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The efficiency of amygdalin and doxorubicin combination on hepatocellular carcinoma and stromal myofibroblastic prostatic cells

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ABSTRACT

This study investigated the possible therapeutic potential of Amygdalin either alone or in combination with Doxorubicin against HepG-2 liver cancer cells and on normal stromal myofibroblastic prostatic (WPMY-1) cells. After determination of IC50 concentrations of Amygdalin and Doxorubicin by MTT assay, each cell line was treated for 48 hrs as follows: Untreated control cells, Amygdalin-only treatment, Doxorubicin-only treatment, and a combination of Amygdalin + Doxorubicin treatment in an equal ratio (1:1). In HepG-2 cells, the treatment with Amygdalin or Doxorubicin significantly decreased the number of surviving cells in a dose-dependent manner. In normal WPMY-1 cells, the percentages of surviving cells were constant and unaffected by increasing Amygdalin or Doxorubicin concentrations, demonstrating no IC50 dose impact threshold. In HepG-2 cells, the Amygdalin and Doxorubicin 1:1 combination at their IC50 concentrations showed a synergistic effect (combination index (CI)= 0.69153). In addition, the average normalized mRNA expression of *BCL-2* was downregulated and that of *BAX* was found overexpressed by all treatments in HepG-2 cells, most significant with the combination treatment. In WPMY-1 cells, both *BCL-2* and *BAX* were downregulated after all treatments, in contrast to the probable behavior against cancer cells. The flow cytometry analysis showed that the combination treatment to HepG-2 cells induced a higher percentage of early and late apoptotic cells than >Doxorubicin >Amygdalin. In WPMY-1 cells, only early apoptotic cells were significantly enhanced by Doxorubicin either alone or in combination with Amygdalin, with no intergroup significant changes in apoptotic cell numbers. The study indicates that Amygdalin synergistically enhances the chemotherapeutic potential of Doxorubicin against malignant cells when used in combination through the induction of apoptosis.

Keywords: Amygdalin; Doxorubicin; HepG-2; WPMY-1; Combination therapy; apoptosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a universal health problem. It ranks the seventh most common cancer for both sexes worldwide regarding Age Standardized Rates (ASR) of incidence and mortalities. In Egypt, it ranks 2nd for both men and women with an ASR incidence of 32.5 /100,000 and mortality of 31.1/100,000 population in 2020 (Sung *et al.*, 2021). The number of HCC patients increased two-fold over a decade. This could be attributed to I) improvement in

screening programs and diagnostic tools, II) increasing the survival rate of cirrhotic patients that increases the chance of developing HCC, and III) the previous history of increased incidence and complications of hepatitis C virus (HCV) and Schistosomiasis, the most important risk factors for liver cancer in Egypt, although both diseases have been recently controlled in Egypt (Rashed *et al.,* 2020).

One of the documented new approaches to cancer therapy is by natural phytoproducts with cyanogenic glycosides, a large group of secondary metabolites that are widely distributed in the plant kingdom (Vyacheslav *et al.,* 2018). Amygdalin is one of the cyanogenic glycosides found, for example, in apples, apricots, and almonds (Huebner *et al.,* 2014). Laetrile, another synthetic product of Amygdalin, was suggested previously to treat cancer. It is broken down into two molecules of glucose, one molecule of hydrogen cyanide, and one molecule of benzaldehyde. In the early days of laetrile research, it was assumed that the hydrogen cyanide molecule was the major cancer cell-killing molecule however, concerns about its safety are still obscure, however, concerns about its safety are still obscure (Citrin *et al.*, 2012).

Amygdalin is an aromatic aminoglycoside with the chemical formula $C_20H_{27}NO_{11}$, including one benzaldehyde, one-unit hydrocyanic acid, and two units of glucose (Makarevic *et al.,* 2016). It has been shown to have antioxidative, antibacterial, anti-inflammatory, and immunoregulatory properties (Li *et al.,* 2018). It showed a significant antiproliferative effect against prostate cancer cell lines; LNCaP, DU145, and PC3, as evidenced by a significant decrease in the G2/M phase and S phase cells and a significant increase in the G0/G1 phase cells (Makarevic *et al.,* 2016).

Doxorubicin (or Adriamycin) is a chemotherapeutic drug used to treat cancer. It is an anthracycline antibiotic with antineoplastic activity developed from the *Streptomyces peucetius* bacteria and it is used to treat leukemias and Hodgkin's lymphoma, as well as malignancies of the liver, bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, multiple myeloma, and others (Enrique, 2011). Doxorubicin is reported to cause cardiotoxicity (Elberry *et al.,* 2010), nephrotoxicity (Boutabet *et al.,* 2011) and hepatotoxicity (Yagmurca *et al.,* 2007). This toxicity seems to be due to the generation of reactive oxygen species (ROS) including hydroxyl radicals and superoxide anions thus leading to lipid peroxidation and tissue damage (Rabelo *et al.,* 2001).

Because chemotherapy medications impact cancer cells at different stages of the cell cycle, combination chemotherapy boosts the chances of eliminating all cancer cells. Multiple medications taken at the same time may raise the chance of drug interactions (Saputra *et al.,* 2018). The main aims are to achieve synergistic therapeutic effect, dose, and toxicity reduction to minimize or delay the induction of drug resistance (Stein *et al.,* 2016).

The hepG-2 human cancer cell line is well-known *in vitro* model of a well-differentiated hepatocellular carcinoma (Aden *et al.,* 1975). On the other hand, WPMY-1 cells are widely used as human normal control cell lines consisting of myofibroblast cells. They possess co-expression of smooth muscle-actin and vimentin led to the conclusion that they were myofibroblasts (Makwana *et al.,* 2020).

The general objectives of the ptresent work are to investigate the possible curative effect of Amygdalin on HepG-2 cells in comparison with Doxorubicin and if the combination of both drugs may enhance the general effect of Doxorubicin. The study also aims to study the underlying cellular and molecular mechanisms predisposing to the expected effects, in comparison with the effects on normal WPMY-1 cells.

MATERIALS AND METHODS

Drugs and Chemicals:

Amygdalin (Cas Number. 29883-15-6) and Doxorubicin (adriamycin HCL; Cas. Number. 25316-40-9), were purchased from Sigma-Aldrich, USA with purity ≥99%. DMSO was obtained from Al-Gomhoriya Co., Cairo- Egypt. MilliQ ultra-purified water (Millipore-Merck, France) was used for different assays.

Cell Lines and Cell Culture:

The Holding Company for Biological Products & Vaccines (Vacsera), Giza, Egypt, provided HepG-2 and normal stromal myofibroblast WPMY-1 cell lines (control cells). Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), (100U) 20g/ml penicillin, and 100 g/ml streptomycin was used to cultivate the cells. The incubation was carried out at 37°C in a 5% CO_2 environment. Non-treated cells were grown in a 1:1 mixture of DMEM and Ham's F12 media containing 20 mg/ml EGF, 100 g/ml cholera toxin, 0.01 mg/ml insulin, 500 g/ml hydrocortisone, and 5% Chelex-treated horse serum.

MTT Assay:

MTT assay method of Mosmann (1983) was adopted for the determination of IC50 levels of Amygdalin, Doxorubicin, or their combinations in HepG-2 cells as well as WPMY-1 control cells. Amygdalin was added to the media of both HepG-2, and normal WPMY-1 cell lines at concentrations of 0, 20, 40, 60, 80 and 120 mg/ml, while Doxorubicin was added to the same cell lines at concentrations of 0, 0.21, 0.42, 0.84, 1.7, 3.4 mg/ml (Boutabet *et al.,* 2011), to obtain the optimal IC50 dose for each drug. All plates were incubated in humidified 5% CO2 at 37°C for 24 hrs and others were incubated for 48 hrs. The cell growth of all plates was observed at 570 nm via a microplate reader (LMR-9602, USA) after 24 or 48 hrs, and the IC50 concentrations were calculated. The experiment was repeated in duplicate.

Cellular treatment with IC50 concentrations:

After determination of the IC50 doses for each drug, HepG-2 was treated with the IC50 doses of Amygdalin, Doxorubicin, or their combination for 48 hours. The four treatment groups of both cell lines were: G1: Non-treated control (vehicle only); G2: Amygdalin treatment at IC50 dose; G3: Doxorubicin treatment at IC50 dose. G4: Drug combination: Amygdalin (IC50 dose) + Doxorubicin (IC50 dose) were applied simultaneously to HepG-2 cells at a 1:1, 1:2 or 2:1 combination regimen.

For the molecular and flow cytometry analyses, HepG-2 cells were treated or not with IC50 doses of Amygdalin, Doxorubicin or their 1:1 combination for 48 hrs. As well, WPMY-1 normal cells were also treated or not for 48 hrs with Amygdalin, Doxorubicin or their 1:1 combination with IC50 doses similar to those used against HepG-2 cells.

Drug combination study:

To study the synergism between Amygdalin and Doxorubicin against HepG-2 cells, a combination index (CI) was calculated using the data obtained from their MTT assays. This was not applied to WPMY-1 cells due to the lack of an effective dose threshold (IC50) when the two drugs were administered. The drug combination studies on HepG-2 cells were based on concentration-effect curves generated as a plot of the fraction of unaffected cells versus drug concentration by the Chou and Talalay method (1984) using CompuSyn software, PD Science,

LLC, USA. The CI values indicate a synergistic effect when < 1, an antagonistic effect when > 1, or an additive effect when equal to 1.

Gene expression analysis using quantitative real-time PCR:

RNA was extracted from all treated and non-treated human HepG-2 and WPMY-1 cells using a total RNA isolation kit (Analytik Jena-Germany) according to the manufacturer's protocol.

cDNA preparation:

Single-stranded complementary DNA (cDNA) was obtained from 1µg of purified RNA using the Sensiscript Reverse Transcriptase (QIAGEN, Germany) synthesis Kit, according to the manufacturer's directions using random hexamers. In 200µl PCR tubes with flat caps (MicroAmp Reaction Tube), a reaction mix was prepared for total RNA to be reverse transcribed.

SYBR Green RT-PCR assay:

All RT-PCR runs were performed on the Qiagen RT-PCR instrument (Rotorgene 5plex, Germany). Samples belonging to the same group were always run together to prevent any inter-run variation. Real-time PCR reactions were performed using HotStar Taq DNA Polymerase modified form of QIAGEN Taq DNA Polymerase. GAPDH gene expression was used as an endogenous control. Primers' sequences are listed in Table (1). All primer pairs were synthesized by Jena Bioscience GmbH (Jena, Germany).

Table 1. Primer Sequences of BCL-2 and BAX and GAPDH.

Gene	Primer's sequences	Size of PCR product
BCL-2	F: 5'-GGATGACTTCTCTCGTCGCTACCGT-3'	**118 bp**
	R: 5-'ATCCCTGAAGAGTTCCTCCACCAC-3'	
BAX	F: 5`-CCAGGACGCATCCACCAAGAAGC-3`	**136 bp**
	R: 5`-TGCCACACGGAAGAAGACCTCTCG-3`	
GAPDH	F: 5`-ATGGAGAAGGCTGGGGCTCACCT -3`	**209 bp**
	R: 5`-AGCCCTTCCACGATGCCAAAGTTGT -3`	

F: Forward; R: Reveres; bp: Base pairs.

qPCR data analysis:

The CT cycle (Threshold cycle) was utilized to compare the expression levels of control and all treatment groups. The gene expression level was then determined using Applied Biosystems Step OneTM Instrument software (Yuan *et al.* 2016). Using the following formula, the results were expressed as the ratio of reference gene to target gene: CT = CT (target genes) - CT (cycle numbers at the threshold level of log-based fluorescence normalized to endogenous control genes) (Kuang *et al.*, 2018).

Annexin V-FITC/PI Staining Assay by Flow Cytometry:

The staining of the mammalian DNA for flow cytometry was achieved according to Vindelov, (1977). After treatment of HepG-2 and WPMY-1 cells with the IC50 doses of Amygdalin, Doxorubicin, or their combinations, apoptosis levels were measured using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences Pharmingen, USA) and analyzed by Fluorescence-activated cell sorting (FACS) caliber flow cytometer (Becton Dickinson, Sunnyvale, CA, USA), with emission filters of 488nm. For this assay, the average

number of evaluated nuclei per specimen was 20,000 and the number of nuclei scanned was 120 per second. DNA histogram derived from flow cytometry was obtained with a computer program for Dean and Jett mathematical analysis (Dean and Jett, 1974).

Statistical analyses:

Group data expressed as means±S.D. were analyzed using the two-tailed t-test or ANOVA analyses. Data expressed as percentages were analyzed using Chi-squared (X2) analysis (Statistical Package for Social Science (SPSS)), version 16, USA.

RESULTS

1. Toxicity of HepG-2 cells treated with Amygdalin or Doxorubicin for 24&48 hrs:

Figure (1) indicates that the percentages of surviving cells after 24 and 48 hrs of incubation with serially increasing Amygdalin or Doxorubicin concentrations drop when concentrations rise in a dose-dependent manner. The IC50 concentrations were calculated as 66.75 µg/mL after 24 hours and 22.45µg/mL after 48 hrs for Amygdalin, and 0.87 µg/ml after 24hrs and 0.3 µg/ml after 48hrs for Doxorubicin.

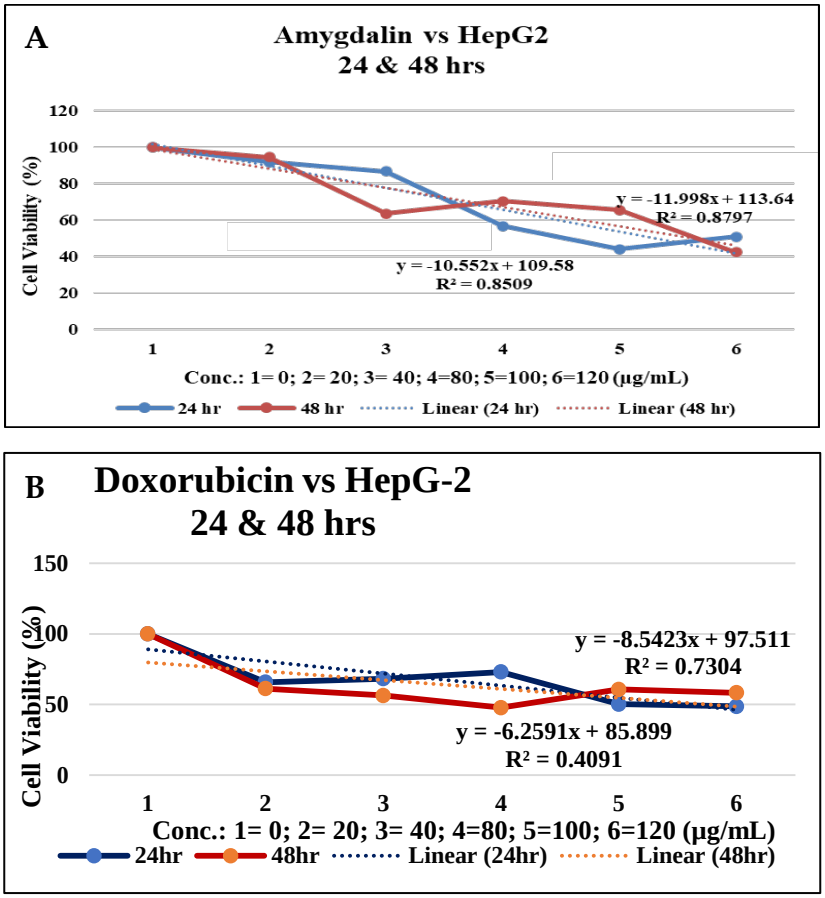

Fig. 1. Combined histograms for the cell viability results of A): Amygdalin and B): Doxorubicin treatments on HepG-2 cells after incubation for 24 and 48 hrs. The percentage of dead cells was measured by MTT cytotoxicity assay.

2. Toxicity of WPMY-1 cells after treatment with Amygdalin or Doxorubicin for 24 & 48 hrs:

The results of Figure (2) show that the percentages of survival cells after incubation of WPMY-1 cells with serial concentrations of Amygdalin or Doxorubicin for 24 and 48 hrs are constant and unaffected with the increased concentrations showing no dosage impact threshold. Also, no toxic inhibition concentrations (IC50) were estimated from the linear plots or R^2 squared regression equation in Figure 2 (A-C) after 24 or 48hrs. Constant cellular toxicity was obtained with Doxorubicin after 48 hrs where about 40% of cells died in all treatment groups (Fig. 2C).

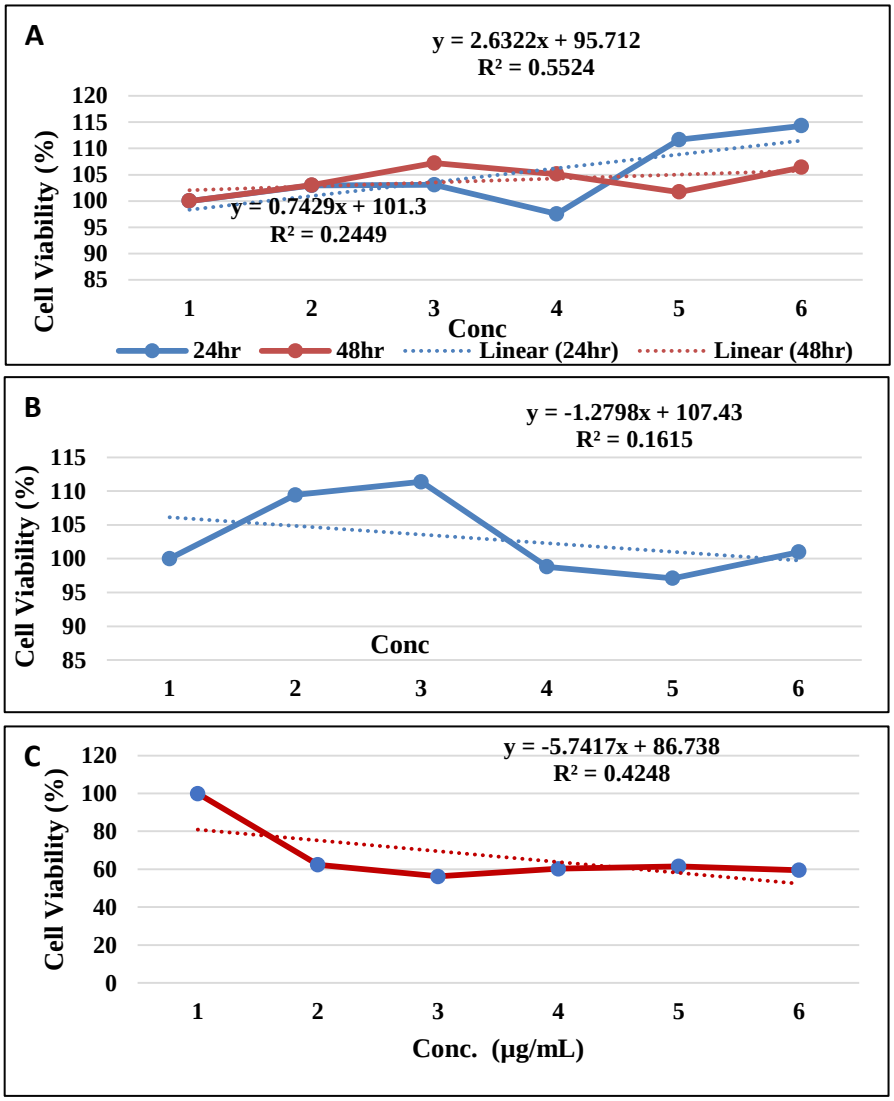

Fig. 2. A): Combined histograms for the cell viability results of Amygdalin treatment on WPMY-1 cells after incubation for 24 and 48 hrs. The percentage of dead cells was measured by MTT cytotoxicity assay showing almost constant cell survival; B): A histogram for the cell viability results of Doxorubicin treatment on WPMY-1 cells after incubation for 24 hrs and 48 hrs (C). Conc. 1= 0; 2= 20; 3= 40; 4=80; 5=100; 6=120 (µg/mL).

The percentage of dead cells was measured by MTT cytotoxicity assay showing almost constant cell survival without cytotoxicity.

3. The combination index analysis (CI):

When Amygdalin and Doxorubicin mixed at IC50 doses of 1:1, 1:2 and 2:1 and then administered to HepG-2 cells for 48 hours, the standardized isobolograms and a combination index (CI) plot were used to determine the kind of drug-drug interaction.

The normal WPMY-1 cells were not used in this combination study because they showed no dose-effect threshold in the MTT cytotoxicity assay. The 1:1 combination ratio has shown a clear synergistic effect between Amygdalin and Doxorubicin (CI=0.69153) against HepG-2 cells. On the other hand, the other two combination ratios (1:2, and 2:1) indicated obvious additive effect levels (CI=1.00076 and CI=1.11797 respectively) (Table 2 and Fig. 3).

Table 2. CI Data for Non-Constant Combo: (Amyg+DOX).

Dose Amyg	Dose DOX	Effect (Mean absorbance)	CI*	Drug-Drug interaction
1.0	**1.0**	0.5	0.69153	Synergistic <1
1.0	**2.0**	0.48	1.00076	Additive =1
2.0	**1.0**	0.53	1.11797	Additive =1

*: CI=Combination Index (Chou and Talalay, 1984).

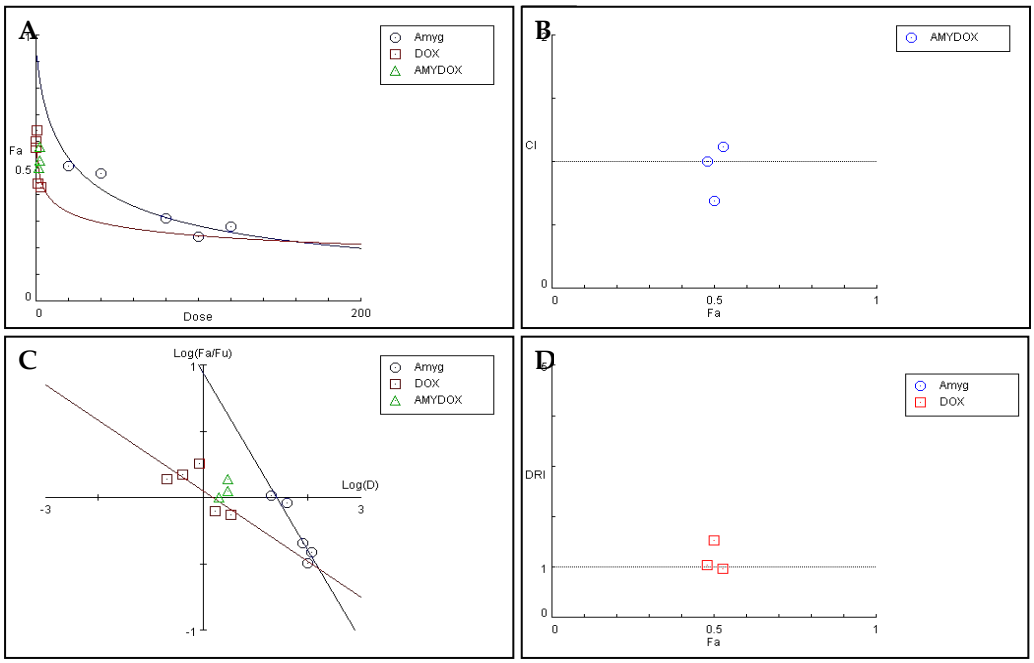

Fig. 3. Combination index histograms. A): the dose effect curve showing combination doses on the vertical axes corresponding to both drugs levels; B): the combination index plot showing significant synergistic effect (CI<1) for one combination, and two additive effects for the other two combinations; C): plot for median effect level for both drugs and for their combination; D): dose-reduction index (DRI) plot for Non-Constant Combo (Amyg + DOX). Fa: Default effect level.

4. qRT-PCR expression levels of BCL-2 and BAX:

In HepG-2 cells, the average BCL-2 expression was downregulated by 1.7-fold in group (2) treated with Amygdalin (P>0.05), and by 2.53-fold (P<0.05) in group (3) treated with Doxorubicin, and by 2.7-fold (P<0.05) in group (4) treated with their combination as compared with the control. In contrast, BAX expression was significantly elevated by 4.2-fold in group (2) treated with Amygdalin, 3.14-fold in group (3) treated with Doxorubicin, and by 5.9-fold in group after treatment (4) with both drugs as compared with the control (P<0.05) (Fig. 4).

In WPMY-1 cells, however, the BCL-2 expression was significantly down regulated by 2.44-fold (P<0.05) after the Amygdalin treatment, and 2.8-fold (P<0.05) by Doxorubicin, and 4.72-fold (P<0.05) after combination treatment as compared with control. In contrast to the data of HepG-2 cells, the relative expression of BAX in WPMY-1 cells s found significantly down regulated in all treatment groups, by 4.2-fold in group 2 (Amygdalin), 3.14-fold in group 3 (Doxorubicin), and 5.9-fold after combination treatment as compared with the control (Fig. 4).

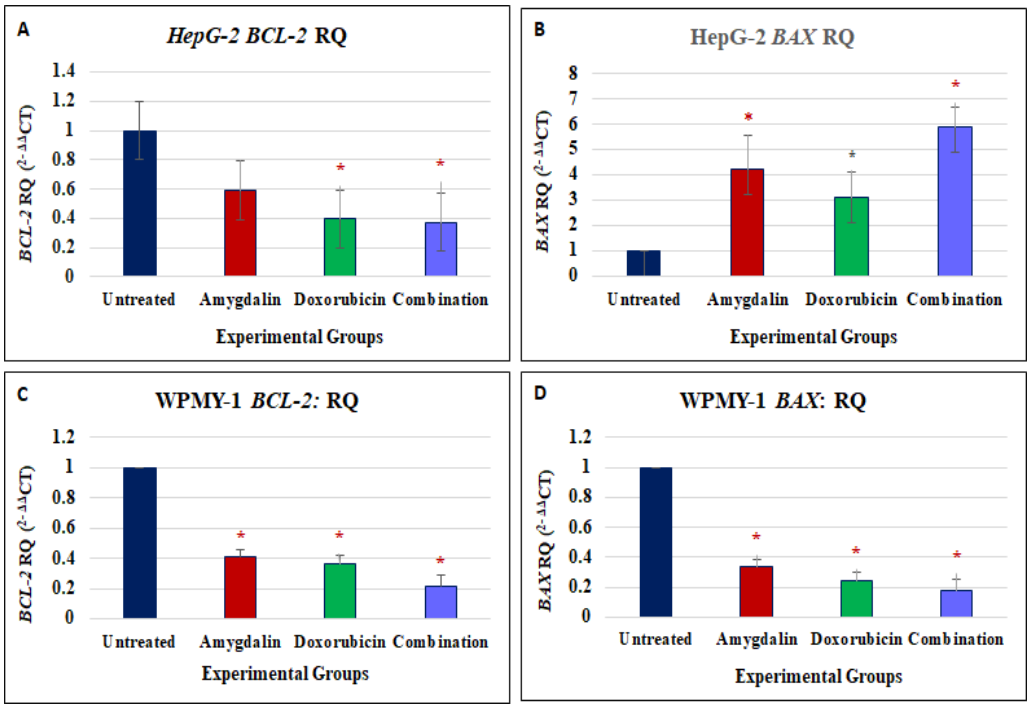

Fig. 4. Average qRT-PCR analysis data of mRNA expression normalized to *GAPDH*. A): *BCL-2* gene expression in HepG-2 groups; B) BAX gene expression in HepG-2 cells; C): BCL-2 gene expression in WPMY-1 cells; D) BAX gene expression in WPMY-1 cells; *: Significance vs. untreated calibrator at P<0.05.

5. Detection of Apoptosis by Flow Cytometry:

The flow cytometric data of HepG-2 cells treated with Amygdalin, Doxorubicin, or their combination revealed a significant increase in the percentage numbers of both early and late apoptotic cells as compared with the control. The apoptotic ratios (%) were 6.1% (untreated control), 47.3% (Amygdalin), 68.8% (Doxorubicin), and 78.2% (combination). The combination treatment significantly induced the highest apoptosis in the treated HepG-2 cells (Table 3 and Fig. 5).

Table 3. Percentage No. of HepG-2 cells positive for Annexin V FITC-A and PIPE-A by flow cytometry showing necrotic, early, viable and late apoptotic cells.

Groups	Treatment (IC50 Doses)	% Cells Positive for PIPE-A (Q1) (Necrotic)	% Cells Positive for Both (Q2) (Late apop.)	% Cells Negative for Both (Q3) (Viable cells)	% Cells Positive for Annexin V FITC-A (Q4) (Early apop.)
G1	Untreated cells	3.5±0.58	1.70±0.09	90.4±1.15	4.40±0.9
G2	Amygdalin	9.2±0.59*	22.3±1.15*	43.5±1.18*	25.0±0.9*
G3	Doxorubicin	١0.1±0.63*	49.7±1.19*	21.١±1.09*	19.1±0.8*
G4	Amyg. + Dox.	10.4±0.92*	60.7±1.05*	11.6±1.20*	17.3±0.9*

Values are percentage means ± S.D; *: Significant vs. G1 at $P<0.05$. Q1, Q2, Q3, and Q4 are flow cytometric panels for Annexin V FITC-A and/or PIPE-A-stained cell

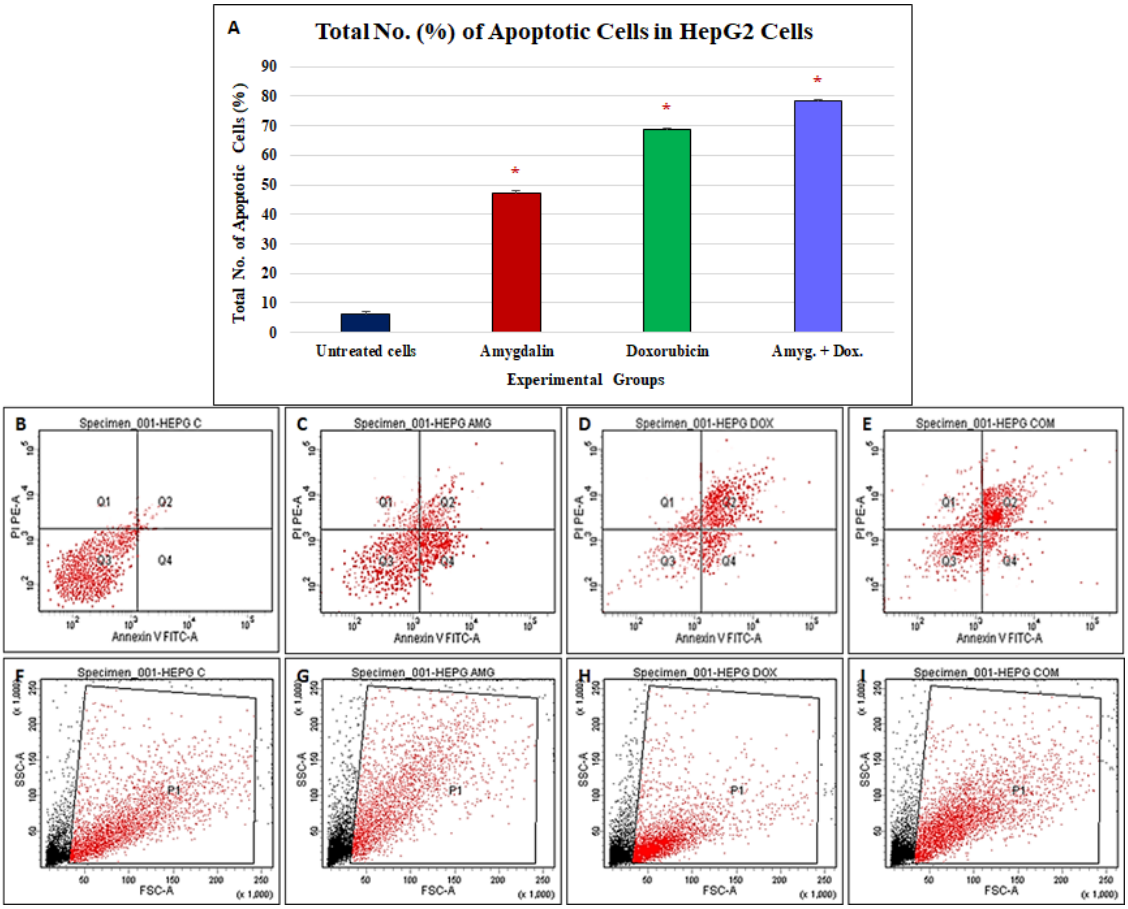

Fig. 5A. Flow cytometry data for the average total numbers (%) of early + late apoptotic cells in HepG-2 untreated cells, treated with Amygdalin, Doxorubicin, or combination of both. The data is significantly different (*) vs. control at $P<0.05$. B-E): flow cytometry dot plot log figures of HepG-2 cells showing percentage numbers of necrotic (Q1; Positive for PIPE-A), late apoptotic (Q2; Positive for both Annexin and PIPE-A), viable cells (Q3; Negative for both Annexin and PIPE-A), and early apoptotic cells (Q4; positive for Annexin V FITC-AI). B): untreated cells; C): cells treated with Amygdalin; D): cells treated with Doxorubicin; E): combination treatment of both drugs. Figures F-I are flow cytometry plot figures for parent (P1) apoptotic cell numbers in corresponding groups as A-D, respectively.

In WPMY-1 cells, only Doxorubicin, or the 1 Amygdalin: 1 Doxorubicin combination revealed a significant increase in the total numbers (%) of apoptotic cells (both early and late apoptotic cells) as compared with the untreated WPMY-1 cells. The total ratios of early + late apoptotic cells in groups (1-4) were 9.7%, 11.4%, 14.9%, and 2.5%, respectively. These data did not show any statistical significance when compared with untreated WPMY-1 cells. Also, there was no intergroup significant differences in apoptotic cell percentages among the three treatment groups (2-4), albeit the treatment with Doxorubicin was the highest **(**Table 4 and Fig. 6).

Table 4. Percentage No. of WPMY-1 cells positive for Annexin V FITC-A and PIPE-A by flow cytometry showing necrotic, early, viable and late apoptotic cells.

Groups	Treatment	% Cells Positive for PIPE-A (Q1) (Necrotic)	% Cells Positive for Both (Q2) (Late apop.)	% Cells Negative for Both (Q3) (Viable cells)	% Cells Positive for Annexin VFITC-A (Q4) (Early apop.)
G1	Untreated cells	09.20±1.15	4.10±0.95	80.10±0.15	5.60±1.01
G2	Amygdalin	14.10±1.14	7.30±0.90	74.50±1.01	4.10±0.90
G3	Doxorubicin	22.60±1.40*	10.9±1.60*	63.4±0.20	3.10±0.91
G4	Amyg. + Dox.	20.00±1.00*	10.0±1.07*	67.5±1.00	2.50±0.95

Values are percentage means ± S.D; *****: Significant vs. G1 at $P<0.05$. Q1, Q2, Q3, and Q4 are flow cytometric panels for Annexin V FITC-A and/or PIPE-A-stained cells.

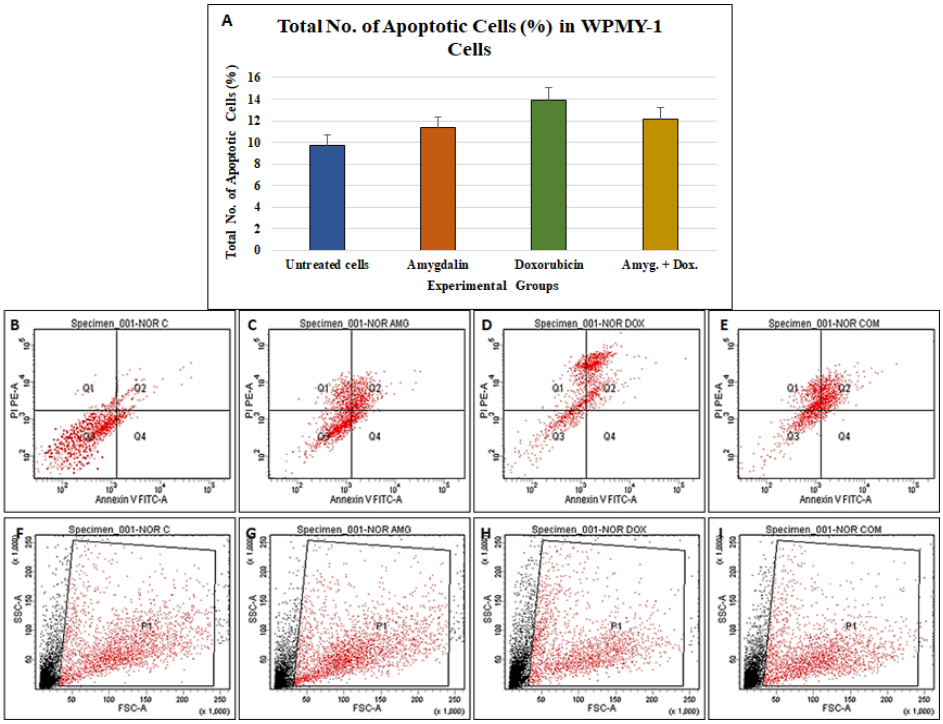

Fig. 6: A). Flow cytometry data for the average total numbers (%) of early + late apoptotic cells in WPMY-1 untreated cells, treated with Amygdalin, Doxorubicin, or combination of both. B-E): flow cytometry dot plot log figures of WPMY-1 cells showing percentage numbers of necrotic (Q1; Positive for PIPE-A), late apoptotic (Q2; Positive for both Annexin and PIPE-A), viable cells (Q3; Negative for both Annexin and PIPE-A), and early apoptotic cells (Q4; positive for Annexin V FITC-AI). B): untreated cells; C): cells treated with Amygdalin; D): cells treated with Doxorubicin; E): combination treatment of both drugs. Figures F-I are flow cytometry plot figures for parent (P1) apoptotic cell numbers in corresponding groups as A-D, respectively.

DISCUSSION

Anticancer drugs obtained from natural resources are recently known to possess strong antitumor potentials *in vitro* and *in vivo* (González Peña *et al.,* 2021). Several studies found anticancer effects for Amygdalin, however, others showed no benefit, and while others concluded that the chemical has a minor effect on cancer cells (Jaszczak-Wilke *et al.*, 2021). To the best of our knowledge, no "measured clinical trials" on Amygdalin have been yet conducted. This indicates that the drug-drug interaction of Amygdalin with other chemotherapeutic drugs has not been conclusively spotted so far (Zhou *et al.,* 2020).

The ability of Amygdalin isolated from different sources to prevent the toxicity of some drugs is not widely described. Wang *et al.* (2021) demonstrated that Amygdalin presented cytotoxic properties *in vitro* and combination with some conventional chemotherapy. Therefore, we assumed that Amygdalin may cooperate synergistically with Doxorubicin, enhancing its inhibiting activity against HepG-2 cancer cells. Consequently, a noncancerous normal WPMY-1 cell line was used here to compare the effects of Amygdalin and Doxorubicin or their combination and to determine their cellular and molecular events in a normal system.

Although Doxorubicin is important for the treatment of both metastatic and original cancers, its side effects are still a serious disadvantage (Lao *et al.,* 2013). Therefore, Doxorubicin can be recommended to be used in combination with other natural compounds. It was used previously combined with resveratrol (Osman *et al.,* 2012), superoxide dismutase (Villani *et al.*, 1992), or other agents like amifostine, which is a broad spectrum cytoprotectant against radio and chemotherapy-related toxicities *in vitro* and *in vivo* while maintaining antitumor activity (Dragojevic-Simic *et al.,* 2013). We have recently coupled Doxorubicin with metformin, a natural biguanide that is a type 2 anti-diabetes prescription, to reduce its side effects and boost its effectiveness against breast cancers *in vivo* (Salim *et al.,* 2021).

In the present study, we evaluated the anticancer effect and the IC50 concentrations of Amygdalin and Doxorubicin when administered with HepG-2 cell lines for 24 or 48 hrs. The percentages of the surviving cells decreased when Amygdalin and Doxorubicin concentrations rose in a dose-dependent manner. Previously, Zhou *et al.* (2012) studied the progress inhibition and initiation of apoptosis carried by Amygdalin and/or combined with β-D-glucosidase and verified a cyto-activity on HepG-2 cells by dose-dependent induction of apoptosis. The present obtained data agreed with the recent findings of Mamdouh *et al.,* (2021) who studied the effect of different concentrations of Amygdalin and Doxorubicin *in vitro* and *in vivo* using HepG-2 and Huh-7 cell lines for 24, 48 and 72hrs, and they found a dose-dependent effect on cellular proliferation.

In normal WPMY-1 cells, Amygdalin applied for 24 and 48 hrs had no dose-effect level revealing no toxicity or IC50 concentration levels estimated. However, all the applied serial concentrations of Doxorubicin caused about 40% mortality of WPMY-1 cells, after 48 hrs but still did not reach half-maximal inhibitory concentration. This may mean that the cytotoxic effect of both Amygdalin and Doxorubicin is probably confined more prominently to tumor cells than normal cells. In support of these data, Yang *et al.,* (2014), showed that Amygdalin, extracted from *S.*

persicae caused follistatin expression in HepG-2 and normal skeletal muscle C2C12 cell lines.

By applying the equation of combination index (CI) of Chou and Talalay (1984), an overall synergistic liver cancer inhibitory effect of a 1:1 combination of Amygdalin and Doxorubicin was detected (CI=0.69153). The other two combination ratios used here (1:2, and 2:1) designated clear additive effect levels (CI=1.00076 and CI=1.11797, respectively). This indicates a possible strong and selective toxic effect of combination synergism on liver cancer cells at 1:1 dose, but the case was not the same on the normal WPMY-1 cells. Recently, it was shown that combining OSMI-1 (OGT inhibitor) with Doxorubicin reduces the IC50 value of Doxorubicin and has a synergistic impact on cancer cells (Makwana *et al.,* 2020). Previously, an anticancer synergy of combinations of vorinostat (HDAC inhibitor), 17-DMAG (HSP90 inhibitor), abacavir (anti-telomerase) or sorafenib (tyrosine kinase inhibitor) solely, combined, or combined with Doxorubicin was evident in lung small cell sarcoma (Dumont *et al.,* 2014).

Although the application of each of Amygdalin and Doxorubicin singly to WPMY-1 cells had almost no cytotoxic IC50 effect, a 1:1 combination of them was tested here to evaluate a possible paracrine action of both drugs on WPMY-1 cells when administered together. Previous studies on the paracrine interactions demonstrated that myofibroblast-conditioned media inhibits WPE1-10 prostatic epithelial cell growth significantly, whereas a conditioned medium from WPE1-10 slightly affected WPMY-1 cell growth. WPMY-1 cells are known to release extremely little MMP-9 but a lot of MMP-2, significantly more than epithelial cells (Webber *et al.,* 1999). For the treatment of cancer, apoptosis induction is one of the important mechanisms proposed for the anticancer therapeutic effects of various drugs (Kirhan *et al.,* 2020). It could be, therefore, explained here as the main mechanism for the profound anticancer effect of Amygdalin, Doxorubicin, or their combination, against HepG-2 cancer cells. In HepG-2 cells, the expression of *BCL-2* was downregulated, while the *BAX* expression was significantly higher after all treatments, most profound with the combination therapy. This indicates that Amygdalin has significantly enhanced the power of Doxorubicin molecular events leading to increased apoptotic induction signals. Members of the *BCL-2* protein family such as *BAX*, *BAK*, *BCL-2*, and *BCL-XL* influence apoptosis or cell cycle entry (Mobarra *et al.,* 2015).

By comparing the same treatments on WPMY-1 cells, the average expression of *BCL-2* was significantly downregulated similar to the effect on HepG-2 cells, while the relative expression of *BAX* was unexpectedly significantly downregulated in all treatment groups, in contrast to its behavior with HepG-2 cells. It is known that the downregulation of *BAX* ceases the induction of apoptosis. Thus, this may explain the lack of the cytotoxic effect of Amygdalin and Doxorubicin on WPMY-1 cells in the present MTT assay and indicates a possible different apoptotic behavior/ or affected pathways of the *BAX* gene expression balance in WPMY-1 cells. Excessive growth and development of normal cells are linked to a disruption of the balance between proliferation and apoptosis signals (Fulda and Debatin, 2006). It is known that the rate of apoptosis is higher in normal tissues than in any benign hyperplastic tissue, whereas hyperplastic tissue has a considerably higher rate of proliferation (Jin and An, 2020). It was shown previously that the positive effect of

Amygdalin's anticancer therapy is in its ability to initiate apoptosis in cancer cells through both intrinsic and extrinsic pathways. The intrinsic apoptotic pathway mediated by the mitochondria is mainly controlled by the balance and interactions between pro-and anti-apoptotic members of the Bcl-2 family proteins (Ghiasi *et al.,* 2014).

To confirm the above results, the flow cytometry analysis data of HepG-2 cells indicated the highest percentage of early and late apoptotic cells after the combination treatment >followed by Doxorubicin >followed by Amygdalin. Queiroz *et al.,* (2014), have measured the apoptotic cells by flow cytometry in MCF-7 cells and postulated that natural products induced apoptosis and autophagy, and inhibited tumor growth through the downregulation of Stat3 activity and *BCL-2* expression, activation of IRβ, Akt and ERK1/2, increased p-AMPK, FOXO3a, P27, BAX, cleaved caspase-3, and decreased phosphorylation of p70S6K and BCL-2 protein expression.

In WPMY-1 cells, only Doxorubicin alone or combined with Amygdalin showed a significant increase in late apoptotic and necrotic cells, but the total numbers of both early and late apoptotic cell percentages in groups 1-4 did not show any statistically significant differences when compared with untreated cells. Also, there were no inter-group significant differences in apoptotic cell percentages among the three treatment groups (2-3), albeit the treatment with Doxorubicin was slightly higher. These data coincide well and could be explained by the downregulation of *BAX* by the same treatment groups and explains the lack of cytotoxic effect of the three treatment regimens on WPMY-1 cells in the present MTT assay. Recently, similar results were obtained by Tamalunas *et al.*, (2021), who studied how Thalidomide affected the biological functions of normal WPMY-1 stromal cells and the contraction of human smooth muscles. WPMY-1 cell growth was moderately affected by Thalidomide.

The most obvious side effect of Amygdalin was thought that it was hydrolyzed in cancer cells, generating deadly hydrogen cyanide (HCN) and killing cells. Unfortunately, recent research has revealed that HCN is also secreted by normal cells with the help of intestinal enzymes, suggesting that it may not be healthy for humans. However, studies on the anti-cancer potential of this chemical are currently being done. *In vitro* investigations have revealed that Amygdalin induces apoptosis by increasing BAX protein and caspase-3 expression while decreasing anti-apoptotic BCL-2 protein expression. It was also shown that Amygdalin boosted the expression of the p19 protein in renal cancer cells, inhibiting cell transfer from G1 to S-phase and thereby inhibiting cell proliferation (Liczbiński and Bukowska, 2018). The present work confirms the selective apoptotic inducing capability of Amygdalin on liver cancer cells.

Conclusions

The present study shows the potency of Amygdalin, either solely or at IC50 (1:1) combination in inhibiting HepG-2 liver cancer cells *in vitro* associated with apoptosis, which is confirmed by different pathways. The mechanism of action was clearly shown to be through induction of apoptosis indicated by increasing the expression of the pro-apoptotic gene, *BAX*, or decreasing the *BCL-2* gene expression (anti-apoptotic gene). The combination treatments by IC50 doses of both Amygdalin and Doxorubicin exerted the most profound cytotoxic effect on the HepG-2 cells as compared with each treatment alone. Thus, the current study further reinforces the potential benefit of Amygdalin to enhance the efficacy of Doxorubicin in liver cancer treatment and provides novel mechanistic

insight into its antiproliferative role.

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كفاءة تركيبة الأميجدالين والدوكسوروبيسين على سرطان الخلايا الكبدية وخلايا البروستاتا الليفية العضلية اللحمية

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المستخلص

تم فى هذه الدراسة تقييم الإمكانات العلاجية المحتملة للأميجدالين إما بمفرده أو بالاشتراك مع الدوكسوروبيسين ضد خلايا سرطان الكبد HepG-2 وخلايا البروستاتا الليفية العضلية اللحمية الطبيعية (WPMY-1). بعد تحديد تركيزات IC50 من الأميجدالين والدوكسوروبيسين بواسطة تحليل MTT، تمت معالجة كل cell line لمدة ٤٨ ساعة على النحو التالي: خلايا control غير المعالجة، والخلايا المعالجة بالأميجدالين فقط، والخلايا المعالجة بالدوكسوروبيسين فقط، ومزيج من علاج الأميجدالين + الدوكسوروبيسين بنسبة متساوية (١:١). بالنسبة لخلايا HepG-2، وجد ان العلاج باستخدام الأميجدالين أو الدوكسوروبيسين أدي بشكل معنوي الي تقليل عدد الخلايا الباقية بطريقة تعتمد على زيادة الجرعة المستخدمة. في خلايا WPMY-1 الطبيعية، كانت النسب المئوية للخلايا الباقية ثابتة وغير متأثرة بزيادة تركيزات الأميجدالين أو الدوكسوروبيسين، مما يدل على عدم وجود تأثير لمستوي جرعة IC50. في خلايا HepG-2، أظهرت تركيبة الأميجدالين والدوكسوروبيسين بنسبة ١:١ بتركيزات IC50 تأثيرا تآزريا (مؤشر التركيب (CI)) = 0.69153). بالإضافة إلى ذلك، تم تقليل متوسط تعبير mRNA الطبيعي الي BCL-2 وتم العثور على تعبير BAX بشكل مفرط في جميع العلاجات في خلايا HepG-2، وأكثرها أهمية مع العلاج المركب. في خلايا WPMY-1، تم تقليل تنظيم كل من BCL-2 وBAX بعد كل العلاجات، على عكس السلوك المحتمل ضد الخلايا السرطانية. أظهر تحليل قياس التدفق الخلوي أن العلاج المركب لخلايا HepG-2 أدى إلى نسبة أعلى من الموت الخلوي المبرمج المبكر والمتأخر>Amygdalin >Doxorubicin. في خلايا WPMY-1، تم تعزيز الموت الخلوي المبرمج المبكر فقط بشكل كبير بواسطة Doxorubicin إما بمفرده أو بالاشتراك مع Amygdalin، مع عدم وجود تغييرات معنوية بين المجموعات في أعداد خلايا الموت المبرمج. تشير الدراسة إلى أن الأميجدالين يعزز بشكل تآزري إمكانات العلاج الكيميائي للدوكسوروبيسين ضد الخلايا السرطانية عند استخدامهما معا من خلال تحريض موت الخلايا المبرمج.