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Original Article

Effects of decursinol angelate on viability and apoptosis in PC-3 prostate cancer cells: In vitro study

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Abstract

Prostate cancer represents the predominant malignant neoplasm observed in the male population and ranks second in terms of mortality attributable to malignant neoplasm among men. Decursinol angelate (DA), derived from the plant *Angelica gigas* Nakai (AGN), has demonstrated anti-cancer effectiveness through the induction of intrinsic and extrinsic apoptosis pathways, inhibition of cancer cell proliferation, having anti-neovascularization, anti-inflammatory anti-oxidative activities and stimulating the immune process. The aim of this study was to determine the IC_{50} dose of DA on human prostate cancer cell line PC-3, as well as to assess its effects on cell viability and apoptosis. PC-3 cells were utilized in this study due to its hormonal therapy resistance characteristics. The treatment commenced with the determination of the IC_{50} of DA and cell viability using the CCK-8 method as a baseline dose. A combination with abiraterone acetate (AA) was performed using an escalated dose based on its IC_{50} to identify whether DA has a synergy with AA in decreasing PC-3 cell viability. Apoptosis levels were measured using flow cytometry. The research includes a control group (C) and three treatment groups: AA group, DA group, and DA+AA group. GraphPad Prism, SPSS version 25 and CompuSyn software were used for statistical analysis. This study reveals that the IC_{50} dose of DA is 13.63 µM. The decrease of PC-3 cell viability exposed to DA occurs in a dose-dependent manner. Additionally, PC-3 cell apoptosis is significantly increased in both the DA group and DA+AA compared to the control. Moreover, no difference in apoptosis level is noted between the DA and AA groups. Notably, there is a synergy between DA and AA, where a specific dose equal to one-fourth of the IC_{50} dose results in greater efficacy in reducing PC-3 cell viability compared to individual treatments of either DA or AA at the IC_{50} doses. This study demonstrates the potential of decursinol angelate as a single drug or combined with abiraterone acetate to reduce viability and increase apoptosis of castrate-resistant prostate cancer cells.

Keywords: Prostate cancer, angelica gigas, abiraterone acetate, phytotherapy, apoptosis

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Introduction

Prostate cancer represents the predominant malignant neoplasm observed in the male population and ranks second in terms of mortality attributable to malignant neoplasm among men in both the European and American regions [1]. All patients who have undergone androgen deprivation therapy (ADT) will eventually progress to the advanced stage, known as metastatic castrate-resistant prostate cancer (mCRPC) [2]. Treatment options for mCRPC are limited and

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palliative in nature. The progression of this disease is often associated with significant morbidity and mortality. Before 2010, chemotherapy using docetaxel was the only treatment that demonstrated efficacy in overall survival, certified in the United States by the Food and Drug Administration (FDA) and commonly utilized as first-line therapy worldwide [3]. Some randomized controlled trials (RCTs) have resulted in the approval of new therapies for mCRPC treatment. These therapies have all shown benefits in overall survival for mCRPC patients who have progressed after receiving docetaxel therapy [4-6]. Additionally, newer-generation hormonal therapies such as abiraterone acetate (AA) and enzalutamide have also demonstrated OS benefits in patients who are either asymptomatic or have mild symptoms and have not previously received chemotherapy [7].

The clinical use of AA has evolved since its proven efficacy in mCRPC. Some studies have demonstrated efficacy and safety for various indications. Initially, its use was investigated for mCRPC patients who had undergone docetaxel chemotherapy, then for mCRPC before chemotherapy, and finally in combination with poly (ADP-ribose) polymerase (PARP) enzyme inhibitor [4,5,8,9]. The COU-AA-301 study, published in 2011, played a crucial role as a pivotal clinical trial that resulted in the initial approval of AA in the United States, where AA exhibited a 35% decrease in mortality risk among patients with mCRPC who previously received docetaxel [10]. However, AA therapy is associated with a high cost, and chemotherapy has serious side effects [11].

Angelica gigas Nakai (AGN), belonging to the *Umbelliferae* family, has been used as a traditional herbal medicine and food ingredient for centuries. The coumarin compound decursinol angelate (DA) is one of the most abundant active compounds extracted from AGN roots, alongside decursin and nodakenin, and this compound has shown strong anti-cancer activity in some studies [12-14]. Its anti-cancer activities are associated with the induction of intrinsic and extrinsic apoptosis pathways in cancer cells, reducing cell proliferation, having anti-angiogenic activity in cancer tissues; as well as having anti-inflammatory and antioxidant activity that stimulates the immune process [15].

Olaparib, a PARP enzyme inhibitor, has revolutionized the treatment of prostate cancer. A study demonstrated that olaparib significantly enhanced progression-free survival (PFS) and overall survival in mCRPC patients with mutations in breast cancer genes *BRCA1*, *BRCA2*, and ataxia-telangiectasia mutated (*ATM)* genes, even after earlier hormonal agent therapy [16]. Nevertheless, olaparib is considered the last choice for prostate cancer treatment due to its associated risks and adverse effects, as well as its expensive cost. Out of the 256 mCRPC patients who had olaparib therapy, 46% experienced anemia, 41% experienced fatigue and nausea, and 18% discontinued the medicine [17]. Both DA and olaparib induce apoptosis by activating pro-apoptosis proteins caspase-3, -8 and -9, and cleaving PARP proteins, suggesting a comparable mode of action [18]. Hence, DA could serve as an alternative substitute in the treatment of mCRPC.

The PC-3 cell line is a model of hormonal therapy resistance prostate cancer cells derived from bone metastases of mCRPC patients and is considered one of the most representative cell types for in vitro study [19]. Nevertheless, there have been no previous studies that fully determined the DA IC_{50} dose for PC-3 cell line and to assess the effects of DA on the viability and apoptosis on PC-3 cells. The aim of this study was to determine the IC_{50} dose of DA on PC-3 cell and to assess its effects on cell proliferation and survival.

Methods

Study design and setting

This in vitro experimental study employed a post-test-only design using human mCRPC PC3 cell lines. All procedures were conducted at the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. The IC_{50} values of DA and AA were utilized as baseline doses to evaluate their effects on apoptosis and viability of PC3 cells. The study was divided into four groups: control, DA, AA, and a combination of DA and AA. Following a 72-hour exposure period, apoptosis levels were measured using flow cytometry and the synergistic effect was determined based on the viability of PC3 cells. A detailed study setting is presented in **Figure 1**.

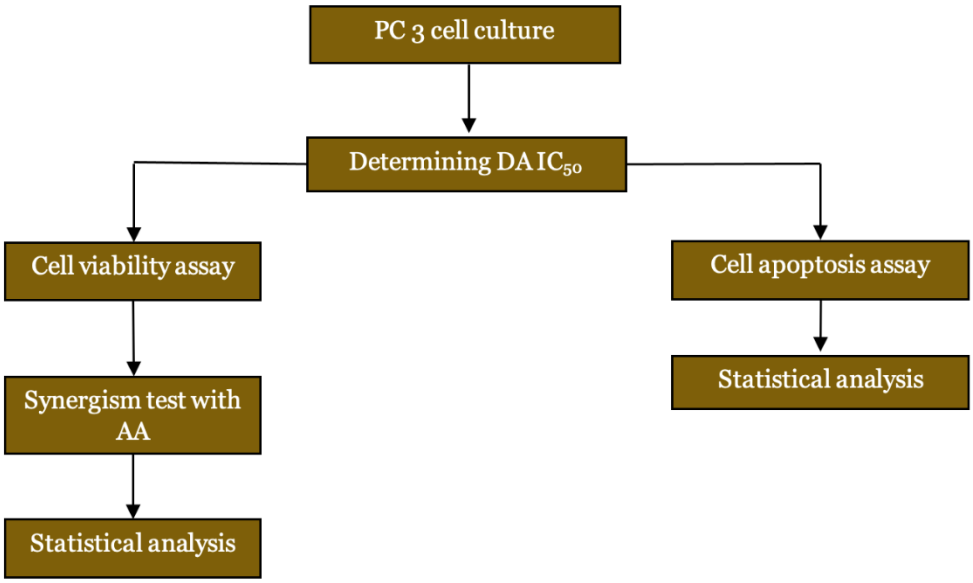

Figure 1. Flowchart of the study. Decursinol angelate (DA) IC_{50} was used as the baseline dose for cell apoptosis assay and cell viability assay for determining synergism with abiraterone acetate (AA) as a standard therapy for mCRPC.

Cell culture

The human PC-3 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, US). After receiving from ATCC, the PC-2 cells were thawed in a water bath with 37°C temperature for two minutes, and were centrifuged in 125 g for 5 minutes speed. The cells were established in a complete medium and transferred to a dish with an additional complete growth medium for 15 minutes until reaching normal pH (7.6). Subculturing was performed when the cell density reached 70%. The culture of cells was conducted in an F-12K medium enriched with fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), penicillin/streptomycin, and phosphate-buffered saline (PBS).

Cell viability assay and IC_{50} measurement

Cell viability assay was conducted to assess DA capability on decreasing PC-3 cell proliferation and its IC_{50} dose. DA was purchased from Sigma Aldrich (Saint Louis, MO, USA). PC-3 cells were placed at 96-well plates with 3,000–4,000 cell density per well and enabled to attach overnight. Subsequently, various doses of DA (0.25×, 0.5×, 1× and 2× IC_{50} dose) and AA (0.25×, 0.5×, 1× and 2× IC_{50} dose) were administered to the cell's plates for 72 hours in complete medium. AA was purchased from Sigma Aldrich (Saint Louis, MO, USA). The AA IC_{50} dose, 66.9 µM, was retrieved from our previous study using the same PC-3 cells [20]. Following treatment, the cells were mixed with the Cell Counting Kit-8 (CCK-8) viability assay (Elabsciences, Houston, TX, USA), 50 µL CCK-8/mL culture media at 37°C and incubated for 2 hours. The cell culture's relative absorbance was measured using an RT-2100C microplate reader (Rayto Life and Analytical Sciences Co., Ltd Shenzen, China) with a wavelength of 450 nm.

Quantitative measurement of cell apoptosis

To analyze whether DA has a role in regulating PC-3 cell death, a cell apoptosis assay was conducted in this study. The apoptosis assay consisted of four groups: one control group (untreated cells) and three treatment groups with IC_{50} of DA, IC_{50} of AA, and a combination of DA and AA IC_{50} obtained from the previous viability test. The PC-3 cells were rinsed with BioLegend Cell Stain Buffer (Elabsciences, Houston, TX, USA) two times before being suspended in Annexin V Binding Buffer (Elabsciences, Houston, TX, USA) at a 1.0×10^7 cells/mL concentration. Following this, 100 µL of the cell suspension was dispensed into a 5 mL reaction tube, and 5 µL FITC Annexin V (Elabsciences, Houston, TX, USA) and 10 µL propidium iodide solution (Elabsciences, Houston, TX, USA) was added. Subsequently, the cells were incubated for 15 mins at room temperature (25°C) with dark environment. Finally, 400 µL of Annexin V Binding Buffer was added into each tube.

Fluorescence-activated cell sorting (FACS) calculations were conducted using gating to differentiate cells based on complexity and cell size. Subsequently, cells were read based on the emitted fluorescence of cells within the gating using Becton Dickinson (BD) FACSCalibur (BD Bioscience, Franklin Lakes, NJ, USA). In this assay, the number of cells analyzed in each cycle was 10,000 cells.

Statistical analysis

The percentage of cell viability values obtained from the CCK-8 assay and the gating percentage from FACS were collected and then analyzed. Data distribution was evaluated through a normality test. The non-parametric Kruskal-Wallis test was employed since the data was not normally distributed, followed by the Mann-Whitney test to examine differences between each group (control, AA, DA and AA+DA). Data were analyzed using the statistical software package IBM SPSS Statistics 25 (IBM, New York, USA).

The IC_{50} dose calculation was performed using the optical density results from the CCK-8 assay, which yielded the percentage viability of each treatment. Subsequently, an assessment was conducted using a curve and logarithmic exponent with the assistance of GraphPad Prism software version 8 (GraphPad Software Inc., La Jolla, CA, USA). Synergistic data analysis in the study was carried out using a lobogram analysis method with the assistance of CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) to obtain a combination index (CI) and dose reduction index (DRI) derived from the isobologram curve. In this study, CI<1 was classified as a synergistic effect, CI=0 as an additive effect and CI>1 suggested an antagonist effect as recommended previously [21].

Results

IC_{50} of decursinol angelate (DA) and abiraterone acetate (AA) for PC-3 cells

Cell viability within each group was quantitatively measured by spectrophotometry at a wavelength of 450 nm after the addition of 10 µL of CCK-8 to each well. The treatment of PC-3 with titrated doses of DA (5 µM, 10 µM, 20 µM, 50 µM, and 100 µM) resulted in absorbance values ranging from 0.319 to 0.415. Viability calculations were performed by dividing the relative absorbance values for each treatment dose by the absorbance value of the control group. The viability values for each treatment group are visualized in **Figure 2**. DA induced a dose-dependent suppression of PC-3 cell viability with an r^2 value of 0.9969. The IC_{50} value of DA against PC-3 cells was determined to be 13.63 µM. IC_{50} dose of the positive control (AA) in this study was determined based on previous research findings on PC-3 cells, which was found to be 66.9 µM [20].

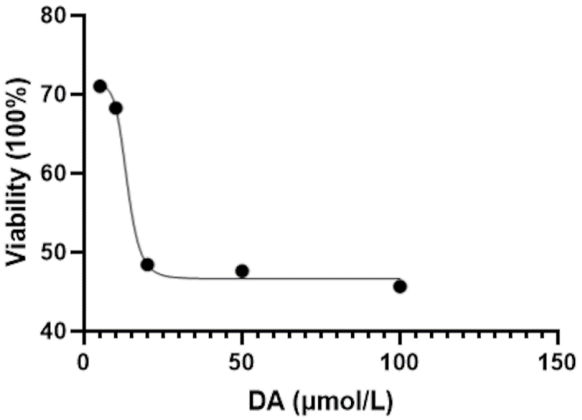

Figure 2. Impact of decursinol angelate (DA) on PC-3 cell line proliferation. Evaluation of PC-3 cell line viability was conducted over a 72-hour period.

Effect of decursinol angelate (DA) on apoptosis of PC-3 cells

Flow cytometry results provided an overview of the distribution of PC-3 cells based on cell size and complexity. Gating processes were conducted to separate PC-3 cells from cell debris.

Subsequently, the treatment groups of PC-3 cells were separated based on their emitted fluorescence intensity into four distinct quadrants (**Figure 3**).

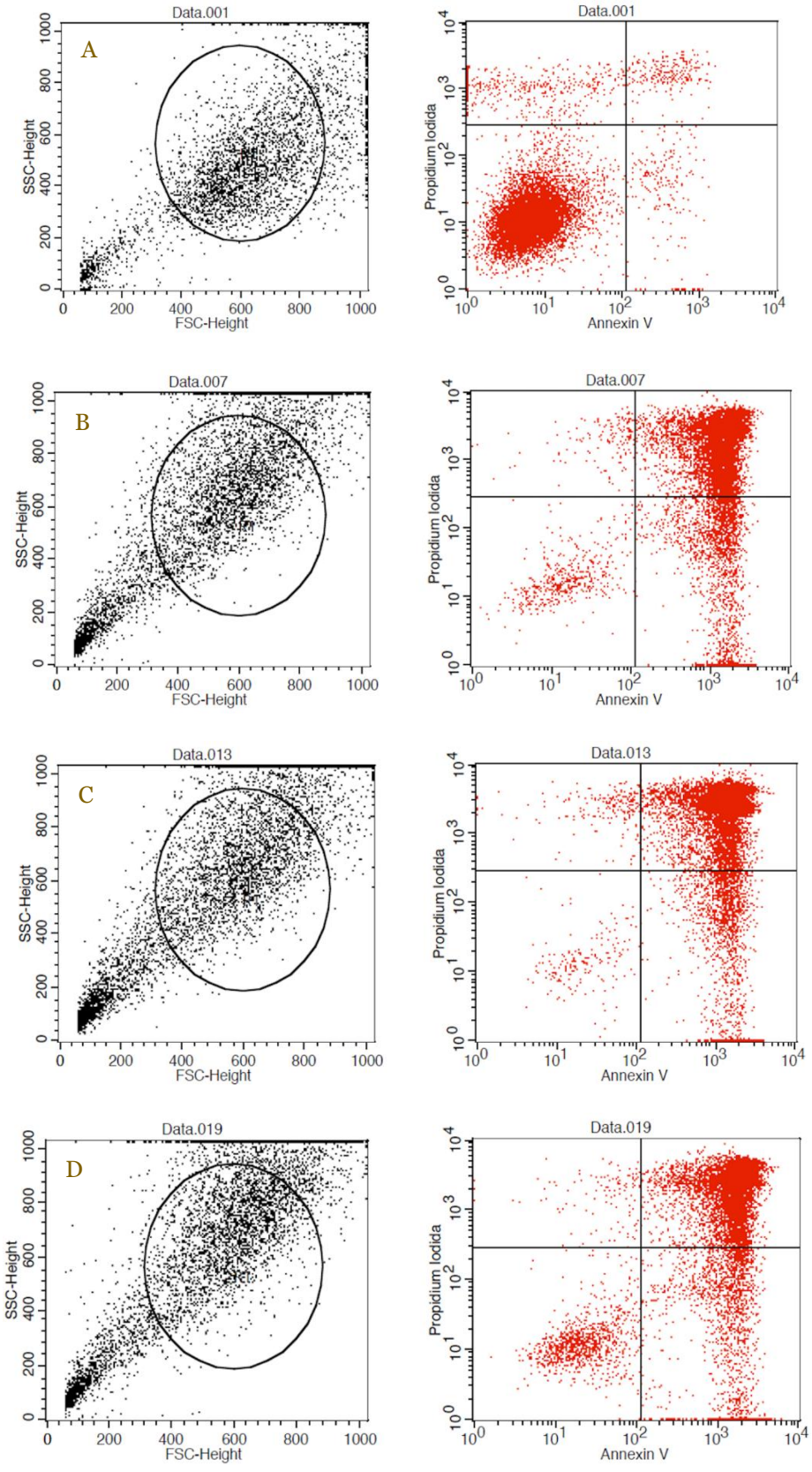

Figure 3. Fluorescence-activated cell sorting (FACS) results of the research treatment. (A) Gating results and quadrants of PC-3 control cells. (B) Gating results and quadrants of PC-3 cells exposed to abiraterone acetate (AA). (C) Gating results and quadrants of PC-3 cells exposed to decursinol angelate (DA). (D) Gating results and quadrants of PC-3 cells exposed to both AA and DA.

The Mann-Whitney test was performed to investigate the differences in total apoptosis (early and late apoptosis) in PC-3 cells among study groups. The Kruskal-Wallis test indicated that at least one group was different from the others (p=0.001) (**Table 1**). Post hoc analyses indicated a significant difference in apoptosis levels in the group that was administered DA, AA and the combination of DA+AA if compared with the control group, as well as the group that received the AA only when compared to a combination of DA+AA (**Table 2**). Interestingly, DA only group was not statistically different between AA or a combination of DA+AA.

Table 1. Comparison of percentage of total apoptosis in PC-3 cells after treatment with abiraterone acetate, decursinol angelate or a combination of both

Groups	Mean±SD	Normality	p-value
Control	5.62±1.21	0.191	0.001
Abiraterone acetate	94.83±0.61	0.999	
Decursinol angelate	89.50±7.54	0.010	
Abiraterone acetate and decursinol angelate	85.87±5.88	0.022	

Table 2. Post hoc analysis of inter-group treatment in PC-3 cells exposed to abiraterone acetate and decursinol angelate

Groups	p-value
Control vs abiraterone acetate	0.004*
Control vs decursinol angelate	0.004*
Control vs abiraterone acetate and decursinol angelate combination	0.004*
Abiraterone acetate vs decursinol angelate	1.000
Abiraterone acetate vs abiraterone acetate and decursinol angelate combination	0.004*
Decursinol angelate vs abiraterone acetate and decursinol angelate combination	0.336

* Statistically significant at p<0.05

Synergy of decursinol angelate with abiraterone acetate in reducing PC-3 cells viability

To further analyze the synergistic effects of AA and DA, the results of cell viability of each treatment group were utilized and analyzed using Compusyn software. The analysis revealed a synergistic effect between AA and DA from fraction affected (Fa) 10% to 97%, with a combination index (CI) of less than 1 (**Table 3** and **Figure 4**). The combination of AA and DA exhibited a synergistic effect with a CI value of 0.156 at ¼ IC_{50}, with respective doses of AA at 16.72 µM (6 times lower than the IC_{50} of AA single drug) and DA at 3.4 µM (128 times lower than the IC_{50} of DA single drug).

Table 3. Synergistic effects at each fraction affected (Fa) of abiraterone acetate (AA) and decursinol angelate (DA) combination with its combination index

Abiraterone acetate dose (µM)	Decursinol angelate dose (µM)	Effect	Combination index (CI)
16.72	3.40	0.28	0.156
16.72	6.80	0.16	0.095
16.72	13.63	0.11	0.071
16.72	27.26	0.12	0.078
33.45	3.40	0.15	0.177
33.45	6.80	0.16	0.187
33.45	13.63	0.16	0.190
33.45	27.26	0.08	0.111
66.90	3.40	0.30	0.645
66.90	6.80	0.16	0.373
66.90	13.63	0.20	0.454
66.90	27.26	0.15	0.360
133.80	3.40	0.28	1.199
133.80	6.80	0.28	1.207
133.80	13.63	0.21	0.937
133.80	27.26	0.09	0.481

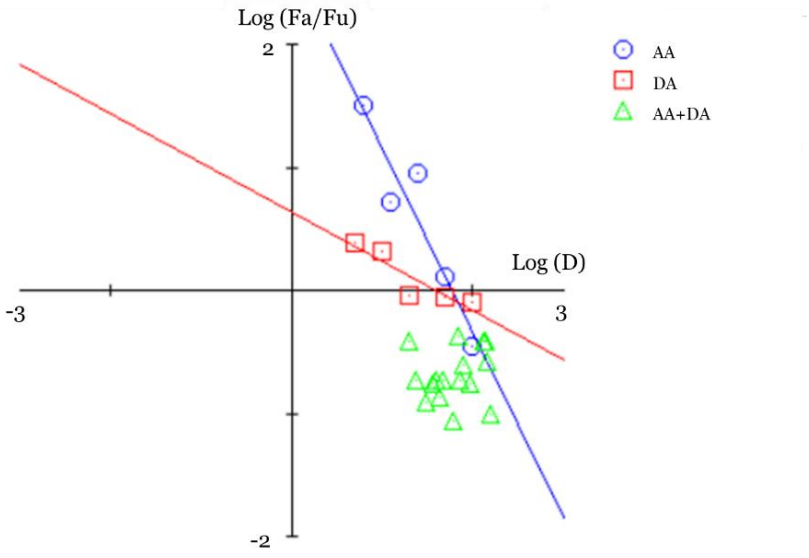

Figure 4. Median effect model graph for combination doses of decursinol angelate (DA) and abiraterone acetate (AA). AA+DA: a combination of AA and DA; D: dose required to produce fraction affected; Fa: fraction affected; Fu: fraction unaffected.

Discussion

Traditional herbal medicine from Asia is gaining popularity, with AGN being one of the most widely used plants in Korea. There have been several studies investigating the anti-cancer effects of AGN and its primary components decursin and DA across diverse cancer types, such as melanoma, leukemia, sarcoma, prostate cancer, and cervical cancer [14,15,18,22,23]. The anti-cancer effects of AGN and decursin have been associated with the suppression of various viability signaling pathways, including the PI3K/AKT, ERK, NF-κB, and Wnt/β-catenin pathways [15]. However, there has been limited research on DA for the treatment of mCRPC.

This study investigates the effects of DA on viability and apoptosis in mCRPC PC-3 cells. The initial step was to determine its IC_{50}, the concentration of a substance required to induce a half-maximal inhibitory effect (50% reduction) on a specific biological process, such as cell viability within a given cell population [24]. Methods for determining the IC_{50} dose vary; the two most commonly used methods are the microculture tetrazolium technique (MTT) and the more recent CCK-8 assay. The CCK-8 assay for determining the IC_{50} dose is currently more popular because it calculates intracellularly produced cell metabolites. The probability of error in reading the density of reagents is lower using the CCK-8 method compared to the MTT assay [25].

In this study, the IC_{50} value of DA was 13.63 μM, and DA could decrease PC-3 cell viability. No previous studies have sought the IC_{50} dose in PC-3 cells. Some previous studies have reported IC_{50} doses of DA in different cell types; for example, 10 μM against HeLa cells [18] and 75 μM against B16F10 cells [23]. The IC_{50} dose of AA (positive control) used in this study was based on the findings of our previous study, which was 66.9 μM [20]. In another study, the IC_{50} dose was 39.66 μM [26]. Another study used a dose of 30 μM to induce apoptosis in prostate cancer cells [27]. The difference in IC_{50} doses between this study and previous studies may be due to differences in the DA or AA materials obtained from different companies, as well as different extraction methods and various cell types [18,23,26]. Some studies used prostate cancer cells sensitive to hormonal therapy, while others used resistant prostate cancer cells, as well as cells that had undergone neuroendocrine malignancy mutations [20,26,27]. The treatment duration in each study also varied significantly; a study used a cell incubation time of 48 hours [25] while other studies used 72 hours of incubation time [20,26,27]. In this study, the IC_{50} value determination was performed using the CCK-8 assay method. A previous study analyzed IC_{50} values using other methods, such as the MTT assay or trypan blue, which may result in differences in IC_{50} calculations [23].

The present study tested apoptosis in PC-3 cell cultures with DA treatment. The results of this study showed a significant increase in apoptotic PC-3 cell values at the IC_{50} dose of DA, AA

and the combination of both compounds. Although the combination of DA with AA was not better than the single drug AA in terms of the number of apoptosis, the single drug DA did not differ significantly in the values of apoptotic cells compared to AA as the standard therapy for mCRPC. This may suggest that DA has potential as an alternative therapy for androgen-insensitive prostate cancer.

The apoptosis mechanisms elicited by DA involve upregulation of TNF-related apoptosis-inducing ligand (TRAIL) expression [18]. TRAIL is a death-inducing ligand that triggers cell death through catalytic activation mediated by receptors on the cell surface within the cysteine proteases series and will affect the extrinsic apoptosis pathway. Increased TRAIL expression will activate pro-apoptotic proteins caspase-3, -8 and -9, as well as cleavage of PARP protein [18]. Cleavage of PARP will cause a cell unable to repair DNA damage, ultimately leading to apoptosis [28]. Inhibition of PARP in cancer cells caused by DA may make this compound a potential adjuvant therapy in the current management of prostate cancer. Previous studies indicated that the apoptosis triggered by AA compounds impacts the equilibrium between pro-apoptotic factors (Caspase-3, p21, and Bax protein) and anti-apoptotic factors (survivin) [27,29].

To determine the synergistic interaction between DA and standard therapy (AA) in mCRPC, viability tests with CCK-8 were performed, where drug concentrations were gradually increased based on the ratio of each drug's IC_{50}. Our data indicated that a combination of DA and AA could inhibit cell growth better than single drugs. Although the number of apoptotic cell parameters in the DA and AA combination at the IC_{50} dose was not better than single drug treatment, this study found that the combination of DA and AA decreased the PC-3 cell viability at lower doses than each single drug. The CI value calculated with CompuSyn software indicated a good synergy. The present findings underscore the rationale for exploring the therapeutic potential of DA in combination with AA. Recent studies have also shown a synergy between decursin (an isomer of DA) and doxorubicin for multiple myeloma cells, as well as a synergy between decursin and Myc inhibitor on lymphoma cell viability and apoptosis [30,31]. Although our study found no significant difference in apoptosis data between the combination and single drug groups, this result should be interpreted with caution. PC-3 cancer cells are essentially slow-growing cells with a low doubling time. This may require longer observation to determine the level of apoptosis in a time-dependent manner [32].

The primary limitation of this study is the reliance on an in vitro model. Although research using cell culture is indispensable for the preliminary screening of anti-cancer agents, they could not fully replicate the complex interactions and microenvironments that are present in an in vivo setting. Consequently, the observed effects of DA on PC-3 cell viability and apoptosis may not fully translate to clinical efficacy in mCRPC patients. While our findings suggest potential mechanisms of action, including PARP cleavage and caspase activation, these mechanisms were inferred rather than directly demonstrated. Detailed mechanistic studies, possibly involving gene knockdown or overexpression approaches, are necessary to conclusively establish the pathways through which DA exerts its anti-cancer effects. The absence of in vivo studies in this research restricts the ability to assess the pharmacokinetics, bioavailability and systemic toxicity of DA. Future studies should incorporate animal and human models to evaluate these critical aspects and to verify the anti-cancer potential of DA that has been observed in vitro.

Conclusion

DA exhibits significant anti-cancer effects on PC3 cells by decreasing cell viability and increasing apoptosis both as a single agent and in combination with AA. The combination of these two drugs demonstrates a synergistic effect in reducing PC3 cell viability and enhancing apoptosis. The level of apoptosis is not significantly different between DA and AA single drugs. This study suggests that reducing AA dose while used in combination with DA might reduce the side effects of AA without compromising oncological outcome. However, this result should be validated using in vivo studies.

Ethics approval

Not required.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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