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Does Combination of DNR and Casticin show advantage in favor of apoptosis on AML leukemia stem-like cell lines? A preliminary study

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ABSTRACT: Acute myeloid leukemia (AML) is a form of acute leukemia with the highest incidence and the lowest overall survival rates. Insufficiency of targeting leukemia stem cells (LSC) is the main obstacle that causes drug resistance and relapse in AML. Another important problem is chemotherapeutics' toxicity. Developing a combination, including well-known chemotherapeutics in lower dose and new agent that have capacity to target LSC may be more reliable and practical way to overcome these limitations. Previously, we found that Casticin polyphenol induces apoptosis in AML stem-like (KG1a) and parental (KG1) cell lines without affecting healthy cell. Therefore, for the first time, we aimed to find synergistic combination of Daunorubicin (DNR) and Casticin to target apoptosis in both LSC and leukemic blasts with less toxicity. Synergism of DNR-Casticin combinations on KG1a, KG1, HL-60 cells were determined with MTT viability assay by Chou-Talalay method. The apoptotic/necrotic effects of combinations were evaluated with Annexin V- PI kit by flow cytometry. Synergistic combination of 0.25 µM DNR + 0.0625 µM Casticin (combination index, CI<1) decreased cell viability to 45.3% and 63.2% in KG1a, KG1 cell lines, respectively. However, the combination-induced apoptosis (KG1a: 5 %; KG1: 5.8%) were not higher than 0.25 µM DNR-induced (KG1a: 9.4%; KG1: 8.1%) or 0.0625 µM Casticin-induced (KG1a: 3.8%; KG1: 5.1%) apoptosis (p>0.05). Our study showed that synergistic combination of DNR-Casticin causes important decrease in cell viability. Although we did not detect increase in apoptosis with the combination, we presume that other cell death pathways may be included. The highest apoptosis was obtained by the treatment of 2 µM Casticin alone in KG1a (21.7%), KG1 (26.5%), HL-60 (14.6%). Therefore, we think that Casticin polyphenol might be the possible candidate for new targeted therapy studies for AML.

KEYWORDS: Acute myeloid leukemia; apoptosis; combination; leukemia stem cell

1. INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of acute leukemia in children and adults with genetic, epigenetic, and phenotypic heterogeneity [1,2]. Sequential treatment with Cytarabine and daunorubicin/idarubicin remains to be the standard induction protocol in AML treatment. Daunorubicin (DNR), which suppresses DNA replication via topoisomerase II inhibition, is one of the most widely used agents in the treatment of AML [3,4]. Chemotherapy regimens show promising results in patients with newly diagnosed AML but generally relapses occur within 2 years in many patients. Hence, 5-years survival rates are still very low [5-7].

Conventional chemotherapeutics generally target rapidly dividing cells, but the most important point is targeting of leukemic stem cells (LSC) during a chemotherapy treatment [8, 9]. Therefore, it is considered that the main obstacle in AML treatment arises from LSC, which cause drug resistance and relapse that lead to poor survival rates. LSC (CD34+, CD38-) are the small and variable fraction (only 1–4%) of the total AML cells, which have the ability self-regenerate, unlimited repopulation potential and remain in the G_0/G_1 quiescent phase for a long time [10,11]. For instance, Bailly et al. have shown that AML cell lines with high a CD34+ population, such as KG1 and KG1a, are 10-15 times more resistant to DNR treatment than CD34- AML cells [12]. Besides, the local and systemic toxicity are the limitations of the current therapeutics, especially cardiotoxicity is an important obstacle that should be overcome [13,14]. Nevertheless, current studies recommended DNR to be included in targeted AML treatment

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approaches [15,16]. According to these studies, high-dose DNR administration significantly increases remission and survival rates in AML patients with FLT3-ITD, NPM1, and DNMT3A mutations.

Apoptosis is a well-documented cell death program, and it also plays an important role in cell development and hematopoietic system regulation. Escaping from apoptotic cell death is an important feature of the cancer cells, which provides uncontrolled growth of the cells. For this reason, triggering apoptosis is an important therapeutic approach in cancer treatment [17-19]. On the other hand, necrosis is originally considered as passive, accidental, unregulated cell death resulting in inflammatory cascade. Therefore, chemotherapeutic-induced necrosis may provoke cancer cell proliferation and drug resistance [17,18,20-22]. Hence, agents that trigger apoptosis with a low level of necrosis are important for cancer treatment.

To overcome drug resistance and relapse, phytochemicals are promising sources for targeted drug research. For decades, several drugs have been developed from natural compounds [23-26]. Casticin (3', 5-dihydroxy-3, 4', 6, 7-tetramethoxyflavone) is a predominant component of the fruit of Vitex trifolia L. and has been widely used in traditional Chinese medicine. A body of studies have reported that Casticin induces cell death in AML blast cell lines [27-29]. In addition, a micro-array study revealed that among the other well-known polyphenols, Casticin has the highest anti-proliferative effects on lymphoblastic leukemia cell lines [29]. Our previous study with Casticin showed that Casticin induces apoptosis in both leukemia stem-like KG1a and its parental KG1 cell lines without affecting healthy human peripheral blood mononuclear cells [30].

Contrary to the fact that DNR loses its importance over time, it is considered as an agent that should be included in targeted AML treatment approaches [15,16]. Higher dosage, non-selective toxicity, development of drug resistance, and relapse are important factors that limit the effectiveness of DNR in AML treatment [13,14,31,32]. In addition, LSC and leukemic blasts must be targeted simultaneously for the ultimate AML treatment [33-35]. Therefore, there is a need for an effective treatment strategy that specifically target LSC and leukemic blasts without damaging healthy cells.

Developing a combination with a well-known chemotherapeutic in lower dose and new agent that have the capacity to target LSC would be increase the possibility to reach a successful AML treatment. Therefore, we aimed to find a possible synergistic effect with DNR-Casticin combinations for the first time to increase the therapeutic efficacy and reduce DNR's side effects. We used Chou-Talalay method and MTT viability assay to find synergism index value (CI). To analyze the apoptotic and necrotic effects of DNR-Casticin combinations, we performed Annexin V- PI flow cytometry analyses.

2. RESULTS

2.1. Cell viability

The effect of Casticin (50-0.125 µM) and DNR (4-0.03125 µM) were examined for 24 and 48 hours. Considering the average plasma half- life of DNR (26.7 h) [36]. DNR IC50 values were calculated for 24 h, which were 0.56 µM, 0.97 µM, and 0.06 µM for KG1a, KG1 and HL-60 cell lines, respectively (Fig 1). Casticin IC50 value was determined as 2 µM for 24 h in all three cell lines (Fig 2).

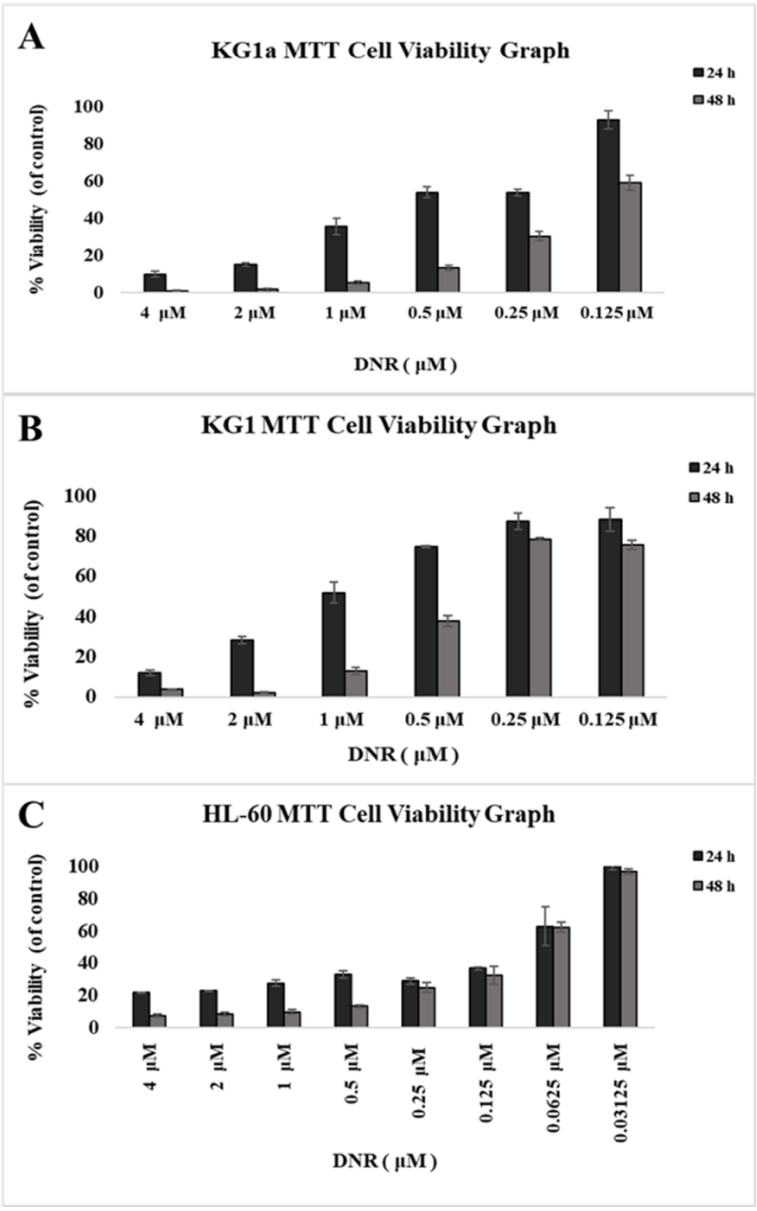

Figure 1. Effects of DNR on KG1a(a), KG1(b), HL-60 (c) cell viabilities. Percentage of cell viabilities relative to vehicle- treated (0.008%DMSO-1%FBS solution) control group. The results are expressed as Mean ±Standard deviation of 3 experiments.

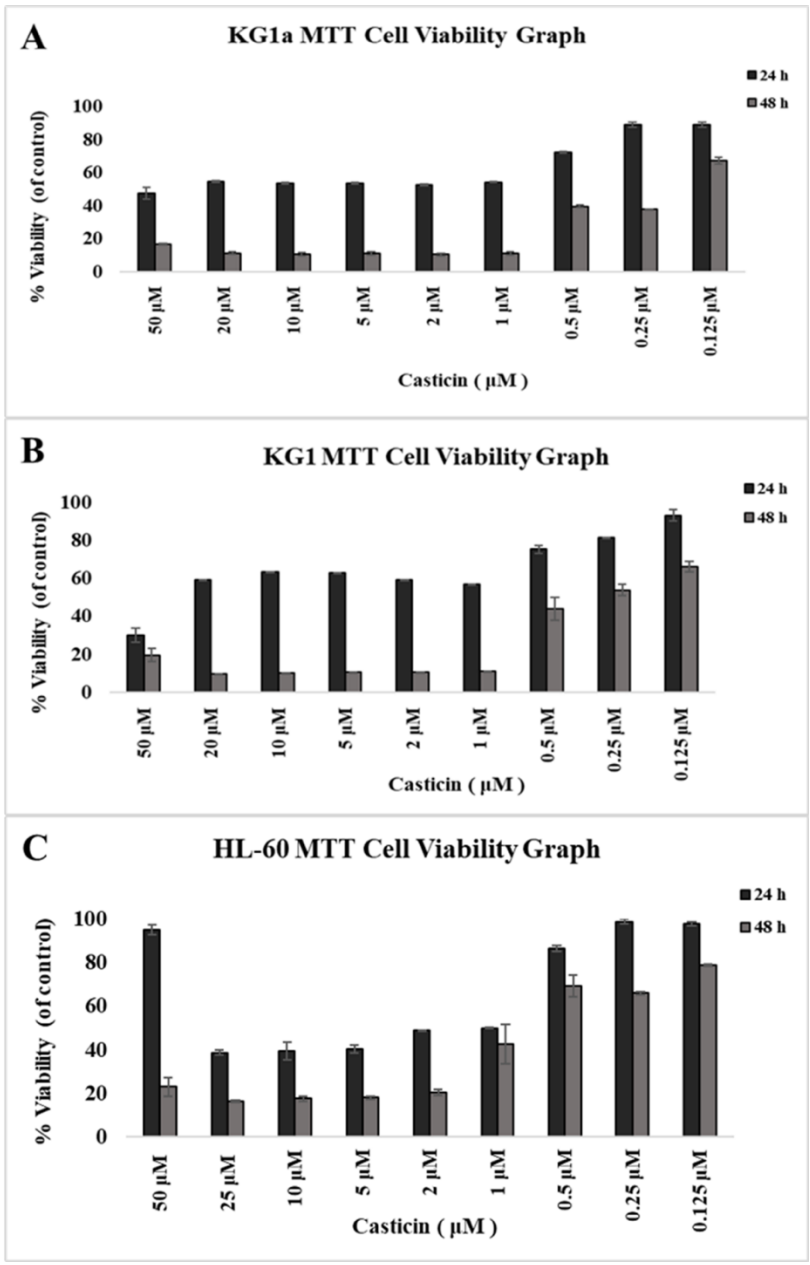

Figure 2. Effects of Casticin on KG1a(a), KG1(b), HL-60 (c) cell viabilities. Percentage of cell viabilities relative to vehicle- treated (0.008%DMSO-1%FBS solution) control group. The results are expressed as Mean±Standard deviation of 3 experiments.

2.2. **Chou-Talalay analyses**

Potential synergistic effects of DNR and Casticin combinations in AML cell lines were investigated. According to the Chou-Talalay method, the effects of constant ratio (1:2 or 1:8) of the combinations on cell viability were analyzed by MTT test for 24 hours (Table 1). A synergistic effect was only found for 0.25 µM DNR-0.5 µM Casticin group in KG1a (CI<1), but synergism was not found for KG1 cell lines (CI>1) in any ratios (Supplementary data 1 and 2). After then, we decided to use "non-ratio" Casticin-DNR combinations for further Chou Talalay studies. As a result of 0.25 µM DNR to the KG1a cell line, the viability was 61.1% compared to the control group, and the viability was 85.9% after 0.0625 µM Casticin treatment.

Table 1. Combination ratios

DNR (µM)	CASTICIN (µM)	RATIO
0.5	1	1:2
0.25	0.5	1:2
0.125	0.25	1:2
0.0625	0.5	1:8
0.125	1	1:8

The treatments of 0.25 µM DNR and 0.0625 µM Casticin to KG1a cell line resulted in 61.1% and 85.9% cell viability compared to their control groups, respectively. Treatment of 0.25 µM DNR+0.0625 µM Casticin combination decreased the viability up to 45.3% compared to the control group. Hence, this non-ratio combination also showed a synergistic effect (CI<1) for KG1a cell line. The viability results for KG1 cell line were 82.6% and 81.8% after 0.25 µM DNR and 0.0625 Casticin treatments, respectively. Similarly, 0.25 µM DNR+0.0625 µM Casticin combination decreased the viability to 63.2% compared to the control group and showed a synergistic effect (CI<1) for KG1 cells (Fig 3).

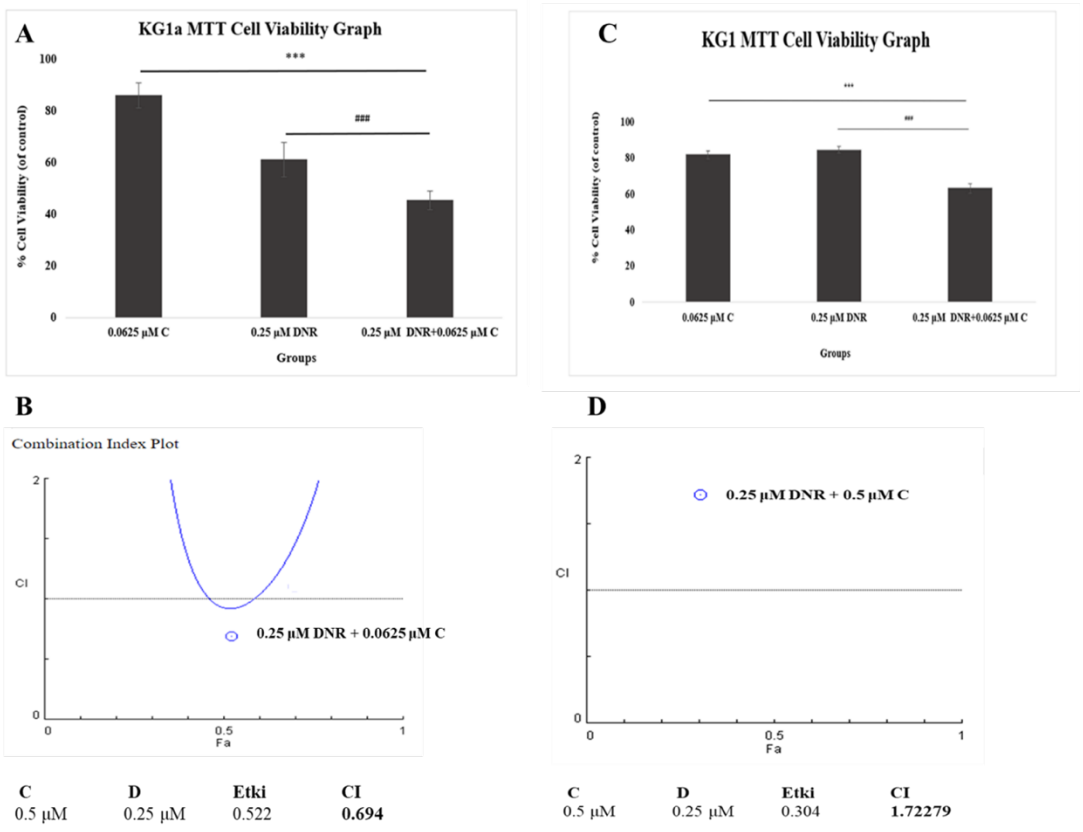

Figure 3. The effect of combined treatment of Casticin (0.0625 µM) and DNR (0.25 µM) agents ON KG1a and KG1 cell lines for 24 hours. The relative cell viability percentages according to vehicle- treated (0.008%DMSO-1%FBS solution) control group (a, c) by MTT test and CI values according to the Compusyn method (b, d). The results are expressed as Mean±Standard deviation of 3 experiments.

2.3. **Apoptotic/necrotic cell death analyses**

To confirm the synergistic effects of Casticin 0.0625 µM-DNR 0.25 µM combination for cell lines, apoptotic cell death ratios were assessed. As a result of 0.25 µM DNR treatment of KG1a and KG1 cell lines, 10.9% and 8.1% apoptosis, respectively and low level of necrosis (0.8%) were detected for both cell lines. The apoptotic percentages of 0.0625 µM Casticin treatment were 7.3% and 9.3% and necrosis percentages were 0.3%; 0.8% for the cell lines, respectively. However, in consequence of Casticin (0.0625 µM)-DNR (0.25 µM) combination treatment did not produced any significant change on either in apoptosis (5%; 5.8%) and necrosis (0.8%; 0.9) for the cell lines ($p>0.05$) (Fig 4, 5).

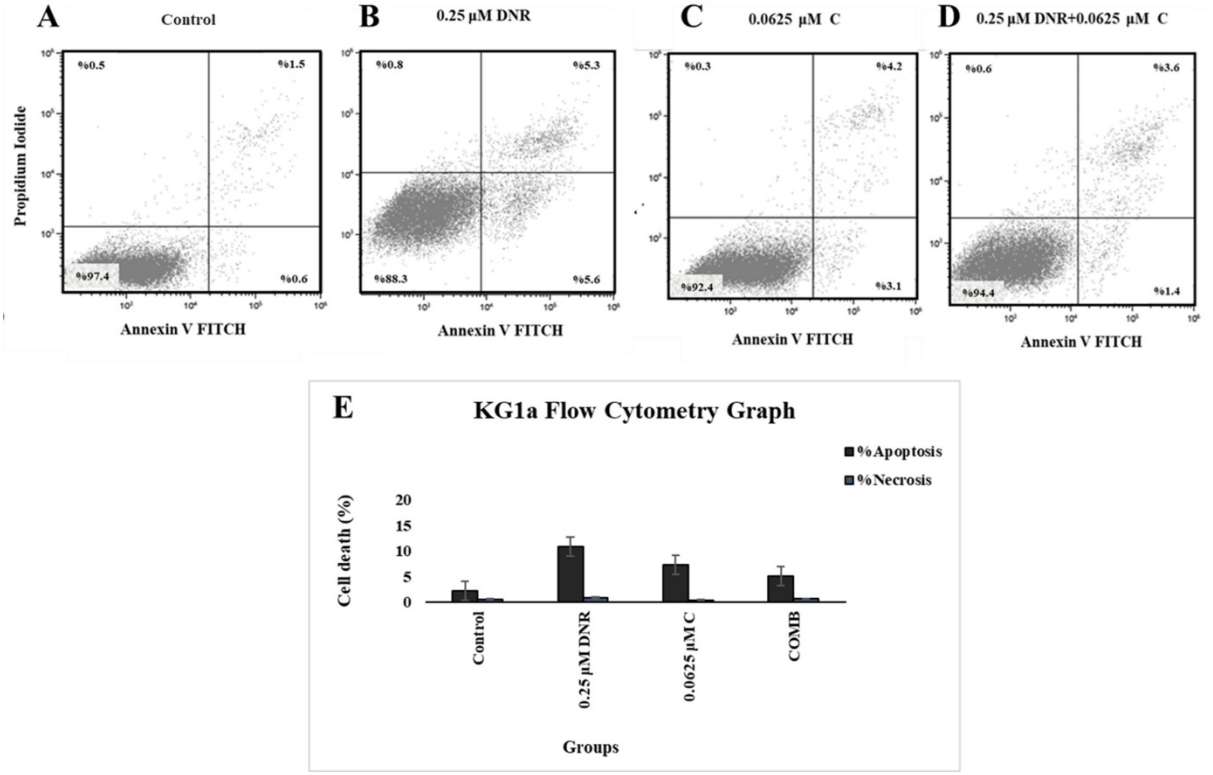

Figure 4. Flow cytometric Apoptotic/Necrotic cell death analyses of KG1a cell line. Flow cytometric diagram of vehicle- treated (0.008%DMSO-1%FBS solution) Control (a), 0.25 µM DNR-treated (b), 0.0625 µM Casticin-treated (c), and combination (COMB) (Casticin (0.0625 µM)-DNR (0.25 µM)- treated (d) KG1a cells. Quadrants represents necrotic cells (upper left), viable cells (bottom left), late apoptotic cells (upper right), early apoptotic cells (lower right). Apoptotic cell death ratios among the groups (e) Comparison of apoptotic cell death between single agent-treated groups (0.25 µM DNR/0.0625 µM Casticin) and combination group (COMB). The results are expressed as Mean±Standard deviation of 3 experiments.

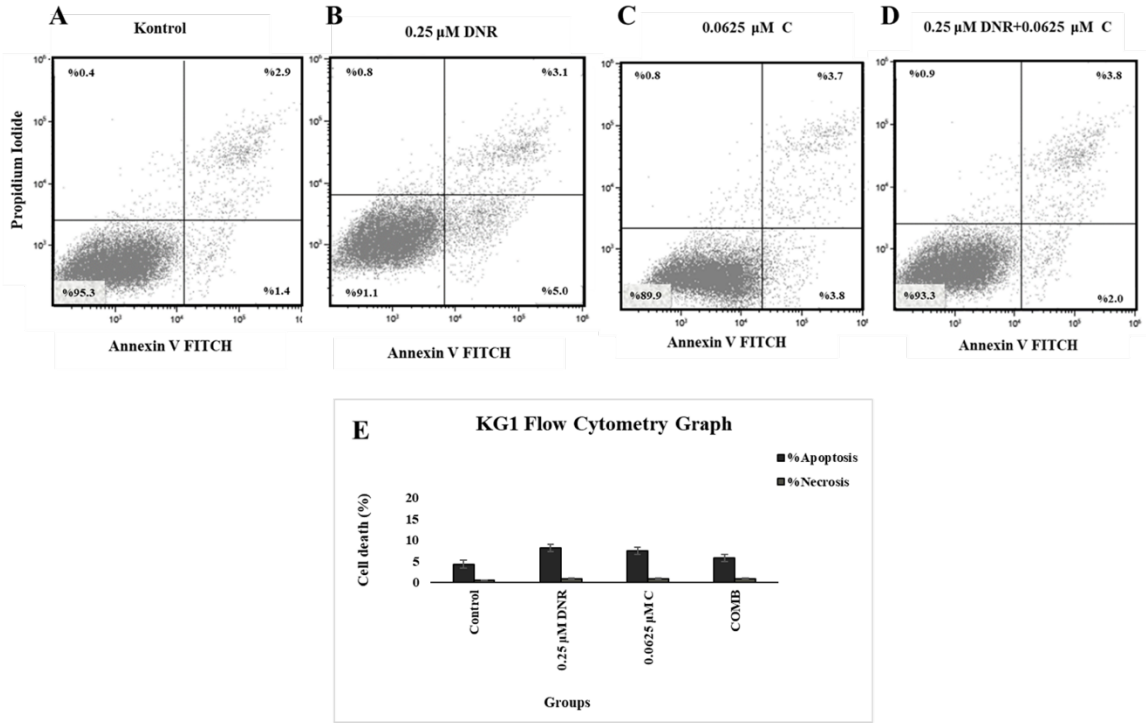

Figure 5. Flow cytometric Apoptotic/Necrotic cell death analyses of KG1 cell line. Flow cytometric diagram of vehicle- treated Control (a), 0.25 µM DNR-treated (b), 0.0625 µM Casticin-treated (c), and combination (COMB) (Casticin (0.0625 µM)-DNR (0.25 µM)- treated (d) KG1 cells. Quadrants represents necrotic cells (upper left), viable cells (bottom left), late apoptotic cells (upper right), early apoptotic cells (lower right). Apoptotic cell death ratios among the groups (e) Comparison of apoptotic cell death between single agent-treated groups (0.25 µM DNR/0.0625 µM Casticin) and combination group (COMB). The results are expressed as Mean±Standard deviation of 3 experiments.

These results directed us to create and examine different combinations of constant DNR concentration (0.0315 µM) with various Casticin concentrations on stem cell-like KG1a cell line for 24 h and 48 h (Table 2).

Table 2. DNR-Cast combination concentrations applied for 24 and 48 h in KG1a cell line

Concentrations	
DNR (µM)	**Cast (µM)**
0.03125	0.0625
0.03125	0.125
0.03125	0.25
0.03125	0.5
0.03125	1
0.03125	2

Increases in apoptotic percentages were evaluated for different concentrations of DNR-Casticin combinations (Table 3). The results showed that the highest apoptotic percentages were obtained from 1 µM and 2 µM Casticin treatments. Combinations of DNR (0.0625 µM or 0.03125 µM) with Casticin did not lead to any increase in apoptotic percentages in comparison with single Casticin treatments ($p>0.05$).

Table 3. DNR-Cast combination concentrations in the KG1a cell line

Concentrations	
DNR (µM)	**Cast (µM)**
0.03125	1
0.03125	2
0.0625	1
0.0625	2
0.125	1
0.125	2
0.25	1
0.25	2

Results were 7.5% apoptosis and 0.8% necrosis for 0.0625 µM DNR treatment, 16.6% and 21.7% apoptosis with 1.3% and 1.2% necrosis for 1 µM and 2 µM Casticin and 13.5% and 12.6% apoptosis with 1.2% and 1.4% necrosis for 0.0625 µM DNR+1 µM Casticin or 0.0625 µM DNR+2 µM Casticin combinations to the KG1a cell line, respectively. Results were 14.9% apoptosis and 1.6% necrosis for 0.03125 µM DNR treatment, 18.4% apoptosis with 1.4% necrosis for 0.5 µM Casticin and 18.4% apoptosis with 0.9% necrosis for 0.03125 µM DNR+0.5 µM Casticin combination in KG1a cell line, respectively (Fig 6).

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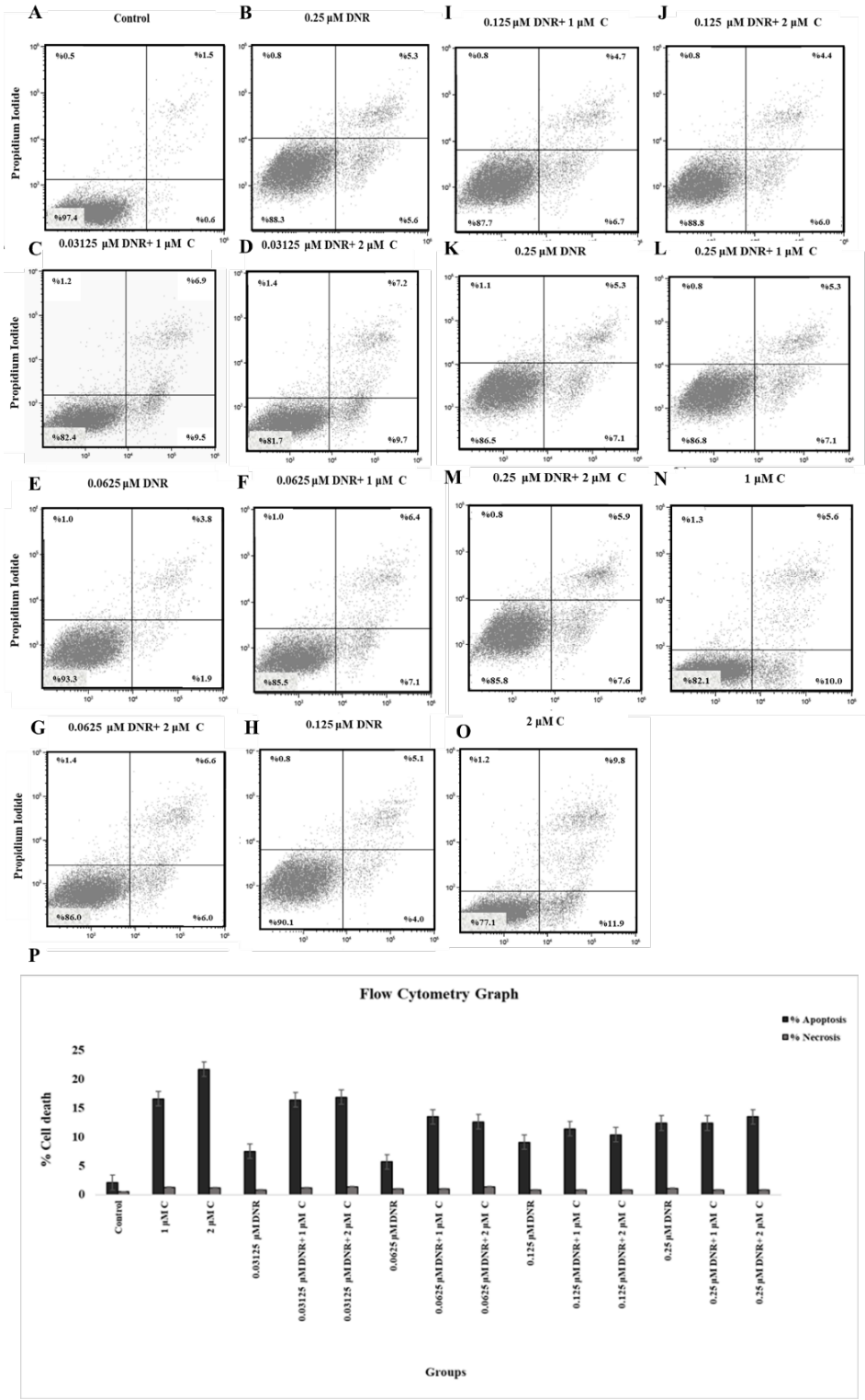

Fig 6. Flow cytometric Apoptotic/Necrotic cell death analyses of KG1a cell line. Flow cytometric diagram of vehicle- treated Control (a), DNR (0.03125-0.25 µM)-treated (b, e, h, k) and Casticin (1-2 µM) -treated (n, o), and their combinations (c, d, f, g, j, l, m) in KG1a cells. Quadrants represents necrotic cells (upper left), viable cells (bottom left), late apoptotic cells (upper right), early apoptotic cells (lower right). Apoptotic cell death ratios among the groups (e) Comparison of apoptotic cell death between single agent-treated groups and combination groups. The results are expressed as Mean ±Standard deviation of 3 experiments.

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The treatments with the combinations for 24 h and 48 h did not significantly increase apoptosis in comparison with the treatment of Casticin (1 µM and 2 µM) ($p>0.05$) (Fig 7, 8).

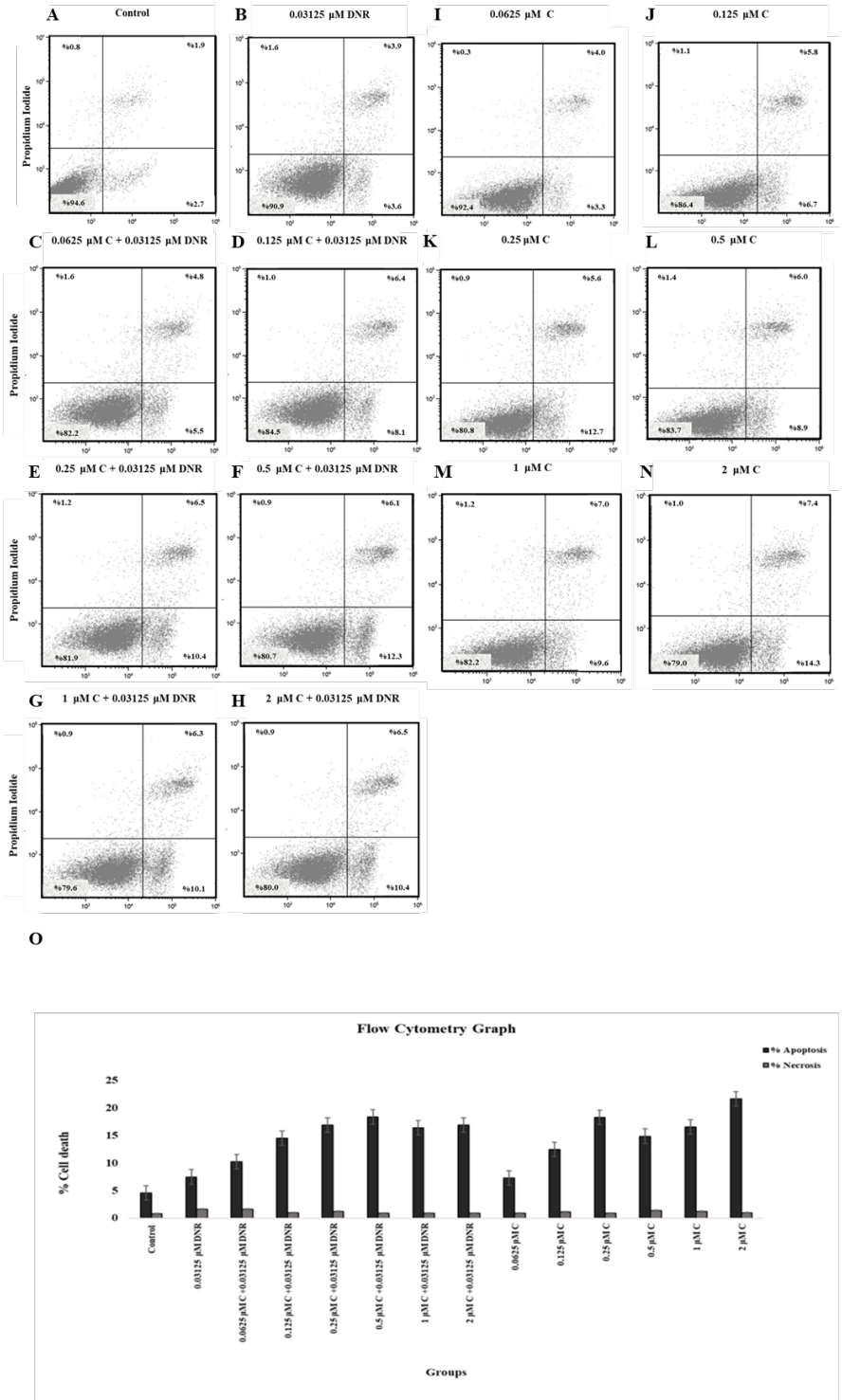

Fig 7. Flow cytometric Apoptotic/Necrotic cell death analyses of KG1a cell line. Flow cytometric diagram of of vehicle- treated Control (a), DNR (0.03125µM) -treated (b) and Casticin (2-0,0625 µM) -treated (i, j, k, l, m, n), and their combinations (c, d, e, f, g, h) in KG1a cells. Quadrants represents necrotic cells (upper left), viable cells (bottom left), late apoptotic cells (upper right), early apoptotic cells (lower right). Apoptotic cell death ratios among the groups (o) Comparison of apoptotic cell death between single agent-treated groups and combination groups. The results are expressed as Mean±Standard deviation of 3 experiments.

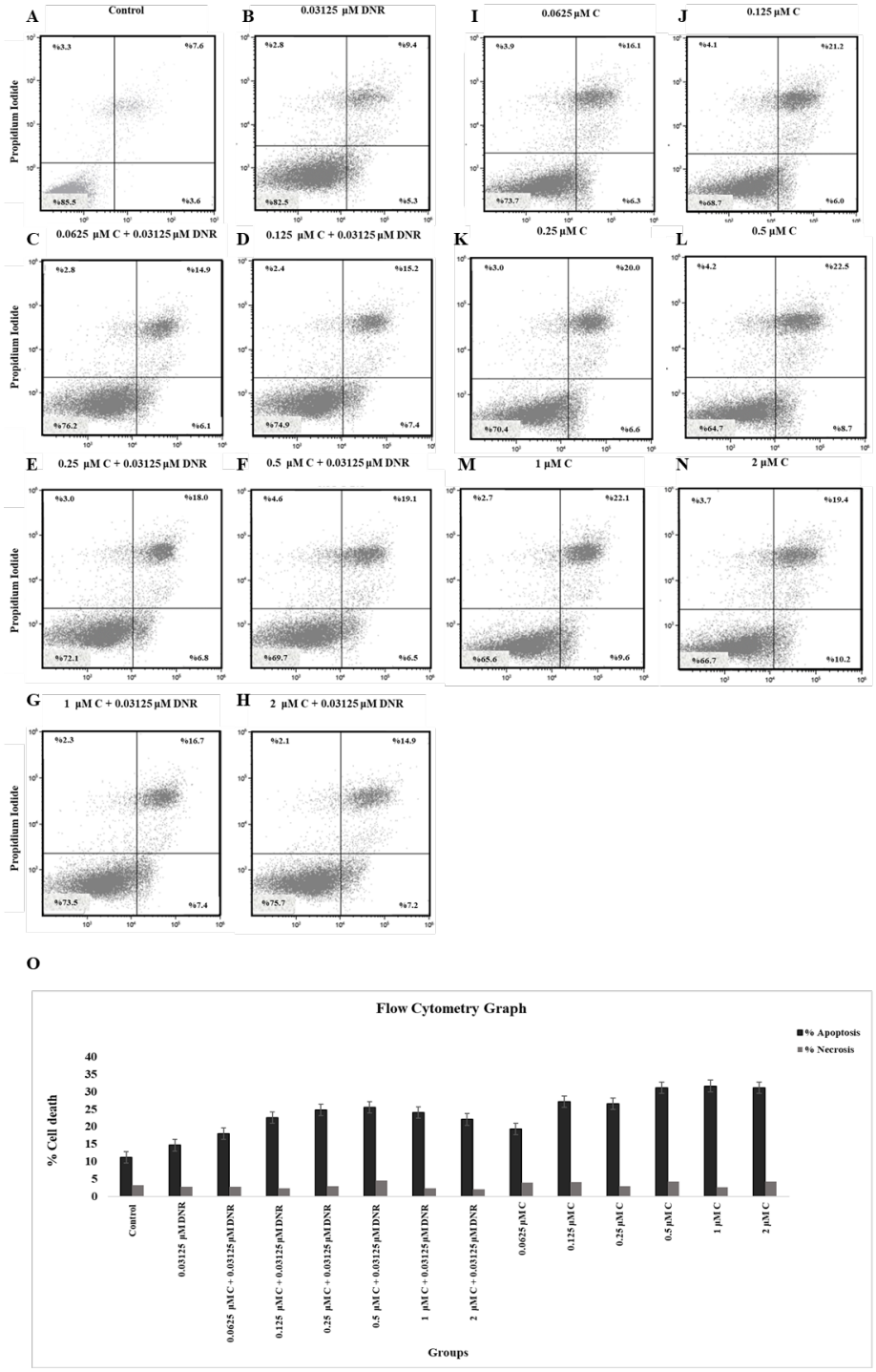

Fig 8. Flow cytometric Apoptotic/Necrotic cell death analyses of KG1a cell line. Flow cytometric diagram of vehicle- treated Control (a), DNR (0.03125µM) -treated (b) and Casticin (2-0,0625 µM) -treated (i, j, k, l, m, n), and their combinations (c, d, e, f, g, h) in KG1a cells. The cells were treated with vehicle solution or agents for 48 h, then stained with Annexin V- FITC and Propidium Iodide. The experiments were repeated at least 3 times. Quadrants represents necrotic cells (upper left), viable cells (bottom left), late apoptotic cells (upper right), early apoptotic cells (lower right). Apoptotic cell death ratios among the groups (o) Comparison of apoptotic cell death between single agent-treated groups and combination groups. The results are expressed as Average (±Standard deviation) of 3 experiments.

Although addition of Casticin to DNR provided an increase in apoptosis percentages, the highest ratio of apoptosis was provided by the treatment of 2 µM Casticin alone for KG1a cell line. Therefore, we decided to continue the experiments and confirm the effects of 2 µM Casticin on KG1 and HL-60 cell lines, as well (Fig 9). All results indicated that 2 µM Casticin has a significant apoptotic effect on the stem-like (KG1a), leukemic blast KG1 (26.5%), and HL-60 (14.6%) cell lines with 1.7% and 1.2% necrosis.

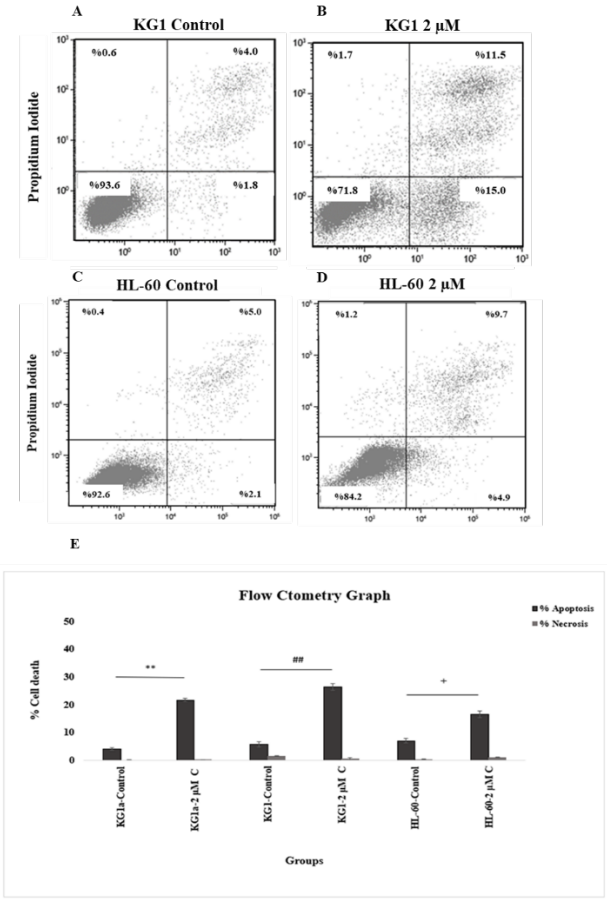

Fig 9. Flow cytometric Apoptotic/Necrotic cell death analyses of cell lines. Flow cytometric diagram of KG1-Control (a), KG1 2µM Casticin-treated (b), HL-60-Control (c), HL-60 2µM Casticin-treated (d) cell lines. The cells were treated with 2µM for 24 h, then stained with Annexin V- FITC and Propidium Iodide. The experiments were repeated at least 3 times. Quadrants represents necrotic cells (upper left), viable cells (bottom left), late apoptotic cells (upper right), early apoptotic cells (lower right). Apoptotic and necrotic cell death percentages among the groups (e). The results are expressed as Average (±Standard deviation) of 3 experiments. *, #, +: Apoptosis percentages of Control vs. 2µM Casticin, respectively. **: $p<0.01$; ##: $p<0.01$; +: $p<0.05$.

3. DISCUSSION

AML is the most common form of acute leukemia, characterized by the accumulation of malignant myeloid precursor cells, and called as a stem cell disease due to the arrest in the differentiation phase in the bone marrow [1,2]. Cytarabine and DNR or idarubicin (anthracycline agents) are still used as the standard treatment in AML. However, AML patients have still poor survival rates, which are arising from the stem cell characteristics of AML and cytotoxic effects of the agents [8,9,13,14]. For more than 50 years, it has been considered that LSC are the potential origin of AML, therefore efficiently targeting of these cells has a great importance in therapy success [37,38].

Current studies still recommend DNR as an agent that should be in targeted AML treatment approaches [15,16]. Higher dosages of DNR, cardiotoxicity, development of drug resistance, and relapse are important factors limiting the effectiveness of DNR [32,34,39,40]. Therefore, there is a need for

effective combination treatments that target both LSC and leukemic blasts without damaging healthy cells.

It is important to consider plasma levels of DNR in patients to reflect clinically relevant results. Pharmacokinetic studies reported that as a result of bolus administration, initial DNR plasma concentrations are between 1-2 µM and decline rapidly [41-43]. Generally, DNR plasma levels fall into the range of 0.1-0.4 µM, approximately [41, 44]. Hence, using DNR concentration below 1 µM provides results associated with the clinical utilization. Our calculated DNR IC50 values for 24 h were 0.56 µM, 0.97 µM, and 0.06 µM for KG1a, KG1 and HL-60 cell lines, respectively. Bailly et al. showed that cell lines with a high CD34+ population such as KG1 and KG1a are 10-15 times more resistant to DNR treatment than CD34- AML cells (HL-60, U937) [12]. Consistent with the literature, in our study, AML stem cell model KG1a (DNR IC50: 0.56 µM) and its relatively mature form, AML blast model KG1 (DNR IC50: 0.97 µM), were found to be 9 and 16 times more resistant to DNR than CD34- HL-60 (DNR IC50: 0.06 µM) cell line.

Natural compounds are generally preferred as an inducer of cell death in cancer cells with less damaging features on healthy cells. Therefore, phytochemicals are gained attention as the promising sources for the anti-leukemic drug research, especially to overcome chemoresistance and relapse [24,34,45-47]. In the literature, combination of the well-known chemotherapeutics with promising phytochemicals is a preferred approach to reach a successful chemotherapy [48,49].

Several studies have reported that some improvement and/or synergism via combining well-known natural flavonoids (curcumin, quercetin, resveratrol, apigenin, genistein, epigallocatechin-3-gallate etc.) with chemotherapeutics (cytarabine, arsenic trioxide, etoposide, cyclophosphamide, DNR, doxorubicin etc.) for AML [50-55]. In our previous study, we revealed that Casticin has apoptotic effect in both stem-like and mature parental cells at 2µM concentration [30], which is lower concentration than several polyphenols in the literature [26,29,56-59]. Therefore, for the first time, we assessed synergistic DNR-Casticin combinations to increase the therapeutic efficacy and reduce DNR's side effects.

Even though Chou-Talalay provides a favorable method to find possible synergistic concentrations, it has an important limitation. Since Chou-Talalay accepts that the single agents and their combinations have linear concentration-effect relation, usage of agents that have non-linear concentration-effect relation, might be challenging [60,61]. Because of that, in our study, to find synergic dose of DNR-Casticin combination was challenging. We could not obtain concentration suggestions that include CI lower than 1 for DNR-Casticin combination. We consider that non-linear concentration-effect relationship of Casticin as a natural polyphenol was the main reason.

Our results explained to us an inconsistency of Chou-Talalay method for some natural polyphenol combinations that have non-linear concentration-effect relations. Therefore, we decided to continue to the study with non-constant combinations of DNR-Casticin.

Targeting apoptosis or using apoptosis-sensitizer agents with certain chemotherapeutics have been main goal of AML treatment for decades due to apoptosis-resistant feature of LSCs [62-66]. On the other hand, due to its ability to provoke immune response and drug resistance, chemotherapy-induced necrosis is not generally desirable [21,67,68]. Based on the knowledge, we thought that the viability test alone is insufficient to assess the synergistic effect. Therefore, in the light of the literature, we continued with the evaluation of apoptotic and necrotic cell death to confirm the synergism [52,69,70]. For instance, Mahbub et al. determined synergistic or additive effect of polyphenols (quercetin, apigenin, emodin, rhein, and cis stilbene) in combination with chemotherapeutics (doxorubicin, etoposide) by evaluation of ATP decrease and caspase 3,8,9 activation [69].

Combinations of chemotherapeutics with polyphenols produces synergistic or antagonistic effects and these effects depend on compatibility and concentrations of chemotherapeutics and polyphenols. For instance, Mahbub et al. reported that combination treatments of the polyphenols to the leukemia cell lines (Jurkat, CCRF-CEM, THP-1, KG1a) caused synergistic effects, but some of them caused antagonistic effect with the mentioned polyphenols. Therefore, they suggested exclusion of some of the polyphenols during the chemotherapy [69,70]. In addition, Chueahongthong et al. reported that combinations of doxorubicin and curcumin produce synergistic and additive effects in leukemia cell lines (KG1, KG1a, EoL-1 and U937) by evaluation of MTT viability test [71].

In this study, we have found a synergism (CI<1) for Casticin (0.0625 µM)- DNR (0.25 µM) combination for the first time. Although the combination treatment decreased cell viability to 50-60 %, there was no significant increase in apoptotic/necrotic cell death compared to the single agent treatment. The contradiction between the results makes us presume that the combination of DNR and Casticin may lead to activation of the other cell death pathways, which requires further studies.

To improve apoptotic effect, we further examined several non-ratio DNR-Casticin combinations. However, no significant apoptotic increase with the non-ratio combinations were obtained. Therefore, we concluded that Casticin-DNR combination is not beneficial in the sense of apoptotic cell death. We obtained the highest percentage of apoptosis by single 2 µM Casticin treatment in cancer stem cell model KG1a (21.7%), its relatively mature form KG1 (26.5%) and HL-60 (14.6%) cell lines. The most important result of the study is assessing of Casticin, DNR and their combinations by Chou-Talalay method and apoptotic evaluation of non-ratio combinations together. In consequence, our study showed that Casticin-DNR combinations do not provide an advantage in terms of apoptosis, for the first time. Therefore, we suggest that Casticin polyphenol might be the advantageous candidate for LSC-targeted therapies for AML.

4. CONCLUSION

A combination that can trigger maximum apoptosis in both LSC and leukemic blasts with low toxicity is crucial for ultimate AML treatment. In this study, although we have found a synergistic CI value for DNR and Casticin combination for the first time, we did not see its improver effect on apoptosis compared to the single treatments. Since we have found an important decrease in cell viability with the treatment of DNR-Casticin combination, we presume that the synergistic combination may have a capacity to trigger other cell death pathways. On the other hand, we found that single Casticin treatment triggers a higher rate of apoptosis in stem cell and blast models of AML, rather than in combination with DNR. Casticin can cause apoptotic cell death in both AML cancer stem cells and leukemic blasts at a lower concentration (2 µM) than several polyphenols in the literature. Therefore, we think that Casticin polyphenol might be the possible candidate for new targeted therapy studies for AML.

5. MATERIALS AND METHODS

5.1. Cell culture

HL-60 cells were kindly gifted from Prof. Zeynep Yüce (Department of Medicinal Biology, Faculty of Medicine, Dokuz Eylül University, Izmir, Turkey) and cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum, penicillin (100 U/ml)/streptomycin (100 µg/ml) and 2 mM L-glutamine at 37°C in 5 % CO_2 incubator. KG1, KG1a, and PBMC cells were obtained from ATCC. KG1, KG1a cells cultured in 20 % fetal bovine serum involved Iscove's Modified Dulbecco's Medium (IMDM) (ATCC, 30-2005) at 37°C in 5 % CO_2 incubator.

KG1a cell line consists of stem-like AML cells, and it is considered as a valuable model for in vitro LSC research, since they do not spontaneously differentiate into granulocyte and macrophage-like cells and do not respond to colony stimulating factor. KG1 cell line, the relatively mature form of KG1a, is capable to differentiate into macrophages and shows a good response to colony-stimulating factors [72-74].

5.2. Cell viability

Viability and IC_{50} values were analyzed by a colorimetric MTT assay that measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,4, diphenyltetrazolium bromide by metabolic active cells. Main stock of DNR (59.1 mM) and Casticin (50 mM) were prepared in DMSO. Further dilutions were made by 1% FBS- cell culture medium to analyze cytotoxic effects of Casticin (50-0.125 µM) and DNR (4-0.03125 µM) by MTT assay (ROCHE, Cat no:11 465 007 001). In control group, 0.008%DMSO-1%FBS solution was used as vehicle solution. Briefly, 100 µl of cell suspension (includes $2x10^5$ cells) with vehicle solution/DNR- and/or Casticin- treated were seeded in a 96-well plate. After 24 h/48 h incubation, 10 µl of MTT solution (5 mg/ml) was added to each well and the plates were incubated for further 4 h. The produced formazan crystals are solubilized via overnight incubation of 100 µl solubilization buffer (10% SDS in 0.01 M HCl.) in humidified atmosphere (+37°C, 5-6.5% CO2). The absorbances were measured at 570 nm by Biotek (ELX800, USA) microplate reader.

5.3. Chou-Talalay method

Potential synergistic effects were evaluated based on the Chou-Talalay method, which considers both the potency and shape of the dose-effect curve. Firstly, we obtained single agents' (DNR or Casticin) dose-response curves and calculated half-maximal inhibitory concentrations (IC50 or Dm). Then, according to the method, we obtained dose- effect plots of two agents at 1:2 and 1:8 constant ratios and at non-constant ratios. All data were used to evaluate the combination index values (CI). According to the method, we employed Fa-CI plot and isobologram analysis to determine whether the interactions between two compounds were synergistic or not. Then, the interactions were evaluated regarding the positions of the dots in the Fa (Fa – fraction affected)-CI plot line (CI < 1 synergism; CI = 1 additive effect and CI > 1 antagonism).

5.4. Flow cytometry

Apoptotic and/or necrotic cell death was determined by flow cytometric analysis of cells with Annexin V-FITC and propidium iodide (PI) stains using an assay kit from BD PharMingen (San Diego, CA). Briefly, after DNR and/or Casticin treatment, cells were collected, washed with cold PBS, suspended in binding buffer, and stained with Annexin V-FITC and PI, respectively. After this step the cell suspensions were analyzed by counting 10,000 cells per sample in a flow cytometer (Navios Flow Cytometer Beckman Coulter, USA) and evaluated by its software (CellQuest Pro software).

5.5. Statistical analysis

All experiments were performed 3 times in triplicate. Cell viability and flow cytometry data are expressed as the Mean ± SD. Results were compared by ANOVA or student-t test by SPSS Version 24. $p<0.05$ was considered statistically significant.

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