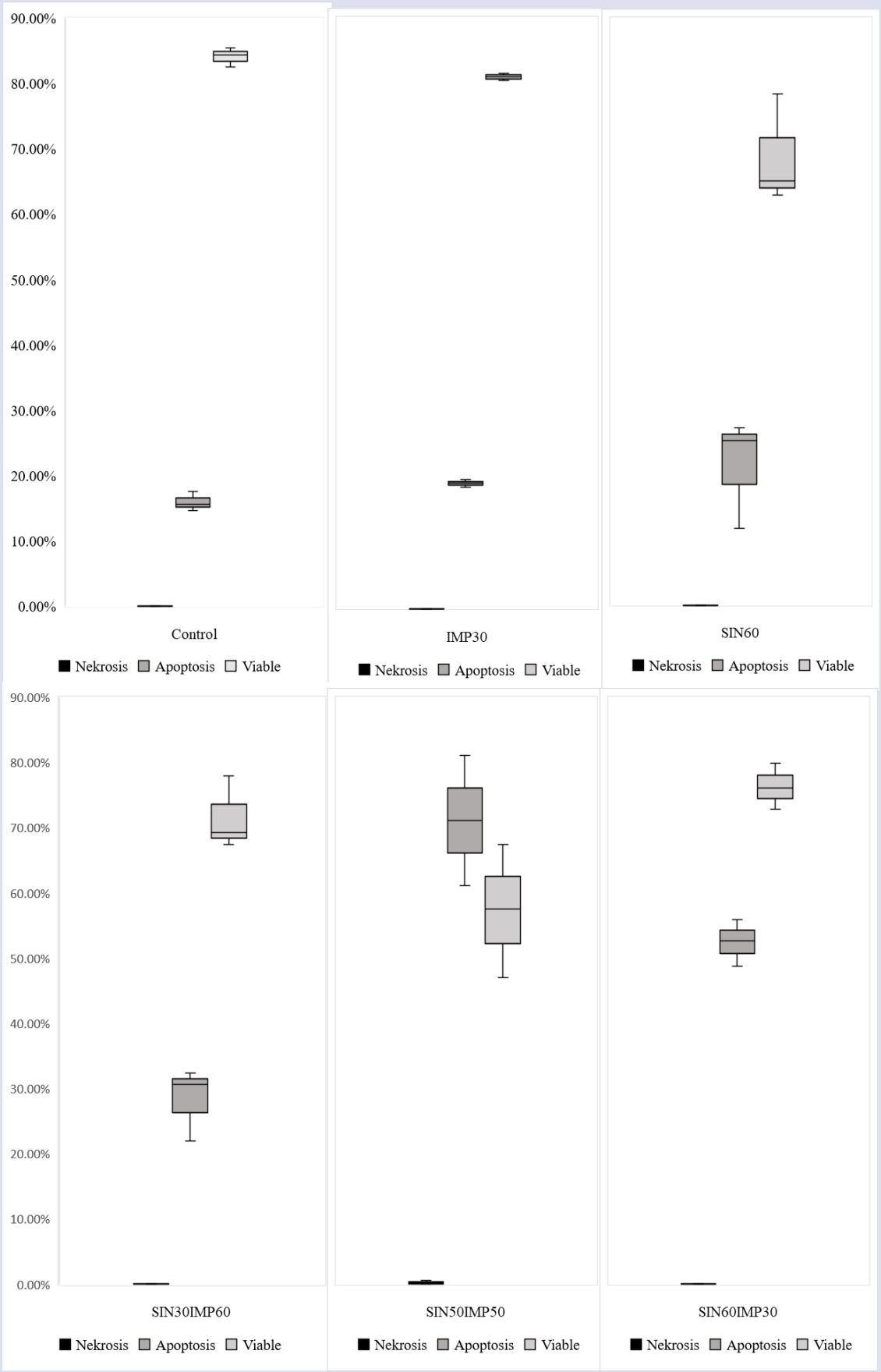

Figure 4: Percentage of Q1 (necrotic cell), Q2 & Q3 (apoptosis), and Q4 (viable cell) in A549 of each group, triplicate after 48 h of sinensetin and imperatorin treatment.

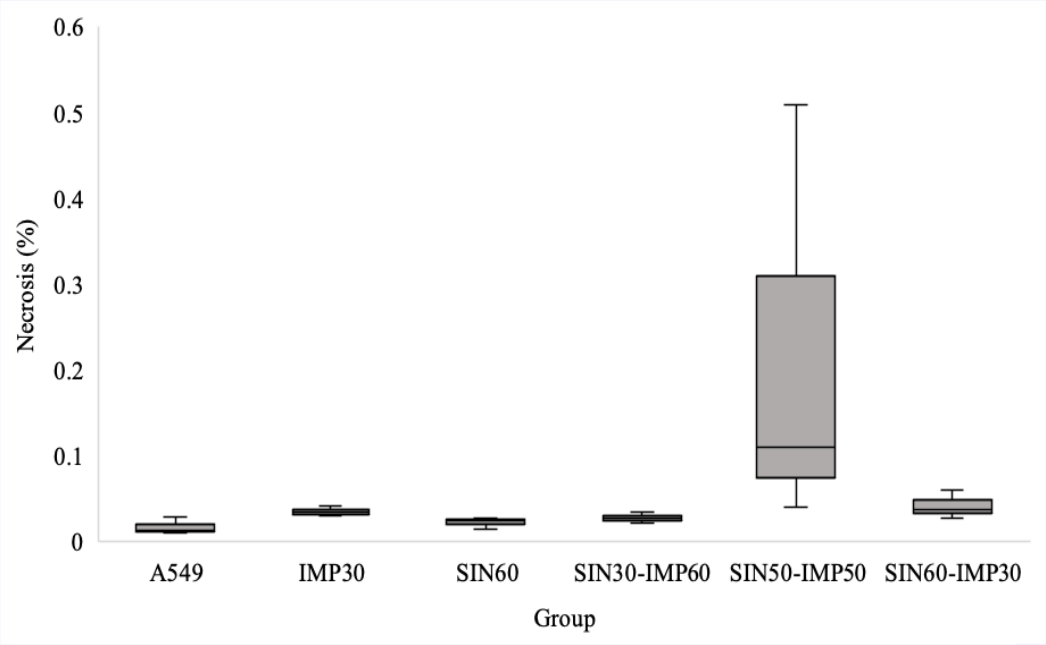

Figure 5: Distribution of necrotic area (Q1) in A549 lung cancer cells after 48 h of treatment with single and a combination of Sinensetin and Imperatorin. p*<0.05, p**<0.01.

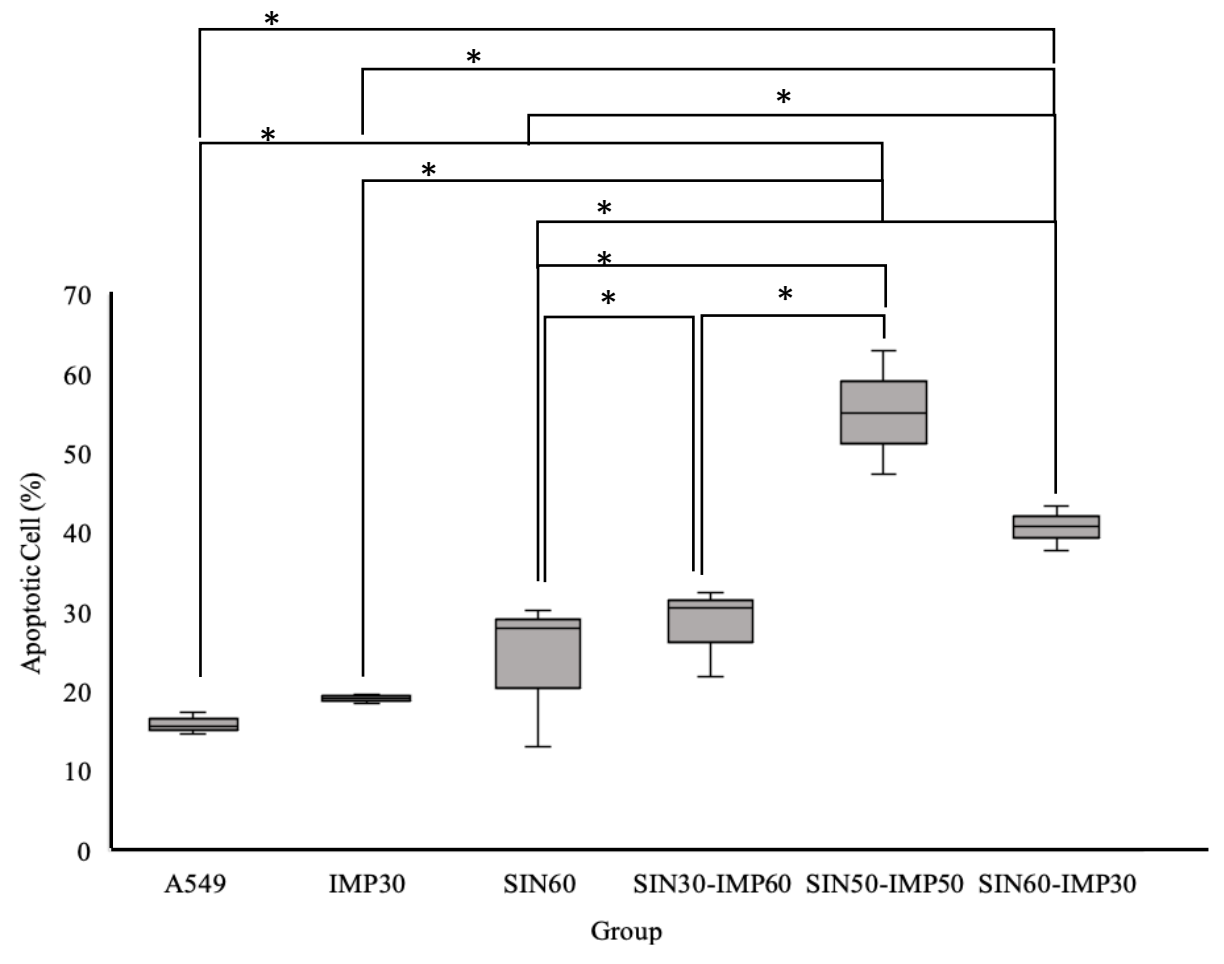

Figure 6: Distribution of the apoptotic (early and late apoptotic) area (Q2+Q3) in A549 lung cancer cells after 48 h treatment of single and a combination of Sinensetin and Imperatorin. p*<0.05, p**<0.01.

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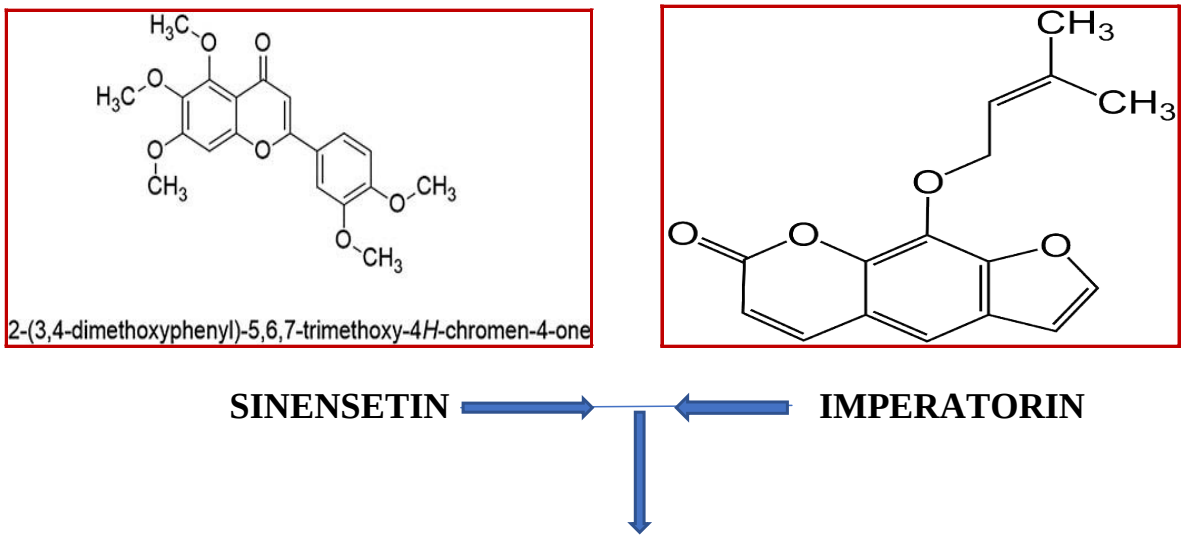
GRAPHICAL ABSTRACT



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