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Investigation on chemosensitizing effect of cycloartane triterpenoids isolated from *Gardenia sessiliflora* on non-small cell lung cancer

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ABSTRACT

The mainstay treatment for non-small cell lung cancer (NSCLC) is chemotherapy. However, developing multidrug resistance to chemotherapy is still the main reason for relapse and poor clinical outcomes. P-glycoprotein (P-gp) is related to multidrug resistance and inhibitors of P-gp have been reported as chemosensitizers. Therefore, the aim of this present study was to evaluate the chemosensitizing effect of pure compounds isolated from *Gardenia sessiliflora* in NSCLC using the A549 cell line. The P-gp function was determined by measuring the intracellular accumulation of [^{3}H]-digoxin or [^{3}H]-paclitaxel, substrates of P-gp. Moreover, cell viability and cell death were evaluated by MTT and apoptosis assays, respectively. The screening results of eight pure compounds revealed that compound 8 showed the most significant increase in accumulation of [^{3}H]-digoxin and [^{3}H]-paclitaxel. Compound 8 at 10 μM did not affect cell viability, whereas paclitaxel significantly decreased cell viability. Interestingly, the combination of compound 8 and paclitaxel significantly decreased cell viability and demonstrated a greater increase in the apoptosis population of A549 cells than a single treatment of paclitaxel. The results of this study indicated that cycloartane triterpenoids compound 8 isolated from *Gardenia sessiliflora* increased the anti-cancer effect of paclitaxel in A549 cells via the inhibition of P-gp-mediated drug efflux.

Keywords: chemosensitizer; cycloartane triterpenoids; *Gardenia sessiliflora*; non-small cell lung cancer; P-glycoprotein

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1. INTRODUCTION

Cancer is one of the major public health problems, and its incidence is responsible for the rapidly growing rate of mortality globally (Sung et al., 2021). Lung cancer remains the leading and most frequent cause of cancer mortality, with an estimated 1.8 million deaths (18%) (Dela Cruz et al., 2011; Sung et al., 2021). According to its pathological classification, lung cancer is composed of two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Adenocarcinoma, the most common type of NSCLC, is derived from type II alveolar cells of small airway epithelial (Sainz de Aja et al., 2021). The mainstay of chemotherapy for NSCLC involves a combination of platinum-based therapy with a tubulin-binding agent such as paclitaxel, docetaxel and vinca alkaloids, gemcitabine, or

pemetrexed (Chang, 2011). Although the response to chemotherapeutic agents is effective in initial treatments, resistance develops eventually (Zahreddine and Borden, 2013).

Human tumor resistance to multiple drugs is one of the major treatment limitations, hindering successful chemotherapeutic treatments and leading to poor prognosis and tumor recurrence. One of the major cellular mechanisms related to MDR is the increased expression of ATP-binding cassette (ABC) efflux transporters (Szakács et al., 2014). There are three major transporters: ABCB1 (P-gp), ABCC1 (MRP1), and ABCG2 (BCRP) that are recognized in association with MDR cancer (Xiao et al., 2021). P-gp over expression, which reduces intracellular chemotherapeutic drugs, is found in chemoresistance cancer cells (Alfarouk et al., 2015; Nanayakkara et al., 2018). Due to the importance of P-gp in MDR, numerous efforts have been made by researchers to investigate P-gp inhibitors and identify chemosensitizers (Kim, 2002). Despite the development of P-gp inhibitors, none have currently been approved for cancer treatment. Therefore, the identification of new inhibitors is required.

Phytochemical agents such as flavonoids, triterpenoids, alkaloids, steroidal saponins, and coumarins have been identified as potential P-gp inhibitors (Wink et al., 2012). *Gardenia sessiliflora* Wall. ex C.B. Clarke is a plant of the genus *Gardenia*, belonging to the Rubiaceae family. Leaves and apical buds of most *Gardenia* plants have been used as antispasmodic, carminative, diaphoretic, expectorant, and antihelminthic agents (Youn et al., 2016). Previous studies reported that *Gardenia* plants are rich in cycloartane triterpenes, sesquiterpenes, flavonoids, and benzoic acid derivatives and exhibit various biological activities, such as cytotoxicity and angiogenesis (Youn et al., 2016). A preceding study on compounds isolated from *G. sessiliflora* revealed the presence of triterpenoids and flavonoids with cytotoxic effects on several cancer cell lines, including MCF-7, P-388, KB, and HT-29. In addition, the compounds also showed anti-viral activities (Thanasansurapong et al., 2020). Moreover, several extracts and compounds from various *Gardenia* species have demonstrated cytotoxic, anti-viral, antiulcer, analgesic, diuretic, and hypotensive biological effects (Silva et al., 1997).

Several preceding studies reported that flavonoids and triterpenoids isolated from other plant species showed inhibitory potencies on ABC transporters in cancer cell lines and displayed synergistic interactions with anti-cancer agents (Gonçalves et al., 2020; Yan et al., 2014). However, no investigation has been conducted regarding the effect of flavonoids and cycloartane triterpenoids isolated from *G. sessiliflora* on ABC transporters. Therefore, this study investigated the potential effect of flavonoids and cycloartane triterpenoids isolated from *G. sessiliflora* on the P-gp function, which plays a major role in multidrug resistance in NSCLC (Jaromi et al., 2021). The inhibition of P-gp-mediated drug efflux, which improves the conventional chemotherapeutic treatment of the NSCLC adenocarcinoma cell line, was evaluated.

2. MATERIALS AND METHODS

2.1 Materials

RPMI 1640 medium (Cat. No. 31-800-022), TryPLE select enzyme (Cat. No. A1217701), 0.4% trypan blue stain (Cat. No. 15250-061), and fetal bovine serum (FBS) (Cat. No. 10270-106) were purchased from Gibco BRL (GRAND Island, NY, USA). Penicillin/streptomycin (SV30010) was supplied by Fisher Scientific (Waltham, Massachusetts, USA). Dimethyl sulfoxide (DMSO) (Cat. No. D8418), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cat. No. M5655), and paclitaxel (Cat. No. T7191) were obtained from Sigma Aldrich (St. Louis, MO, USA). [^{3}H]-paclitaxel (Cat. No. MT2081) was provided by Moravek, Inc. (Brea, CA, USA), with [^{3}H]-digoxin (Cat. No. NET222250UC) obtained from Perkin Elmer (Boston, MA, USA). FITC Annexin V apoptosis detection kit I (Cat. No. 556547) was purchased from BD Biosciences (San Jose, CA, USA). Eight purified compounds were isolated from *G. sessiliflora,* as described in our previous study (Thanasansurapong et al., 2020). Compounds were dissolved in DMSO as stock solutions and stored frozen under light-protected conditions.

2.2 Cell culture

Human A549 cell lines (ATCC CRM-CCL-185), identified as lung adenocarcinoma cells, were purchased from the American Type Culture Collection (Manassas, VA) and routinely grown in RPMI-1640 medium containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C with a 5% CO_2 incubator (Shellab, Cornelius, OR, USA). The cells were subcultured using enzymatic digestion with 1.25X TryPLE select enzyme when reaching ~80% confluence. The morphology of the cell was taken using the phase contrast light microscope (Nikon Eclipse TS 100, Nikon Instruments Inc., Japan).

2.3 Assay of intracellular drug accumulation

A549 cells were seeded in a 24-well plate for 4-5 days. The cells were washed twice with a warm transport buffer and further preincubated with the buffer for 15 min at 37°C. The cells were then treated with a buffer containing [^{3}H]-digoxin or [^{3}H]-paclitaxel alone or test compounds for 2 h. After incubation, the buffer was removed, and the cells were rinsed rapidly three times with an ice-cold buffer. Cells were solubilized by adding 10% SDS to 0.4 M NaOH for 24 h. The samples were neutralized with HCl and transferred to vials containing liquid scintillation to measure the accumulated radioactivity using a beta counter (Perkin Elmer, Germany), as previously described (Soodvilai et al., 2011). The radioactivity was recorded in counts per minute (CPM) per square centimeter of the surface area of the confluent monolayer.

2.4 Cell viability assay

The MTT assay was used to assess cell viability. A549 cells were plated in 96-well plates and then incubated in humidified air with 5% CO_2 at 37°C. When the cells reached 100% confluency, they were treated with the test compounds. After incubation, the MTT solution (0.5 mg/mL in medium) was added to each well and incubated for 3 h. The developed purple formasan crystals were solubilized with 100 µL DMSO. Absorbance was determined at 562 nm and 630 nm using the Varikosan® multimode reader (Thermoscientific, US). Cell viability was expressed as a percentage of the vehicle control.

2.5 Apoptosis assay

FITC Annexin V apoptosis detection kit (BD Biosciences, CA, USA) was used to detect cell apoptosis. After being

treated with compounds, the cells were collected by trypsinization and stained with FITC Annexin V and propidium iodide (PI). The number of live cells and early apopotsis and late apoptosis were then analyzed using a CytoFLEX flow cytometer (Beckman Coulter, IN, USA). Data acquisition and analysis were performed by the CytExpert for CytoFLEX acquisition and analysis (Beckman Coulter, IN, USA).

2.6 Statistical analysis

All values were represented as mean±S.D. The statistical significance of any differences in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by the Tukey test using GraphPad Prism 5 software (GraphPad, CA, USA). The level of significance was set at $p<0.01$.

3. RESULTS

3.1 Inhibitory effect of the compounds on P-gp-mediated drug transport in NSCLC

First, the inhibitory effects of three flavonoids (compounds 1-3) and five cycloartanes (compounds 4-8) isolated from *G. sessiliflora* were determined. The chemical structures of the compounds are shown in Figure 1. The inhibitory effect of the compounds on P-gp-mediated transport was monitored by measuring the accumulation of ^{3}[H]-digoxin, a substrates of P-gp, in A549 cells. Incubation of the cells with a buffer containing ^{3}[H]-digoxin plus 100 µM of compounds 5 and 8 significantly increased the ^{3}[H]-digoxin cellular accumulation (Figure 2A). The inhibitory effect of compounds 5 and 8 on P-gp-mediated ^{3}[H]-digoxin transport was comparable to that of verapamil (100 µM), a typical P-gp inhibitor. Next, the inhibitory effect of compounds 5 and 8 on the P-gp transport function was confirmed by monitoring the accumulation of ^{3}[H]-paclitaxel. Paclitaxel is a chemotherapeutic drug which is a selective substrate of P-gp. The results demonstrated that compound 8 showed the most inhibitory effect on [^{3}H]-paclitaxel in A549 cells (Figure 2B).

3.2 Concentration-dependent effect of compound 8 on P-gp-mediated [^{3}H]-paclitaxel transport

Since compound 8 showed the most inhibitory effect on paclitaxel accumulation, its concentration dependent effect was determined. The results showed that incubating the cells with compound 8 (10-50 µM) for 2 h significantly increased the ^{3}[H]-paclitaxel accumulation in a concentration dependent manner (Figure 3A). Next, we tested whether the inhibitory effect of compound 8 was mediated by reduced cell viability. The effects of compound 8 (1-50 µM) on cell viability were determined. Cells were incubated with compound 8 for 2 h with cell viability, then measured using MTT assay. The results revealed no significant cytotoxic effect at a concentration of 1-50 µM for compound 8 (Figure 3B).

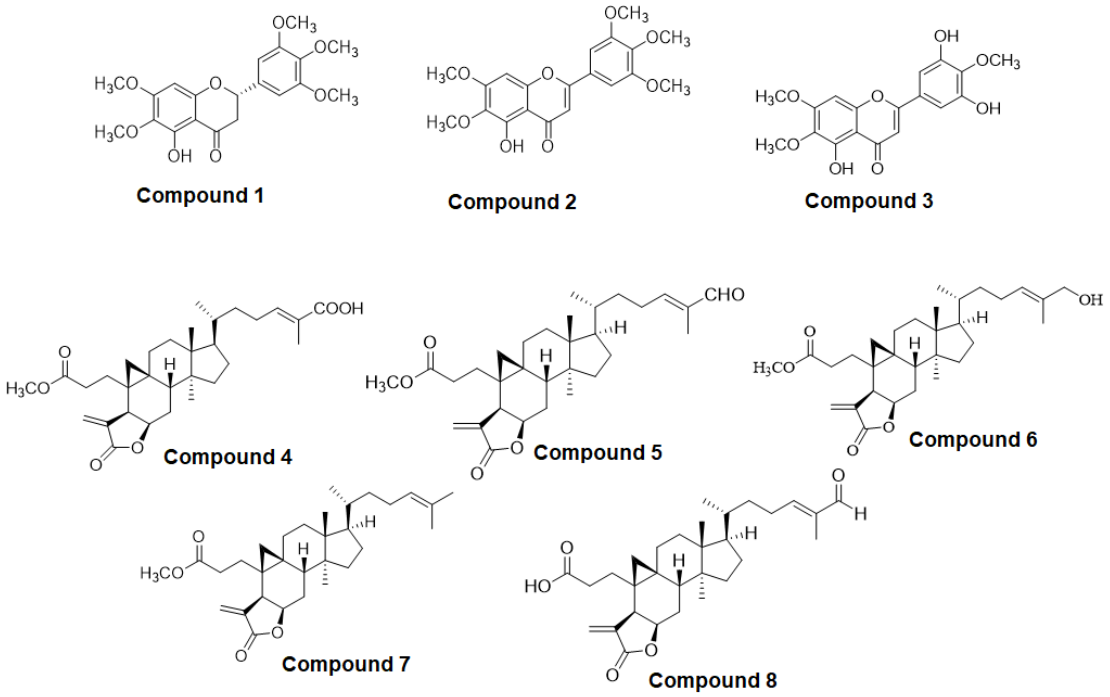

Figure 1. Chemical structure of the pure compounds isolated from *Gardenia sessiliflora*

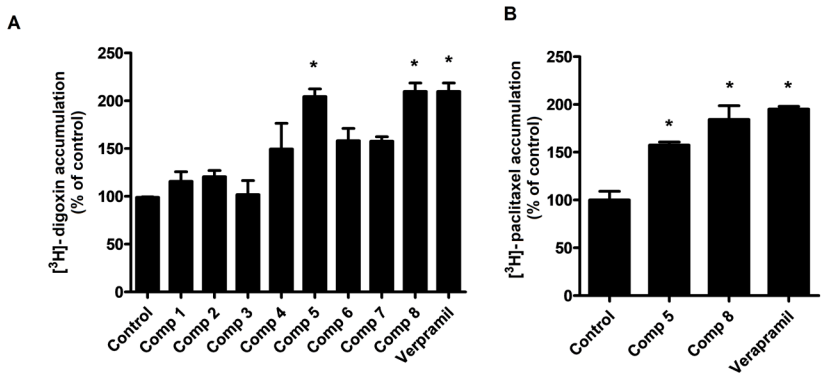

Figure 2. Screening the effects of compounds 1-8 isolated from *Gardenia sessiliflora* on the P-gp transport function in A549 cells
Note: *p<0.01 compared to the untreated control.

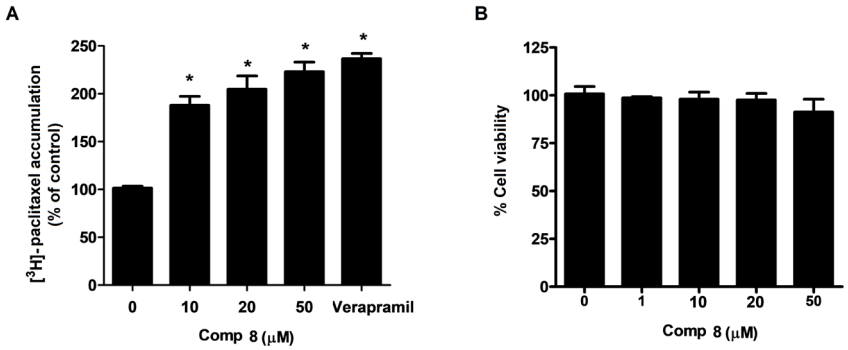

Figure 3. Concentration-dependent effects of compound 8 on P-gp-mediated transport in A549 cells; cells were incubated with compound 8 for 2 h with the 3[H]-paclitaxel accumulation (A) and cell viability determined using the MTT assay (B)
Note: *p<0.01 compared to the untreated control.

3.3 Effects of compound 8 in the combination treatment with paclitaxel on cell viability

The chemosensitizing effects of compound 8 on paclitaxel treatment were determined in A549 cells. As shown in Figure 4A, a concentration dependent effect on A549 cell viability was found following treatment for 24 h with paclitaxel at 1-500 nM. Increasing the concentration of paclitaxel up to 1 μM did not produce further inhibition. This might imply the resistance of A549 cells to paclitaxel treatment. Treating the cells with compound 8 at 1-10 μM did not affect the cell viability, whereas higher concentrations caused a reduction in cell viability (Figure 4B). Compound 8 (10 μM) showed an inhibitory effect on P-gp for the sensitivity of A549 cells to paclitaxel. The cells were incubated with the vehicle, paclitaxel (50, 100, and 500 nM), or paclitaxel plus 10 μM compound 8 for 24 h, followed by the measurement of cell viability and cell death. Treating the cells with paclitaxel alone significantly decreased viability when compared to the vehicle-treated cells. Cell viability after co-treatment of the cells with compound 8 and paclitaxel significantly decreased when compared to paclitaxel-treated cells (Figure 4C). The chemosensitizing effect of compound 8 was confirmed by determining its apoptosis. As presented in Figure 4D, treating A549 cells with 100 nM paclitaxel significantly increased the apoptotic cells when compared to the vehicle control. Co-treating the cells with 10 μM compound 8 showed a significant increase in apoptotic cells when compared to paclitaxel-treated cells.

4. DISCUSSION

Chemotherapy is the recommended treatment for patients with NSCLC. Standard chemotherapy is a combination of chemotherapeutic agents, including platinum-based agents, with drugs that stabilize the microtubule polymer (Hellmann et al., 2016). Nevertheless, the therapeutic failure of chemotherapy in NSCLC is due to the development of drug resistance, which leads to tumor re-occurence and disease progression. One of the most commonly encountered drug resistance mechanisms is the increased efflux of anti-cancer drugs (Dong et al., 2009). Inhibition of drug efflux is one of the targets for overcoming chemotherapy resistance. The present study revealed that the inhibitory effect of cycloartane triterpenoid isolated from *Gardenia sessiliflora* on paclitaxel efflux improved the anti-cancer effect of paclitaxel.

Among the eight pure compounds isolated from *Gardenia sessiliflora*, compounds 5 and 8 showed the most significant increase in the accumulation of ^{3}[H]-digoxin,

which is a substrate of both BCRP and P-gp (Pavek et al., 2005; Shi et al., 2011), and ^{3}[H]-paclitaxel, a selective substrate of P-gp (Kawahara et al., 2020). Protein expression of P-gp was found in A549 cells but not BCRP (Jaromi et al., 2021). These data implied that the inhibitory effects of compounds 5 and 8 on paclitaxel transport might be mediated by the inhibition of P-gp. Since the inhibitory effect of compound 8 was more pronounced, its chemosensitizing effects were further determined in A549 cells. The inhibitory effects of compound 8 on P-gp could be the result of a decrease in cell viability. Data on cell viability obtained from the MTT assay supported that the inhibitory effects of compound 8 at concentration ≤ 20 μM might not be mediated by the decrease in cell viability. P-gp is an ABC efflux transporter (Xiao et al., 2021), inhibited by a decrease in the cellular ATP level (Amin, 2013). The present study did not rule out the possibility that compound 8 could interfere with the metabolic activity of cells, subsequently reducing the ATP level. This possibility needs to be further verified.

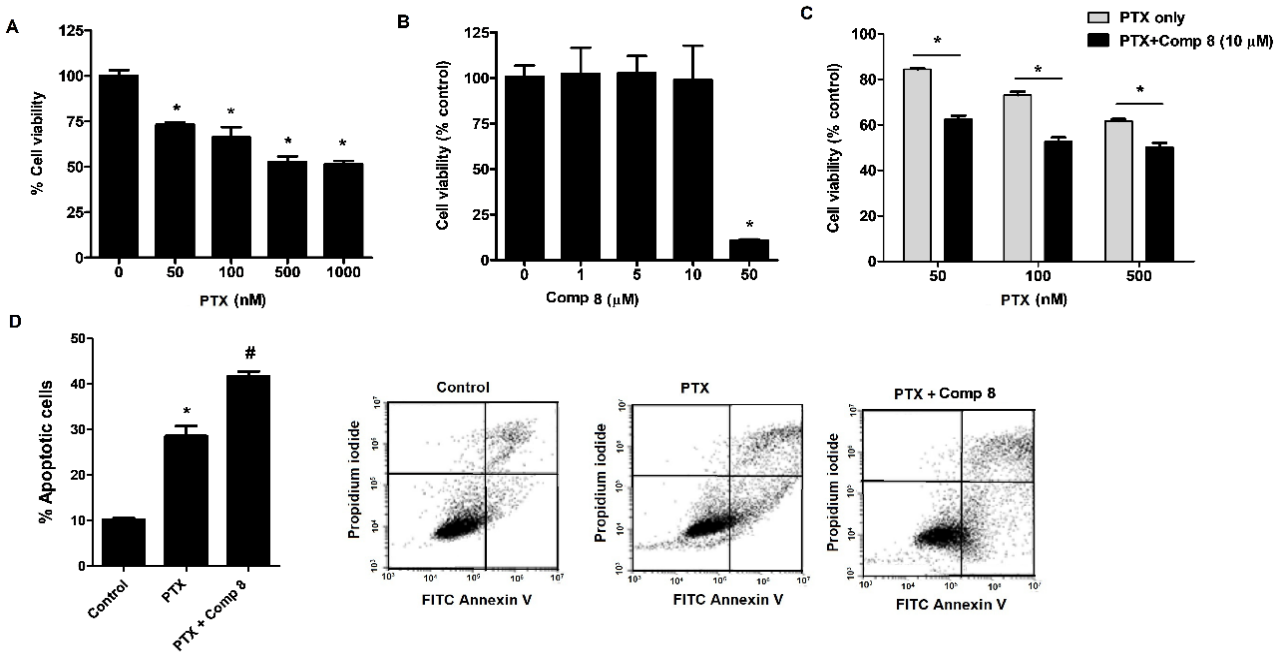

Figure 4. Effects of compound 8 on paclitaxel sensitivity; (A-B) concentration dependent cytotoxicity effect of paclitaxel (PTX) and compound 8 on cell viability at 24 h incubation, (C-D) effect of compound 8 (10 μM) on the sensitivity of A549 cells to paclitaxel (PTX) induced apoptosis
Note: *p< 0.01 compared to the control, whereas #p<0.01 compared to the PTX-treated cells.

As P-gp plays a crucial role in the unresponsiveness of cancer to chemotherapy, the inhibition of P-gp is one of the targets for improving cancer treatment (Szakács et al., 2014). Our data demonstrated that compound 8 inhibited P-gp-mediated paclitaxel efflux. This result raised the possibility that this compound could increase the anti-cancer activity of paclitaxel in A549 cells. Paclitaxel is one of the chemotherapeutic agents for NSCLC treatment since it causes G2-M phase arrest of the cell cycle and induces apoptosis of the lung cancer cell line (Feng et al., 2013; Georgiadis et al., 1997). The IC_{50} of paclitaxel on the viability of A549 cells obtained in this study (about 500 nM) differed from other studies (10 nM to 2 μM) (Owonikoko et al., 2010; Strachowska et al., 2021). The variation in IC_{50} might be dependent on experimental protocols. We investigated the combination treatment of paclitaxel with 10 μM of compound 8. The results showed a significant P-gp inhibition effect without decreasing the viability of A549 cells. The significant decrease in cell viability and increase in apoptosis in the combination treatment were likely due to P-gp inhibition, which subsequently increased the intracellular paclitaxel accumulation. Thus, compound 8 could improve the anti-cancer activity of the conventional chemotherapeutic agent, paclitaxel, in A549 cells at least via P-gp mediated transport. Despite several studies and efforts to develop suitable P-gp inhibitors, none are used in current clinical settings for cancer treatments due to the incidence of side effects and lack of clinical response (Lai et al., 2020). The chemical structures related to compound 8 may be further developed as P-gp inhibitors and used in combination with conventional chemotherapy in NSCLC.

5. CONCLUSION

The present study revealed that cycloartane triterpenoids compound 8 increases the anti-cancer effect of paclitaxel in A549 cells by increasing the intracellular accumulation level of paclitaxel via the inhibition of P-gp. The findings in this study may provide useful information for the development of compound 8 as an adjuvant in conventional chemotherapeutic agents.

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