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EXPERIMENTAL EVALUATION OF DOXORUBICIN DRUG AS ANTICANCER AGENT USING A NATURAL COMPONENT: AN IN VIRTO STUDY

By

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Abstract

HCC is the sixth commonest cancer worldwide and the third one to cause death. In Egypt, liver cancer forms 1.68% of total malignancies. HCC constitutes 70.48% of liver tumors. HCC represents the main complication of cirrhosis. Chemotherapy has an undesirable side effect, so the drugs are used instead. Bee venom and curcumin have been used as folk medicine since ancient times. This study examined the anticancer potential of bee (*Apis mellifera*) venom and curcumin compared with a synthetic anticancer of Doxorubicin (Dox) drug, and tested the effect of combination between the tested three components to decrease the chemotherapy side effect. Cytotoxicity activity of Bee venom and curcumin were determined using MTT assay. Cell cycle was determined by flow cytometry and apoptotic detection by using Annexin V-FITC Assay. RNA was extracted to evaluate the expression of P53, Bcl2 & NF-κB genes using semi-quantitative RT-PCR after 24hrs. HepG2 cell line was more save to test bee venom and curcumin onHepG2 cell line. Curcumin and bee venom had IC_{50} as 15.15 & 31μg/ml with HepG2 cell line. Combination between Bee venom, curcumin, and doxorubicin drug had apoptotic effect against HepG2cell line through the elevation by increasing the level of p53 & NF-κB genes and decrease the Bcl-2 gene expression.

Keywords: Hepatocellular carcinoma (HCC), Doxorubicin, Bee venom, Curcumin, Combination therapy.

Introduction

Mortality rates due to HCC have increased in many countries over recent decades. In this study, we updated worldwide mortality trends for HCC from 1990 to 2014, and predicted trends for some countries to 2020, with unfavorable trends in Northern and Central Europe, North and Latin America. East Asia showed an improvement; however mortality rates in this region were 2-to 5-fold higher than in most European countries and the Americas. Steady declines to 2020 are predicted for East Asia but not for Europe and the Americas (Bertuccio *et al*, 2017). In Egypt, liver cancer forms 11.75% of the malignancies of all digestive organs and 1.68% of the total malignancies. HCC constitutes 70.48% of all liver tumors among Egyptian (Holah *et al*, 2015). HCC is a malignancy with extremely troubling prospects and a five-year survival rate announced underneath 9%. Hepatocellular carcinoma has increased significantly worldwide, especially in developing countries in Asia, such as China and sub-Saharan Africa. Although, the larger part of HCC cases are a consequence of infections because of the hepatitis B and C viruses, hazard factors such as both alcoholic and non-alcoholic, obesity, iron overload, cirrhosis, and in addition dietary hepatocarcinogenesis, such as aflatoxins and nitrosamines have likewise been implicated as critical key reasons of HCC (Darvesh *et al*, 2012). Progressed HCC is highly refractive to presently accessible chemotherapies, and patients suffering from HCC regulatory develop drug resistance during treatment. Several chemotherapeutic agents have been tested in patients with cutting-edge HCC however, the results were disappointing and life-threatening side effects frequently limit prolonged treatment (Bagi and Andresen, 2010). One of the chemotherapeutics tested in HCC patients, Doxorubicin is an an-

thracycline antibiotic that exerts its cytotoxic effects via intercalating between base pairs of the DNA double helix structure and inhibiting the function of topoisomerase II, the enzyme that is responsible for the proper conformation and stability of DNA helix (Mahjoub *et al,* 2017) that showed rather low efficacy however, with a response rate of 10-15% and doxorubicin remains the best single specialist as of now accessible (Bagi and Andresen, 2010).The use of traditional drugs is declining and naturally extracted drugs are used instead. Several pharmacological anticancer applications have been found in the bee venom (Alizadehnohi *et al*, 2017). Although the curcumin has possesses valuable pharmaceutical properties and medicinal benefits (Mahjoub *et al*, 2017). Many authors have recognized therapeutic potential; as an anti-inflammatory, antioxidant and anti-cancer agent (Sharma *et al*, 2016).Bee venom from the venom gland located in the abdominal cavity contains several biologically active peptides that including approximately 50% of melittin and other of mast cell degranulating peptide, apamin, adolapin, and phospholipase A2 enzyme as well as non-peptide components such as norepinephrine, histamine ,and dopamine and non-peptide components including lipids, carbohydrates and free amino acids. The effects of bee venom induce poison of apoptosis, necrosis, cytotoxicity, and growth inhibition on cancer cells. BV inhibits proliferation of cancer cell and tumor growth that involve stimulating of the local cellular immune responses in lymph nodes (Oršolić, 2012).Recent studies have demonstrated that natural products can inhibit tumor growth and its metastasis leading to induction of cell apoptosis. Thus, it seemed to consider using these natural products as an alternative new medicine for human cancer. Throughout the past two decades, melittin as a major peptide in bee venom has concerned a huge attention for their potential effect in treating cancer. Melittin is an amphiphilic peptide consisting of 26 amino acid. It is isolated from bee *Apis mellifera* venom that disturbed the membrane (Trumbeckaite *et al*, 2015). Curcumin is a yellow pigment extracted from rhizome of turmeric is the crystallization of polyphenols with low molecular weight. Turmeric belongs to a permanent herb called *Curcuma longa L.* which is prevalent in tropical and subtropical regions. The biological characteristic of turmeric is known to be attributed to the content of curcuminoids found in a dense structure of turmeric. Curcumin has an extensive variety of applications as nutritional agent of treatment, food ingredients, and medicine in various diseases (Darvesh*et al.,* 2012).Curcumin contains a high plutonobic molecule capable of interacting with many of the molecular targets involved in inflammation. Curcumin has ability to down-regulating of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) enzymes. The production of cytokines also inhibits inflammation of the tumor necrosis factor-alpha (TNF-a) and monocyte chemo-attractant protein (MCP) .Cox-2 and iNOS are most likely inhibited by curcumin suppression of the nuclear factor kappa B (NF-kB) activation NF-kB. The eukaryotic transcription factor is involved everywhere in the regulation of inflammation, cellular proliferation, and transformation .Curcumin is believed to suppress the activation of NF-kB and pro-inflammatory gene expression by inhibiting phosphorylation of inhibitor factor I-Kappa B kinase. The suppression of NF-kB activation later down regulates COX-2 &iNOS expression, thereby inhibiting the inflammatory process and tumors (Jurenka, 2009). P53, BCL-2, & NF-κB ex-pression genes played an important role in the control of apoptosis. P53 is a protein which plays a critical role in the intrinsic apoptosis pathway activation (Fesik, 2005). Bcl-2 may act upstream of P53 pathway. The induction of NF-κB mRNA and protein were found to be stuck in presence of Bcl-2. Cross-like between Bcl-2, p53 and NF-κB genes is a significant determinant of

drug-induced apoptosis (Giménez-Bonafé *et al*, 2009).

This work evaluated the possible anticancer effects of some natural components such as bee venom and curcumin that compared to a synthetic anticancer drug (doxorubicin). The parameters included cytotoxicity, cell cycle, and the expression of some genes related to apoptosis by semi-quantitative RT-PCR technique to show the effect of combination on human cell line.

Material and Methods

Doxorubicin 50mg/25mL (MYLAN Pharmaceutical Co.) was diluted in RPMI-1641 media (Bio-Whittaker™ Classical Media, Lonza) to prepare test concentrations. Egyptian of bee (*Apis mellifera*) venom and curcumin were su-pplied from Sera plant VACSERA. Both were dissolved in 2.5mL sterile double distilled water to contain 10mg/mL and sterilized using 0.22m syringe filter (Millipore-USA) and serially diluted in RPMI-1641 media. HepG2 (Human liver cancer cell line) was originally supplied from USA Type culture collection (ATCC). Trypsin 0.25% was provided by VACSERA-Cell Culture Unit. Cells were seeded at a density of 2×105/ml and routinely cultured in RPMI-1641 medium in tissue culture flasks (Griener, Germany).

Maintenance of cell line and culture conditions: Hep G2 (Human liver cancer cell line) was provided by VACSERA-Cell Culture Unit, Egypt. Cells were cultured in RPMI medium in T-75 flasks (Griener, Germany) supplemented with 10% fetal bovine serum. The cell cultures were maintained at 37^{o}C, 95% humidified atmosphere with 5% CO_2. Monolayer cells were trypsinized once they reached 80% confluency (Masters, 2000).

Cell counting: Accurate cell number in the suspension was calculated using the haemocytometer (Soliman *et al*, 2013). Briefly, double fold dilution of the original cell suspension was prepared by adding 0.5mL of undiluted cell suspension to 0.5mL of 0.4% trypan blue dye.The mixture was mixed well and immediately aspirated to fill the haemocytometer counting chambers.

Cytotoxicity using colorimetric assay (MTT): Hep G2 cell line was seeded in the 96- well tissue culture plate with 100µL of RPMI medium. 200µL of tested materials serial dilution was done using two fold dilutions. The plates were then incubated with the various concentrations of each bee venom and curcumin for 24 h at 37ºC. The plates were read using ELISA reader at wavelength 570 nm and the absorbance was correlated with the cell number. The cytotoxic effects of the tested venom and curcumin on the Hep G2 cell lines were expressed as the IC50 value. Inhibitory concentration (IC50) of the compounds was assessed by Master-plex software (2010).

Morphology by inverted microscope. Untreated and treated Hep G2 cell line with bee venom and curcumin were examined for changes, 24h post treatment using an inverted phase contrast microscope.

Flow cytometry cell cycle analysis using Propidium iodide DNA staining: For cell cycles analyzed by FACS, HepG2 cell line was seeded in DMEM with 10% FBS, then fixed in cold 70% ethanol on 6-well plates and allowed to attach overnight. While vortexing, the pellet was added drop wise. All cells were fixed for 30min at 4°C and minimized clumping. After ensuring fixation, the cells were washed in PBS and spun at 850g in a centrifuge. Supernatant was discarded especially after spinning out of ethanol. Cells were treated with ribonuclease I (100mg/ml stock solution). 50µl of a 100µg/ml sock of RNase was added to ensure only DNA not RNA was stained. 200µl propidium iodide was added (50µg/ml stock solution).

Apoptosis analysis by using Annexin V-FITC Assay: 1-5 x 105 cells were collected by centrifugation, re-suspended in 500µl of 1X Binding Buffer. 5µl of Annexin V-FITC & 5µl of propidium iodide (PI 50mg/ml) were added. Incubation was done at room temperature for 5 min in the dark. Annexin

V-FITC binding was analyzed by flow cytometry (Ex= 488nm; Em= 530nm) using FITC signal detector and PI staining by phycoerythrin emission signal detector. For adherent cells, they were gently trypsinized and washed once with serum-containing media before incubation with Annexin V-FITC. Stained cells were analyzed for apoptotic cells % by Partec flow cytometer and FloMax software.

RNA Extraction and Quantitative RT-PCR: Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse-transcribed complementary DNA was synthesized using the Prime Script RT reagent kit (TaKaRa, Otsu, Japan). Real-time polymerase chain reaction was performed using SYBR Premix ExTaq (TaKaRa, Otsu, Japan) on an ABI Step One Real-Time PCR System (Applied Biosystem, Carlsbad, CA, USA). The amount of mRNA for each gene was normalized by GAPDH, and the relative expression levels were calculated using the 2-ΔΔCt method. The primer sequences of the oligonucleotides used for PCR were as follows in relative quantification for the genes (β-actin, P53, Bcl-2, and NF-kB).

Primer sequences for genes

Gene	Primers sequences	Annealing temperature
β-actin	F 5'GTGACATCCACACCCAGAGG 3'	52°C
	R 5'ACAGGATGTCAAAACTGCCC 3'	52°C
P53	F 5' CCCCTCCTGGCCCCTGTCATCTTC 3'	53°C
	R 5'GCAGCGCCTCACAACCTCCGTCAT 3'	51°C
Bcl-2	F 5'CCTGTG GAT GAC TGA GTA CC 3'	49°C
	R 5'GAGACA GCC AGG AGA AAT CA 3'	47°C
NF-*k*B	F 5'-CGCAGAGCACGTGGGACCTG -3'	55°C
	R 5'- GGCGACGACTGTAGCGCCTC-3'	60°C

F: Forward strand and R: Reverse strand .One mixed and left 10 min in dark at 25°C the absorbance of the supernatant was measured photometrically at 540 nm.

Statistical analyses: All experiments were performed in triplicate unless specified, represented as the mean ± SEM. Analysis was performed using unpaired Student's t test. Groups were compared by one-way ANOVA with Dunnett's posttest. P values <0.05 were considered significant and p <0.01 highly significant. Cytotoxicity using colorimetric as-say (MTT) showed that doxorubi**Cin** drug and bee venom inhibited cancer cells growth. Liver cell line (*Hep-G2)* was treated with combination of DOX and bee venom against anticancer chemotherapy growth inhibitory, effects were performed and evaluated using MTT assay.

Results

The results are shown in tables (1, 2, 3, 4, 5 & 6) and figures (1 to 7).

Table 1: Effect of doxorubicin drug with bee venom against drug doxorubicin only on inhibitory effect on Hep-G2 liver cancer cell line

IC50	*DOX*	B.V	*DOX+B.V*
(ug/mL)	217	31	66.36

Cytotoxic effect of bee venom and doxorubicin on *Hep-G2* was expressed as IC_{50} value; the drug concentration reduced the absorbance of treated cells by 50% with respect to untreated cells. Inhibitory concentration (IC_{50}) was assessed by master pellex software. Bee venom and DOX showed significantly high growth inhibitory effects on *Hep-G2* liver cancer cell line in a concentration dependent manner compared to bee venom alone. Bee venom was selected to detect pro-apoptotic such as (P53) gene and anti-apoptotic (bcl-2) gene expression levels in cell lines post treatment. IC_{50} measured for *Hep-G2*, and for doxorubicin drug was 217µg/ mL, bee venom was 31µg/mL, and doxorubicin with bee venom was 66.36µg/ mL. DOX was more toxic than bee venom alone or with bee venom (Fig. 1, Tab. 1).

Table 2: Effect of curcumin, curcumin with DOX, and combination of curcumin & DOX with bee venom against doxorubicin drug on inhibitory effect on Hep-G2 liver cancer cell line

IC50 (ug/mL)	Curcumin	Curcumin + DOX	*CBD*
	15.15	77.30	348.00

CBD= respective on Hep-G2 cell line.

Cytotoxic effect of the bee venom as well as doxorubicin on *Hep-G2* was expressed as IC_{50} value; drug concentration reduced the absorbance of treated cells by 50%. Inhibitory concentration (IC_{50}) of compound assessed by master pellex software. Combination of CBD showed significantly high growth inhibitory effects on *Hep-G2* liver cancer cell line in a concentration dependent manner compared to curcumin only or with doxorubicin drug. So, curcumin and bee venom were selected to detect the pro-apoptotic (P53) gene and anti-apoptotic (bcl-2) gene expression levels in cell lines post treatment.

IC_{50} for curcumin was15.15µg/mL, curcumin & doxorubicin drug was 77.5 µg/ mL and combination of curcumin and doxorubicin with bee venom was 348.00 µg/mL. So, *CBD* 348.00 µg/mL was more toxic than curcumin only or with DOX. So, curcumin and bee venom determined their effect on pro-apoptotic and anti-apoptotic gene expression level post treatment (Fig. 2)

Table 3: Effect of cell cycle concentration on HepG2 cell line by different samples

ser	Sample code	%G0-G1	%S	%G2/M	%PreG1	Comment
1	D (DOX)	66.02	21.84	12.14	10.29	PreG1apoptosis&Cell growth arrest@ G2/M
2	Cur (Curcumin)	62.86	18.92	18.22	16.4	PreG1apoptosis&Cell growth arrest@ G2/M
3	B (BV)	64.32	22.46	13.22	11.29	PreG1apoptosis&Cell growth arrest@ G2/M
4	D+B	64.28	21.13	14.59	11.39	PreG1apoptosis&Cell growth arrest@ G2/M
5	D+C	56.41	17.33	26.26	24.31	PreG1apoptosis&Cell growth arrest@ G2/M
6	D+C+B	59.62	24.2	16.18	13.38	PreG1apoptosis&Cell growth arrest@ G2/M
7	cont. HepG2	69.32	27.91	2.77	2.47	

In HepG2 cells, DOX sample cell population was G0-G1, G2/M, S, & Pre-G1 phases from 66.02ug/ml, 21.84ug/ ml, 12.14ug/ml, and 10.29ug/ml. then cur in G0-G1, G2/M, S, & Pre-G1phases from 62.86ug/ml, 18.92ug/ml, 18.22ug/ ml, & 16.4 ug/ml. BV indicated in G0-G1, G2/M, S, & Pre-G1 phases from 64.32ug/ml, 22.46ug/ml, 13.22 ug/ml, & 11.29ug/ml. Combination of DOX & bee venom in G0-G1, G2/M, S, & Pre-G1phases from 64.28ug/ml, 21.13ug /ml, 14.59ug/ml, &11.39ug/ml. and in combination of DOX & curcumin (D+C) in G0-G1, G2/M, S, & Pre-G1 phases from 56.41ug/ ml, 17.33ug/ml, 26.26ug/ml, & 24.31ug/ml. depended combination of G0-G1, G2/M, S, & Pre-G1phases from 59.62ug/ml, 24.2ug/ml, 16.18ug/ml, & 13.38ug/ml. Control Hep G2 cell line showed G0-G1, G2/M, S, & Pre-G1phases as 69.32ug/ml, 27.91ug/ ml, 2.77ug/ml, & 2.47ug/ml respectively..

Induction of cell cycle arrest in G0/ G1, S, G2/M and apoptosis phases after 24h of incubation of HepG2 cell line (Fig. 3), accumulated in G0/G1 phase (purple columns) of cell cycle, but proportion of cells in S phase (violet columns) decreased on six combination in independent experiments. When compared to arrest in G2/M phase (yellow columns) and apoptosis (aqua columns) in HepG2 cell differed from proportion of cells in G0/G1 phase of cell cycle.

Table 4: Effect of different stages of apoptosis (cell death program) on HepG2cell line.

Ser	Sample	Total	Early	Late	necrosis
1	D (DOX)	10.29	3.86	4.36	2.07
2	Cur (Curcumin)	16.4	6.52	7.89	1.99
3	B(BV)	11.29	4.48	5.07	1.74
4	D+B	11.39	4.17	5.27	1.95
5	D+C	24.31	10.55	9.76	4.0
6	D+C+B	13.38	5.49	5.87	2.02
7	cont HepG2	2.47	0.89	0.75	0.83

early, late, and necrosis stages was 11.39, 4.17, 5.27, & 1.95. The DOX with curcumin gave high significant in total, early, late, and necrosis stages as 24.3, 10.55, 9.76, & 4.0. Combination between bee venom, curcumin, and DOX gave best effect on live cancer cell line in total, early, late, and necrosis stages as 13.38, 5.49, 5.87\ & 2.02 respectively.

Effect of (DOX) in total, early, late, and necrosis stages arranged10.29, 3.86, 4.36, & 2.07. also, in Cur (Curcumin) in total, early, late, and necrosis stages arranged 16.4, 6.52, 7.89, &1.99.and bee venom as antioxidant B(BV) gave good in total, early, late, and necrosis stages as 11.29, 4.48, 5.07, & 1.74. Combination of DOX & bee venom in total,

Table 5: Relative Bcl2, p53, and NFkB gene expression in HepG2 cell line according to sample data

Ser	Sample code	P53	Bcl2	NFkB
1	D (DOX)	8.962536	0.306488	7.826363
2	Cur (Curcumin)	11.37421	0.147549	11.16162
3	B (BV)	10.11092	0.522225	8.08324
4	D+B	7.62008	0.586194	10.97516
5	D+C	15.16802	0.194713	14.48009
6	D+C+B	9.507292	0.329361	10.78922
7	cont. HepG2	1	1	1

compared with those of house-keeping gene as relative fold expression change. A high value depicts a higher expression level in P53, but a less one showed a lower expression in tumor relative to liver samples (NFkB & Bcl2).

Gene expression level of pro-apoptotic gene as p53 and anti-apoptotic gene as Bcl-2 and NFkB were examined in cell lines post treatment with IC_{50} of curcum in, DOX, & bee venom compared to control by PCR (Fig. 5). Duplicated data were normalized

Table.6a: Gene expression level of Bcl2 in quantitation data with control and test cell

A	Sample data	Bcl2							
		Control cells			Test cells				FLD
Ser	Sample code Conc. uM	B Actin	Bcl2	ΔCTC	B Actin	Bcl2	ΔCTE	ΔΔ CT	2^ ΔΔCT
		HC	TC	HC-HE	HE	TE	TC-TE	ΔCTE/ΔCTC	Eamp=1.849
1	D (DOX)	27.69	28.96	2.5	25.19	33.77	-4.81	-1.924	0.306488
2	Cur (Curcumin)	27.69	28.96	1.5	26.19	33.63	-4.67	-3.11333	0.147549
3	B (BV)	27.69	28.96	1.58	26.11	30.63	-1.67	-1.05696	0.522225
4	D+B	27.69	28.96	1.45	26.24	30.22	-1.26	-0.86897	0.586194
5	D+C	27.69	28.96	1.45	26.24	32.82	-3.86	-2.66207	0.194713
6	D+C+B	27.69	28.96	1.45	26.24	31.58	-2.62	-1.8069	0.329361

Table 6b: Gene expression level of p53 in quantitation data with control cell and test cell

B	Sample data	p53							
		Control cells			Test cells				FLD
Ser	Sample code Conc. uM	B Actin	p53	ΔCTC	B Actin	p53	ΔCTE	ΔΔ CT	2^ ΔΔCT
		HC	TC	HC-HE	HE	TE	TC-TE	ΔCTE/ΔCTC	Eamp=1.849
1	D (DOX)	27.69	34.27	1.25	26.44	29.81	4.46	3.568	8.962536
2	Cur (Curcumin)	27.69	34.27	1.58	26.11	28.02	6.25	3.955696	11.37421
3	B (BV)	27.69	34.27	1.06	26.63	30.28	3.99	3.764151	10.11092
4	D+B	27.69	34.27	1.25	26.44	30.14	4.13	3.304	7.62008
5	D+C	27.69	34.27	1.25	26.44	28.74	5.53	4.424	15.16802
6	D+C+B	27.69	34.27	1.25	26.44	29.69	4.58	3.664	9.507292

Table 6c: Gene expression level of NFkB in quantitation data with control cell and test cell

C	Sample data	NFkB							
		Control cells			Test cells				FLD
Ser	Sample code Conc. uM	B Actin	Casp3	ΔCTC	B Actin	Casp3	ΔCTE	ΔΔ CT	2^ ΔΔCT
		HC	TC	HC-HE	HE	TE	TC-TE	ΔCTE/ΔCTC	Eamp=1.849
1	D (DOX)	27.69	33.52	1.18	26.51	29.57	3.95	3.347458	7.826363
2	Cur (Curcumin)	27.69	34.69	2	25.69	26.84	7.85	3.925	11.16162
3	B (BV)	27.69	34.78	1.8	25.89	28.66	6.12	3.4	8.08324
4	D+B	27.69	34.78	1.66	26.03	28.31	6.47	3.89759	10.97516
5	D+C	27.69	34.78	1.98	25.71	26.17	8.61	4.348485	14.48009
6	D+C+B	27.69	34.78	1.92	25.77	27.35	7.43	3.869792	10.78922

Discussion

No doubt, the toxic effect of DOX is a reason for concern that limits its usability. It provides an inadequate effect in addition to its effect on the cancer cells as well as the normal cells. Also, is one of the major problems of chemotherapy is the resistance developed after initial treatment. The problem of using chemotherapeutic drug has main factors of poor prognosis and advancement of liver cancer in the human. Thus, scientists participated in the design of the most powerful treatments to overcome this obstacle. It has been confirmed that defective apoptosis is one mechanism of latent chemical resistance in liver cancer (Jiang *et al*., 2013). This has led to the increased demand of using anticancer drugs developed from natural resources. The biodiversity of bee venom and curcumin makes them a unique source from which novel therapeutics may be developed. Curcumin and bee venom reported having potential cytotoxic effects on tumor cells. However, little is known about their cytotoxicity as crude venoms compared with their major components effects on the liver cancer. In the current study, the anti-proliferative effects of curcumin and BV have been studied on the liver cancer cell. Bee venom inhibits the proliferation of carcinoma cell in a dose dependent way. The bee venom has the ability to induce apoptosis via Bcl-2 & NF-kβ in down-regulation in signaling pathways. Curcumin-mediated growth inhibition and DNA damage in both mitochondria as well as the nucleus involved pro-oxidant mechanisms as evidenced by increased ROS and lipid peroxide levels in HepG2 cells. In a subsequent study showed that curcumin produced mitochondrial hyperpolarization, elevated mitochondrial membrane potential and also increased cytochrome C release in HepG2 cells. Up-regulation of the notch1 receptor and signaling pathways in hepatocarcinogenesis result in increased cellular proliferation as well as inhibition of apoptosis. Jurenka (2009) reported that the possible mechanisms responsible for the induction of apoptosis by curcumin inhibits of growth and induction of apoptosis in a dependent and independent way in liver cancer cells is via down-regulating the expression of Bcl-2 and Bcl-XL and up-regulating the expression of P53. Curcumin also affected p53 by modulating its phosphorylation at serine 15 and its acetylation in a concentration-dependent manner.

In the present study, bee venom inhibited tHepG2 cell proliferation and induced morphological changes that agreed with Premratanachai and Chanchao (2014) who confirmed that bee venom has an inhibitory effect on HepG2cell line after 24hrs treatment. Samarghandian *et al.* (2014) proved that HepG2 liver cancer cells showed morphological changes post 24hrs in a time and concentration dependent.

In the present study, cell proliferation inhibition and induction of morphological changes was observed that was in agreed with Ip *et al*. (2008) confirmed that bee venom posseesed an inhibitory effect on HepG2 cell line 24hrs post treatment. Regarding to measure the cytotoxicity of bee venom to HepG2 cell line the IC50 value was that was alignment with 31µg/m. Alizadehnohi *et al.* (2017) reported that the IC_{50} value of bee venom was 28.5µg/ml which is almost equal to present data and induced decrease of cell viability and apoptosis in human A172 glioblastoma cancer cell line. Several cellular cytotoxicity mechanisms have been reported in different types of cancer cells such as cell cycle modifications, an effect on proliferation, growth inhibition, and induction of apoptosis / necrosis. Also, the anti-proliferative effect of BV has recently demonstrated on C33A cervical cancer cells. The present cytotoxicity of doxorubicin drug in HepG2 cell line treated with the IC50 was recorded with a value 217 µg/ml after 24h that was near to that of bee venom. This disagreed with the IC50 value 164µg/ml after 24h in the A549 lung (Qian*et al*, 2011) cancer cell line. the combination of

bee venom and doxorubicin induced synergetic activity induced a decreased IC50 value compared with that on using both bee venom and Dox in a single form recording IC50 value in the order of 66.36 µg/ml after 24h, that was in accordance with (Jurenka, 2009) recording that the higher level of lung cancerA549 cell treatment with bee venom a Dox mix was attributed to the synergetic activity detected. Furthermore BV treatment did not affect viability of normal cells indicating a degree of specificity for malignant cells. Therefore, it seems that BV components can be good candidate for future clinical trials for cancer therapy. BV constitutes an enormous source of enzymes and bioactive peptides such as melittin and phospholipase A2 and its beneficial actions on tumor cells may be due the effects of a single or several constituents on the tumor cells. Regarding to measure the cytotoxicity of curcumin that use as anti-oxidant for treatment of liver cancer (HepG2) cell line treated, Curcumi showed a higher toxicity to liver cancer cells recording an IC50 value in the order of 15.5µg/ml after 24h. The data agreed with Putri*et al.*(2016) who reported that the curcumin inhibited the proliferation of HepG2 cells in a dose and time dependent manner. The cell viability decreases steadily when the dose of Curcumin increases as well as the incubation time prolongs. The IC50 value of Curcumin against HepG2 cells was 45.7µg/ml. In vitro cytotoxicity evaluation indicates that Curcumin possesses a dose-dependent cell inhibition effect against HepG2 cells with the activation of Caspase-3 (Jiang *et al,* 2013). In the present study, there was a synergetic activity of Curcumin to Dox recording an IC50 value of 77.5µg/ml after 24h., which agreed with Putri *et al.* (2016) recording that IC50 calculated post lung cancer cell line (A549) was 72 72µg/ml. Also, the result synergetic activity of the 3 mix induced an antagonistic activity recording a higher IC50 value 348 µg/ml and that pro and anti-apoptotic gene (P53-BCL-2) Curcumin and bee venom were selected to detect the pro-apoptotic such as (P53) gene and anti-apoptotic (bcl-2) gene expression levels in the cell lines after the treatment.

Cell cycle arrest is a common feature of cells that are undergoing terminal differentiation and defective proliferation. Based on the growth inhibitory and loss of membrane integrity on HepG2 cell lines, the cell cycle indicated that the cytotoxicity caused by doxorubicin might be derived from its potency in inducing marked apoptotic cell death after 24 hrs incubation, apoptotic cell death was the main contributor to toxicity, while the accumulation in the G2/M phase was decreased after 24 hours. Doxorubicin is classified as an antitumor antibiotic, made from natural products produced by species of the soil fungus *Streptomyces.* It acts during multiple phases of the cell cycle and the cytotoxic effect of doxorubicin are generally considered to be cell-cycle specific where accumulation in G2/M phase can normally be detected in doxorubicin treated HepG2 (Qian *et al,* 2011). Another supporter to the effect of cell cycle on doxorubicin drug that interact with the cellular DNA while cells are in either S-phase or G2/M-phase inducing cell cycle arrest at G2/M-phase, also called mitoticcrises/catastrophe . It was reported that agents inducing S-phase accumulation sensitize tumorcells to the killing effect of doxorubicin. Regarding the cell cycle profile it was noticed that our result was in accordance with that Alizadehnohi et al.,(2017) recording that the effect of bee venom and Dox on human lung (A549) induced pre G1 apoptosis and cell cycle arrest at G2/M and cell apoptosis may be attributed to the extrinsic path way and the and polyphenols , phenolic acids and vitamins, epicatechin and gallic acid are the most abundant that affecting death receptors (DR) leading to apoptosis in A549 through the interaction with TNF cytokine family such as tumor necrosis factor (TNF) with death receptor 1 (DR1), Fas Ligand (FASL) with death receptor 1 (DR2) & Apo3 ligand

(Apo3L) with death receptor 3 (DR3). Cell death and apoptosis might be due to the elevated antioxidants and anti-inflammatory, anti-mutagenic, and anticancer activities (Premratanachai and Chanchao, 2014). Currently combination of doxorubicin and curcumin-induced defects in mitotic events were paralleled by a significant increase in tumor cell G2/M arrest, suggesting that curcumin-induced suppression in cell growth. The induction of apoptosis was characterized by the accumulation of cells at the sub-G1 phase and increase in liver cancer cells. The inhibitory role of curcumin was due to the induction of cell arrest and apoptosis. Curcumin itself is a potent anticancer agent. Phase III clinical trials are undergoing to evaluate the effects of curcumin against pancreatic and liver cancers (Hatcher *et al*, 2008). Possible antitumor activity of curcumin and doxorubicin includes induction of tumor apoptosis and inhibition of tumor proliferation, invasion, angiogenesis, and metastasis. Numerous targets regulated by curcumin and doxorubicin consisting of kinases, enzymes, growth factors, cytokines, and transcription factors. In several studies, curcumin-induced p53-dependent apoptosis and G2/M arrest. p53, a tumor suppressor and a key regulator of cell survival and cell cycle progression in p53 mutated cell line the antitumor efficacy of curcumin proceeding via inhibition of HepG2cells must be p53-independent.In the present study, cell cycle and apoptotic profile post application of bee venom-curcumin-Doxorubicin mix to liver cancer showed cell arrest and apoptotic profiles differently than use of each product in a single form. The regulation of apoptosis in normal and malignant cells has become an area of extensive study in cancer research. The apoptotic process is involved in the growth and inhibition of the tumor cells. In the present study, the examination of apoptotic regulatory effect of the tested venoms and their components as well as their combinations in liver cancer through the increase in expression intensity of P53, Bax and decreased Bcl2 resembling that approved by different studies on the relation of bee venom their cancer cell death by apoptotic process. Curcumin-mediated growth inhibition and DNA damage in both mitochondria as well as the nucleus involved pro-oxidant mechanisms as evidenced by increased ROS and lipid peroxide levels in HepG2 cells. In a subsequent study, showed that curcumin produced mitochondrial hyperpolarization, elevated mitochondrial membrane potential (MMPs) and also increased cytochrome *C* release in HepG2 cells. Up regulation of the P53 receptor and signaling pathways in hepatocarcinogenesis increased cellular proliferation and inhibited apoptosis.

In the present study, P53 was up-regulated gene and down regulated NF-kβ and Bcl-2. Tumor suppressor P53 frequently altered target in doxorubicin-resistant HCC, which is one of the key DNA damage sensors that transcriptional activator of pro-apoptotic factors including Bax, Bak. Doxorubicin up-regulates P53 which occurs through its phosphorylation by DDR kinases that inhibit its binding to and phosphorylation by MDM2, part of pathway of constitutive ubiquitination and proteosomal degradation that normally leads to low steady-state levels of P53 An inhibitor of MDM2-P53 binding, Nutlin-3 enhanced P53 stabilization and activation, and increased doxorubicin sensitivity in HCC cells with wild-type P53. Mutation or deletion of P53, or disruption of P53 activation pathways were events in HCC tumorigenesis, providing a possible me-chanism for intrinsic resistance to doxorubicin. Increasing P53 was not the only way to enhance doxorubicin sensitivity as the three hepatoma cell line in HepG2 cells, which have wild-type P53 (Cox and Weinman, 2016).

In the present study, effect of curcumin on the expression of P53 was in these liver cells. It was significantly enhan- ced P53-responsive P21 plays a key role in G2/M arrest of the cell cycle and indicates that expression of the tumor suppressor P21, a po-

tent cyclin-dependent kinase (CDK) inhibitor that response to a variety of conditions including DNA damage and terminal differentiation. Moreover, up-regulation of P21 was required for growth inhibition of human cancer cells. P21induction in response to DNA damage requires the function of the P53 tumor suppressor protein. However, P21 expression was shown to be induced in several cell lines by agents that cause terminal differentiation via P53-independent mechanism. The indu- ction of P21 and Bax by curcumin was associated with apoptosis induction. Curcumin caused cell cycle arrest and apoptosis in several tumor cell lines (Veeraraghavan *et al*, 2010). The activity and stability of bee venom on tumor suppressor protein p53 was negatively regulated by oncogenic proteins MDM2 and MDMX cellular process initiated by MDM2/MDMX binding to the N-termi-nal transactivation domain of P53. The antagonists blocked P53-binding pocket of MDM2/MDMX killed tumor cells *in vitro* by reactivating the P53 pathway. Since MDM2 and MDMX act synergistically in tumor cells, they highly attractive molecular targets for anticancer drug development. Inhibiting interactions between P53 and MDM2 have an antiproliferative effect in some cancer cells. Since the tumor suppressor p53 inhibits tumor growth primarily through its ability to induce apoptosis, reactivation of P53 by trigger massive apoptosis and eliminate the tumor cells.NF-κB of doxorubicin drug have a tumor promoting effect by signaling is activated by DNA damage and can have varying effects on subsequent apoptosis primarily through regulation of its target genes, such as Bcl-XL, NF-κB has an anti-apoptotic effect in response to drugs in DNA such as doxorubicin although might be partially dependent on the cancer cell type. Few studied the NF-κB role in resistance to doxorubicin in HCC although it has been shown to be activated in HCC cells in response to doxorubicin and several studies have indicated that activation of NF-κB is a mechanism by which a diverse set of stimuli generate an anti-apoptotic effect. Anti-apoptotic gene BAG-1 enhanced doxorubicin resistance by potentiating the NF-κB transcriptional activity (Cox and Weinman, 2016). NF-kB in curcumin played a crucial role in signal transduction pathways involved in acute chronic inflammatory diseases and various types of cancer. NF-kB proteins reside in cytoplasm in an inactive state because the transition to nucleus at activation required activation of different kinases, phosphorylation, and degradation of NF-kB (Hatcher *et al*, 2008). Alizadehnohi *et al*. (2017) reported that bee venom on cancer cell growth depended on cancer cell types. The cancer cell growth inhibitory effect was correlated with the down-regulation of various cell proliferative genes regulated by NF-κB. In agreement, BV suppressed DNA binding activity of NF-κB. Also, decrease of NF-κB DNA binding activity was associated with t inhibitory effect of BV on the IκB phosphorylation and nuclear translocation of p50 & p65 in colon cancer cells. The present data showed that BV suppressed the expression of anti-apoptotic proteins like Bcl-2, and increased the expression of pro-apoptotic proteins such as Bax, caspase-3, caspase-8 and caspase-9 regulated by NF-κB or might be a difference in doxorubicin the ability of sequence-specific to suppress Bcl-2 and Bcl-xL expression. In the present results greater suppression of Bcl-2 than Bcl-xL was achieved, their differences did not fully affect chemotherapy. The differential effects of Bcl-2 and Bcl-xL on drug sensitivity might be unique to liver cancer. Despite similarities in function in Bcl-2 family proteins, that Bcl-2 and Bcl-xL were subjected to different regulatory mechanisms. Bcl-2 inhibits induced apoptosis by blocking cytochrome *c* release; but Bcl-xL did not affect insertion into mitochondrial membranes (Takahashi *et al*, 2003). Expression result showed Bcl-2 oncogene was down-regulated. This agreed with Zheng *et al.* (2015) and Alizadehnohi *et al.* (2017) who reported

that in caspase cascade activation led to induction of apoptosis via activation of initiator caspases caspase-2, -8, & -10, and initiator caspases cleave and activate effector caspases-3, -6 &-7.

Conclusion

1- Bee (*Apis mellifera*) venom and curcumin had anticancer effect. 2-curcumin and bee venom has an anti-cancer potential**.** 3-Anticancer potentials could be confirmed by up-regulation and down-regulations of pro and anti-apoptotic ge-nes.

Recommendations

1- More cancer cell lines could be tested for verification of anti-cancer potentials of target test materials (curcumin and bee venom). 2- In-vivo application of anti-cancer potentials of tested materials using murine models. 3-Monitoring of biochemical changes pre and post treatment with anti-cancer agent regarding the antioxidant parameters (GTH, MDA, NO, ROS etc.).

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Explanation figures

Fig. 1: A- Evaluation of IC50 of bee venom and doxorubicin on Hep-G2 cell line, B- Evaluation of IC50 of DOX on Hep-G2 cell line, C- Evaluation of IC50 of B.V on Hep-G2 cell line, and D- Evaluation of IC50 of DOX with B.V on Hep-G2 cell line.

Fig. 2: A-Evaluation of IC50 of curcumin and curcumin with doxorubicin and combination of CBD on Hep-G2 cell line, B-Evaluation of IC50 of curcumin and curcumin with DOX on Hep-G2 cell line, and C-Evaluation of IC50 of curcumin, curcumin plus DOX, and CBD on Hep-G2 cell line.

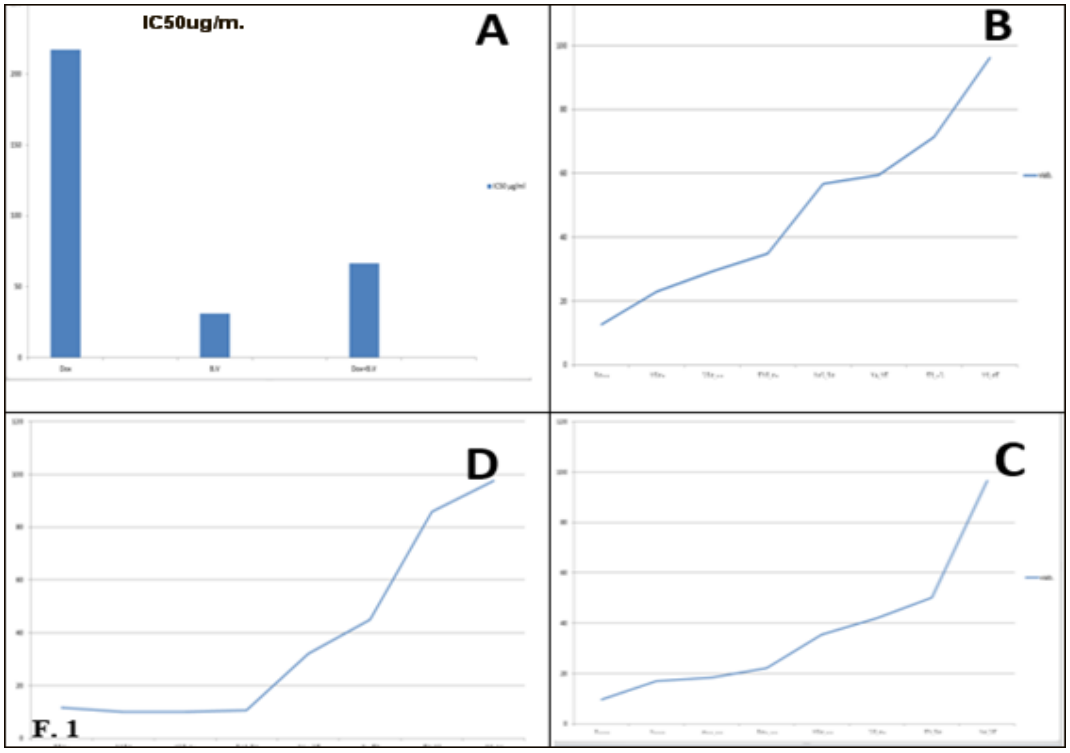
Fig. 3: distribution of HepG2 cell line between cell cycle phases on control and treated samples

Fig. 4: Flow cytometry showed early & late apoptosis and necrotic cells after treatment with several of combination compounds on HepG2 cell line.

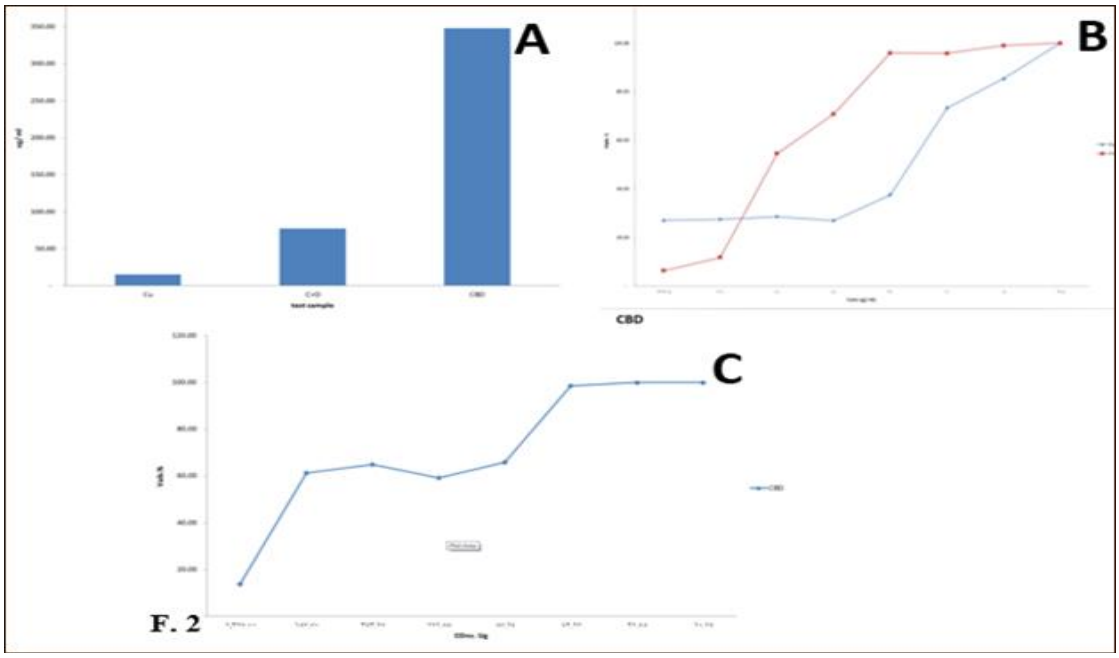
Fig. 5: Continue flow cytometry showed early & late apoptosis and necrotic cells after treatment with several of combination compounds on HepG2 cell line.

Fig. 6: Apoptosis analysis of a HepG2 cancer cell line. Respective cell % in early & late, and necrosis apoptosis for different time period in paragraph.

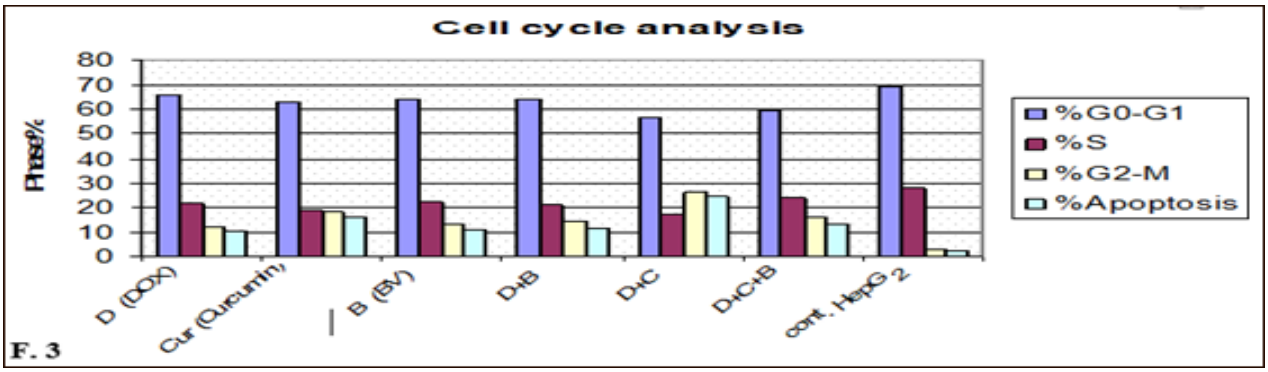
Fig. 7: Analysis of gene expression profiles of p53, Bcl2, & NFkB by using real-time PCR.


IC50ug/m.
A
B
D
C

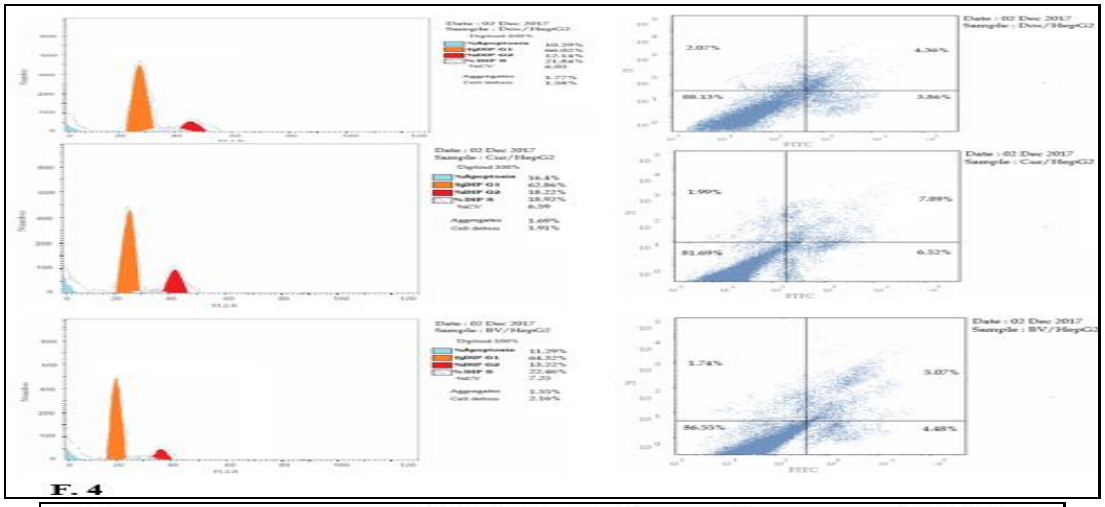
F. 1


A
B
CBD
C

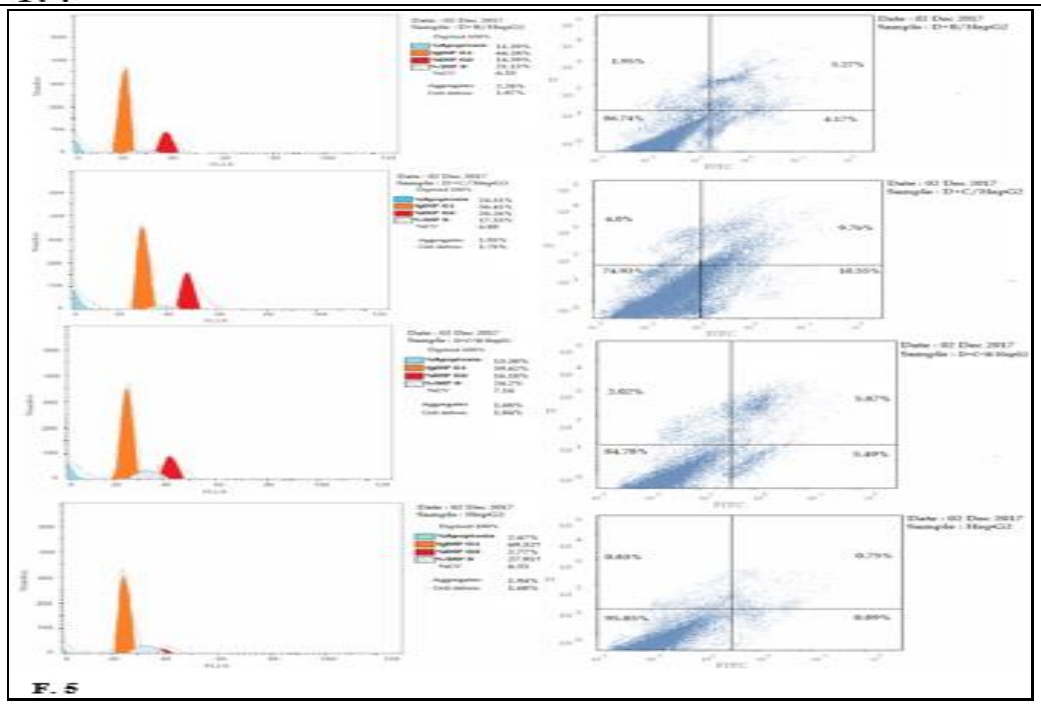
F. 2


Cell cycle analysis
Phase%
80
70
60
50
40
30
20
10
0
D (DOX)
Cur (Curcumin)
B (BV)
D+B
D+C
D+C+B
cont. HepG 2
%G0-G1
%S
%G2-M
%Apoptosis

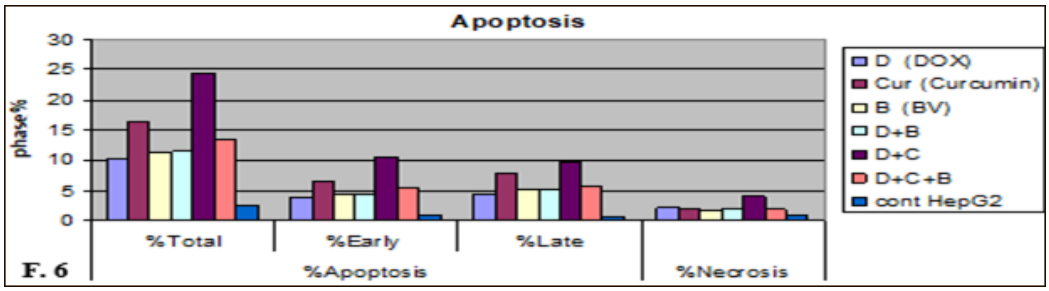
F. 3



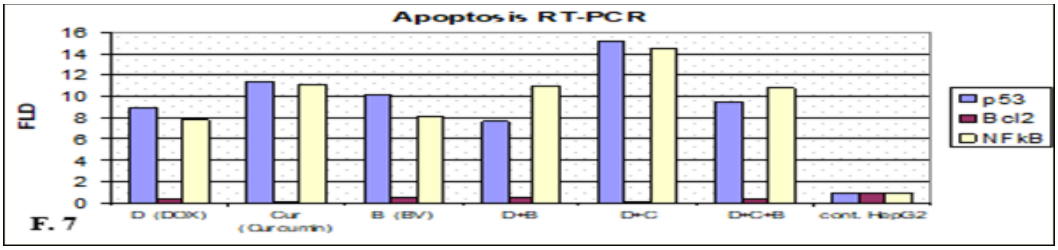
F. 4



F. 5


Apoptosis
phase%
30
25
20
15
10
5
0
%Total
%Early
%Late
%Apoptosis
%Necrosis
D (DOX)
Cur (Curcumin)
B (BV)
D+B
D+C
D+C+B
cont HepG2

F. 6


Apoptosis RT-PCR
FLD
16
14
12
10
8
6
4
2
0
D (DOX)
Cur (Curcumin)
B (BV)
D+B
D+C
D+C+B
cont. HepG2
p53
Bcl2
NFkB

F. 7