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The Effect of Multidrug System on Treatment of Hepatocellular Carcinoma

Ahmad R. Bassiouny, Amira Z. Hassan, Tayssir M. Ghonaim*, and Fatma A. Mahmoud

Biochemistry Departement, Faculty of Science, Alexandria University, Alexandria, Egypt

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ABSTRACT

Background: Hepatocellular carcinoma is the most frequent primary malignancy of the liver. It is the sixth most common cancer and third most frequent cause of cancer-related death worldwide. **Objective:** Epigenetic mechanisms (such as methylation, acetylation, and ubiquitination) are altered in HCC. One of these epigenetic mechanisms is Histone acetylation. The balance between histone transacetylases and deacetylases is often damaged in cancer, leading to changed expressions of tumor suppressor genes and/ or proto-oncogenes. **Methods:** HepG2 cells were treated with curcumin, valproic acid and curcumin + valproic acid than subjected to MTT assay, DNA fragmentation and real time PCR. **Results:** The result from MTT assay showed that IC_{50} of curcumin = 0.91 mM, valproic acid = 2.61 mM, curcumin + valproic acid = 1.12 mM. Real time PCR showed decrease in HDAC1, VEGF, IL- 6 and increase in PTEN, P53 genes expression in cell treated with curcumin or valproic acid individually while treatment of both show no synergism.

Introduction:

Hepatocellular carcinoma (HCC) is the sixth most common cancer and third most frequent cause of cancer-related death worldwide [1, 2]. Many cancer-related genes and epigenetic mechanisms (such as methylation, acetylation, and ubiquitination) are altered in HCC [2]. One of these epigenetic mechanisms is Histone acetylation which affects chromatin condensation and these alterations influence gene transcription [3, 4]. The acetylation state of histones and other proteins is maintained by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes. HATs catalyze the transfer of an acetyl group from acetyl-CoA to lysine residues in proteins and HDAC removes it [5]. Decreased activities of HDACs are associated with inhibition of tumor cell development and growth [6, 7]. The balance between histone transacetylases and deacetylases is often altered in cancer, leading to changed expressions of tumor suppressor genes and/ or proto-oncogenes [3, 4, 8, 9]. HDAC inhibitors induce apoptosis in tumor cells by regulation of expression of pro-apoptotic and anti-apoptotic genes [10]. Furthermore, it may be possible to investigate the correlation between inhibiting specific HDAC-dependent complexes in particular tumor types with clinical outcomes following HDACi treatment, allowing for the identification of novel HDACi

Corresponding author: *Tayssir Ghonaim, Faculty of Science, Alexandria University. Moharrambaik, Alexandria, Egypt. **E-mail**: tayssir_salem@yahoo.com*

response biomarkers [11]. We used in our study One of the drugs that inhibit hdac1 valproic acid and one natural product, curcumin, Valproic acid (2-propyl-pentanoic acid, V) is the first-line therapy in the treatment of epilepsy for more than 30 years. More recently, studies have found that VPA is a potential anti-cancer agent, due in part to its anti-histone deacetylase activity [12]. Curcumin is a rhizome from the herb *Curcuma longa Linn* [13]. Ayurvedic medicine clearly designates curcumin as an effective medicine for various disorders such as asthma, bronchial hyperactivity, allergy, anorexia, coryza, cough, sinusitis, and hepatic disease [14]. Curcumin inhibits proliferation and induces apoptosis in numerous types of cancer cells including liver cancer. Moreover, it has displayed synergistic effects with current cancer therapeutics [15]. The current study evaluated the role of HDAC1 in the regulation of specific genes expression in hepatocellular carcinoma-cell line (HepG2).

Materials and Methods:

Materials:

Curcumin (MW 368.39) and valporic acid (MW 169.67) were purchased from fromTocris bioscience (USA). Fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from Bio-west, South America. (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) "MTT" was pursued from thermo-fisher scientific, USA.

Cell culture:

The human hepatocellular carcinoma cell line, HepG2, was obtained from medical research institute (Alexandria, Egypt). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM/high glucose) supplemented with 10% FBS, and 1% penicillin / streptomycin in a humidified atmosphere of 5% CO2 and at 37˚C [16, 17]. The cultured HepG2 cells were divided into 4 groups: First HepG2 cells as mock-treated cells, second HepG2 cells that were treated with valproic conditioned medium. The third HepG2 cells were treated with curcumin (25 µM), the fourth HepG2 cells were treated with curcumin + valproic acid at the same ratio. All groups were incubated for 6 h and 24 h [18].

Methods

MTT Cell Proliferation Assay:

Cell proliferation of HepG2 cells in all groups was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazoliumbromide) cell proliferation kit (Trevigen Inc., Gaithersburg, MD, USA) as per manufacturer's protocol. Briefly, cells were plated in 96-well tissue culture plates in a range of 103-105 cells/well in a final volume of 100 µL of medium and were allowed to attach overnight. The MTT reagent was added (10 µL per well) and the plate was incubated for 2 to 12 h to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. Detergent reagent is added to each well to solubilize the formazan dye prior to measuring the absorbance of each sample in a microplate reader at 550 – 600 nm. Six wells were used for each group. Cell proliferation was assessed as the percentage of cell proliferation compared to untreated HepG2 as control cells.

DNA fragmentation:

DNA fragmentation was used to determine the induction of apoptosis by observing the biochemical change. Briefly, after cancer cells were treated with IC50 of curcumin, valproic and curcumin + valproic acid for 24 hours, the cells were collected and washed with media. Then cell suspensions were transferred to microcentrifuge tubes (1.5 ml) and centrifuged at

300×g (Wisd Laboratory instrument, Germany) for five minutes to collect the cell pellets. The DNA in the cell pellet was extracted with Flexigene DNA Kit (QIAGEN, Germany); 2 µg of DNA was electrophoresed on 2% agarose gel containing 0.1 mg/ml ethidium bromide. After electrophoresis, DNA fragments were analyzed with a UV-illuminated camera (Syngene, UK) [19, 20].

Quantitative real time PCR:

Isolation of total RNA and reverse transcription (RT)- PCR analysis Total RNA from HepG2 cells was extracted using biozol total RNA extraction® (BioFlux) where solated RNA was subjected to reverse transcription using a HiSenScript^{tm} RH(-)kit for cDNA synthesis. Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed Using 5X HOT FIREPOL^{R} EvaGreen^{R} qPCR mix plus (no ROX) and Samples were normalized using B-actin [21].

Primers Sequence of Studied Genes:

(1)PTEN:
Forward primer:
GATGCAGTGGATGTTGGTTG
Reverse primer:
GCCCAGAAAGGTTAGGGAAC
(2)P53:
Forword primer:
GCCCACTTACAAGTACTAA
Reverse primer:
GTGGTTTCAAGGCCAGATGT
(3):APE1:
Forward primer:
GCTGCCTGGACTCTCTCATC
Reverse primer:
GCTGTTACCAGCACAAACGA
(4)HDAC1:
Forward primer:
GAAATCTACCGCCCTCACAA
Reverse primer:
AACAGGCCATCGAATACTGG
(5)IL6:
Forward primer:
TACCCCCAGAGAAGTTCC
Reverse primer:
TTTTCTGCCAGTGCCTCTTT
(6)VEGF:
Forward primer:
CCCACTGAGGAGTCCAACAT
Reverse primer:
TTTCTTGCGCTTTCGTTTTT
(7)B-ACTIN:
Forward primer-
Reverse primer-

Statistical Analysis:

Statistical analysis was performed using oneway analysis of variance (ANOVA) followed by

The data from the experiments are presented as means ± SD, $p < 0.01$ was considered statistically significant.

Results

MTT proliferation assay of HepG2 cells showed a significant decrease ($p < 0.01$) in the proliferation rate in the HepG2 cells treated with curcmin or valproic acid or curcumin + valproic acid compared to the mock-treated HepG2 cells. Cells treated with curcumin showed a significant decrease ($p < 0.0001$) when compared to the mock-treated HepG2 cells. HepG2 cells treated with curcumin + valproic acid also showed a significant decrease ($p < 0.01$) compared to the mock-treated HepG2 cells.

DNA fragmentation

Evaluation of apoptosis was further carried out by determining the DNA laddering as a result of DNA fragmentation, indicative of the late stage of apoptosis (Figure 4). HepG2 cells treated with the IC 50 of curcumin, valproic acid and curcumin + valproic acid showed characteristics of DNA laddering.

HepG2 cells treated with curcumin or a valproic acid showed significant decrease ($p < 0.01$) in HDAC1 (Figure 5A), APE1 (Figure 5B), VEGF (Figure 5C), IL-6 (Figure 5D), P53 (Figure 5E) , PTEN (Figure 5F), and gene expression compared to mock treated HepG2 cells. HepG2

cells treated with both curcumin and Valproic acid showed a significant decrease ($p < 0.01$) in the expression of HDAC1 when compared with the mock treated HepG2 cells. VEGF, IL-6, P53, APE1 and PTEN gene expression in HepG2 cells treated with both curcumin and Valproic acid with no significant difference ($p > 0.01$) when compared with HepG2 treated with curcumin or valproic acid.

DISCUSSION

Activation of many signaling pathways has been implicated in human hepatocarcinogenesis [(22)]. PI3K/AKT/mTOR signaling pathway Constitutive activation of the PI3K/AKT/mTOR signaling pathway has been established as a major determinant of tumor cell growth and survival in a multitude of solid tumors [(23)]. In the PI3K/AKT/mTOR signaling pathway, binding of growth factors (most notably IGF and EGF) to their receptors activates PI3K [(24)]. PI3K subsequently produces the lipid second messenger PIP3b (phosphoinositoltriphosphate), which activates the serine/threonine kinase AKT, in addition to regulating several transcription factors [(25)]. Activated AKT phosphorylates several cytoplasmic proteins, most notably mTOR and BCL-2-associated death promoter and angiogenesis [(24)]. The activation of mTOR increases cellular proliferation, and inactivation of BAD decreases apoptosis and increases cell survival [(26)]. In normal tissue, this pathway is negatively regulated by the phosphatase and tumor suppressor phosphatase on chromosome 10 (phosphatase and tensin homolog (PTEN)), which targets the lipid products of PI3K for dephosphorylation [(26)]. Studies suggest that aberrant PTEN function may lead to overactivation of the PI3K/AKT/ mTOR pathway in HCC. The PTEN gene is mutated in 5% of HCCs [(27)] and its expression is reduced in nearly half of all HCC tumors [(28)]. Inactivation of AKT signaling by novel approaches could be useful for cancer therapy [(22)]. IL-6 induced activation of the PI3K/Akt pathway is involved in protection against apoptosis [(29)]. The ability of IL-6 dependent Ras/PI3K/Akt pathway to induce uncontrolled proliferation and tumor survival in human cancer cells was investigated. Indeed, various studies showed that IL-6 dependent activation of PI3K/Akt as it promotes proliferation [(30)], protects survival [(31)], and stimulates migration of cancer cells [(32)]. IL-6-induced effects on cytotoxic drug-induced caspase activation and apoptosis was examined [(33)]. Angiogenesis is a cascade of linked and sequential steps leading to tumour neovascularization, its activation is as a result of PI3K/AKT/mTOR signaling pathway. Preclinical data suggested that significant HCC growth is dependent on angiogenesis. One of the most intensely studied growth factors involved in angiogenesis is VEGF [(34)]. Overexpression of VEGF may be induced by the hypoxic tumor environment (mediated by hypoxia-inducible factor 2-a). Increased expression of VEGF and VEGF receptors (VEGFRs; which include VEGFR-1, -2 and -3) has been observed in HCC cell lines and tissues, as well as in the serum of patients with HCC [(27, 35-39)]. Increased levels of VEGF expression have been linked with HCC tumor grade [(40)], poor outcome after resection [(41)], disease recurrence, poor disease-free and overall survival (OS) [(42)], vascular invasion [(43)] and portal vein emboli [(44)]. So, from this aspect, the present study evaluated the effect of HDAC1 inhibitor valproic acid and curcumin on PI3K/AKT/mTOR signaling pathway in hepatoma cell line (HepG2

cells). Numerous studies have evaluated the effects of curcumin and Valproic acid in several rodent as well as human hepatoma cells individually , so we tried in the present study effect to evaluate the effect of combination of curcumin and valproic acid. Treatment of HepG2 cells by curcumin or valproic acid leads to a significant decrease in the expression of HDAC1, APE1, VEGF and IL-6 genes which were increased significantly in the malignant cells and significant increase in PTEN and p53. Pretreatment of curcumin with valproic acid leads to significant decrease in the expression of HDAC1 only but no synergism between them in decrease in APE1, VEGF and IL- 6 or increase in p53 and PTEN. AP endonuclease 1 (APE1), a multifunctional enzyme that is part of the BER pathwayand DNA repair activity. APE1 also known as the redox effector factor 1 (Ref-1) which activates TFs including c-Jun, activator protein-1 (AP-1), nuclear factor kappa B (NF-kB), the tumor-suppressor protein p53, hypoxia-inducible factor 1a (HIF-1a) and paired box gene 8, which are involved in various cellular processes such as cell survival, growth signaling and inflammatory pathways. Acetylation of APE1 has been shown to be involved in early growth response (Egr-1)-mediated activation of phosphoinositol phosphatase and tensin homologue (PTEN) expression. Because p53 acts as a pro-apoptotic factor in the cellular response to stress, whereas APE1 is a prosurvival protein .It seems that the cell uses p53 to downregulate APE1 expression in response to DNA damage, and promotes apoptosis. The direct interaction between APE1 and histone deacetylase 1 (HDAC1), provided a possible molecular mechanism of the gene suppression. Once acetylation takes place, APE1 increases its existence at the nCaRE regions in the genome, where nearby promoters are actively transcribed. Through the direct interaction with APE1, HDAC1 is recruited to the promoter region to deacetylate histones and to suppress gene expression. APE1 is released from the region as HDAC1 removes the acetyl group from APE1 thereafter. This feedback model ensures the temporary nature of the gene suppression. Moreover, hyperacetylation stabilizes the p53 protein, promoting both cell cycle arrest and apoptosis. Zheng *et al.*, 2004 reported that, curcumin up-regulated p53, have recently shown in yeast that depletion of class I and II HDACs by mutation, or via HDAC inhibition with valproic acid (VPA) [(45)], prevented DNA damage signaling and interfered with DNA break repair [(14)]. An important substrate of HDAC1 is the tumor suppressor protein p53. Recruitment of HDAC1 by MDM2 promotes p53 degradation by deacetylation. Thus HDAC1 decreases DNA damage-induced p53 acetylation, and inhibits the induction of p21 and MDM2. HDAC1 depletion led to ubiquitination of MDM2 leading to p53 activation and sustained DNA damage response thereby inducing apoptosis [(46, 47)]. Acetylation abrogates complex formation between p53 and Mdm2, whereas an unacetylatable p53 mutant strongly interacts with Mdm2 resulting in p53 degradation [(48)]. In an overexpression system, Mdm2 formed an HDAC1containing complex binding to p53. Recruitment of HDAC1 might thereby link two enzymatic activities promoting p53 degradation [(9, 47, 49)]. PTEN is aberrantly expressed in many human cancers. PTEN is induced by p53 and stabilizes p53 by protecting it from mdm2-mediated ubiquitination. This positive feedback loop contributes to p53-induced apoptosis and senescence. Moreover, PTEN block the activation of the *PI3K–AKT–mTOR*

pathway. Taken together, these data suggest that the PI3K/ AKT/mTOR pathway has a critical role in the pathogenesis of HCC [(50)]. Indeed, levels of the phosphorylated form of mTOR have been shown to be elevated in 15% of cases of HCC [(51)]. In our study we showed that, once the IL - 6 activity is decreased and expression of p53 increase as aresult of inhibition of HDAC1 and activation of APE1 leading to increase the expression of PTEN. The resulting inhibition of PI3K/ AKT/mTOR pathway and induced apoptosis as consequence, VEGF activity is decreased, which cause inhibition of angiogenesis. In the present work, the applications of curcumin or valproic acid to HepG2 cells lead to a significant reduction in the proliferation rate of the cells. The effect was less significant than curcumin with valproic acid.

5. Conclusion

Curcumin or valproic acid led to growth inhibition of HepG2 cells due to induction of apoptosis and cell cycle arrest through down regulation of PI3K/ AKT/mTOR pathway by HDAC1 inhibition . These data suggest that modulation of HDAC1 activity by curcumin or valproic acid can be considered as a therapeutic target in HCC.

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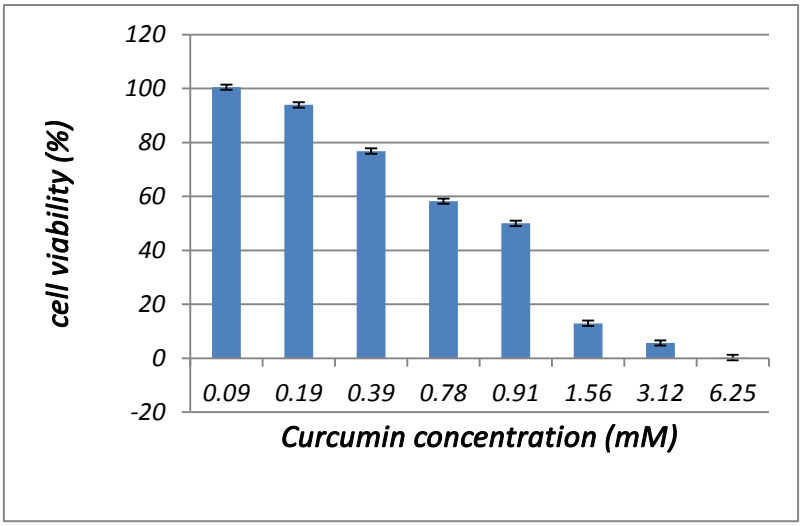


Figure (1): Growth inhibition of HepG2 cells after the treatment with curcumin. Cells were cultured for 24 h before drug treatment in 96 well plates. Cells were treated with curcumin for 24h and cell viability was measured by MTT assay. Data are expressed as mean ± SD (N=3).

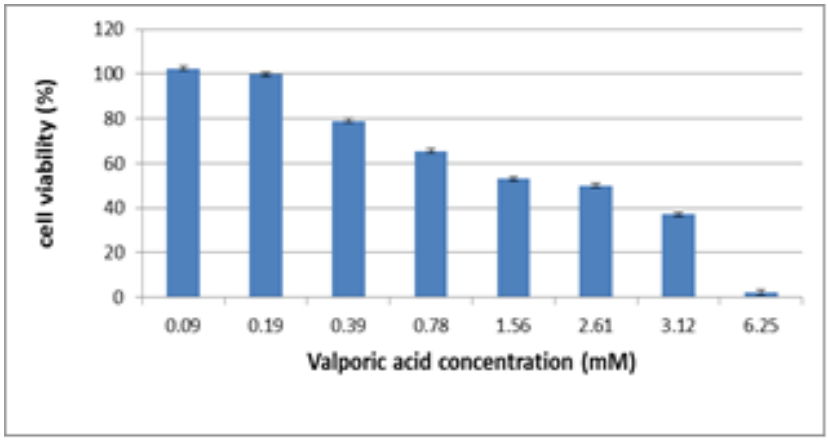


Figure (2): Growth inhibition of HepG2 cells after the treatment with valproic acid. Cells were cultured for 24 h before drug treatment in 96 well plates. Cells were treated with valproic acid for 24 h and cell viability was measured by MTT assay. Data are expressed as mean ± SD (N=3).

Biochemistry letters, 12 (10) 2017, Pages: 110-123

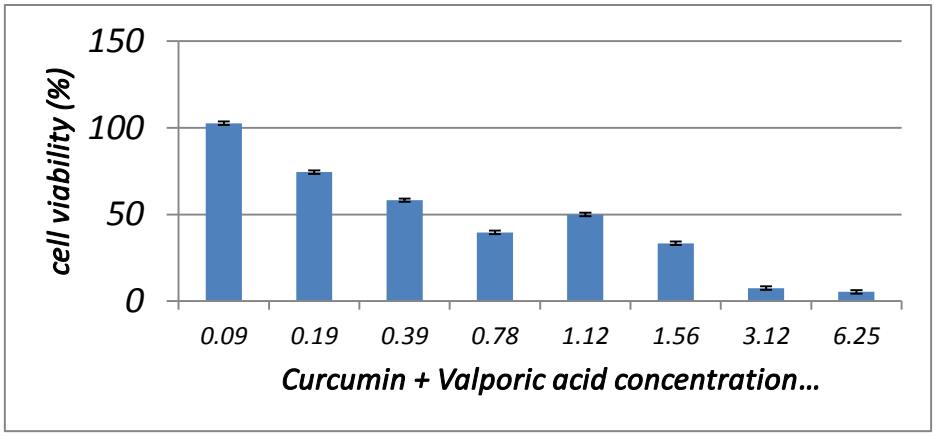
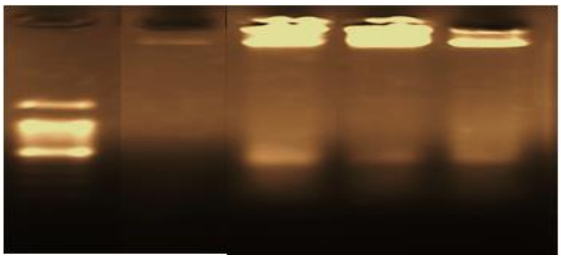


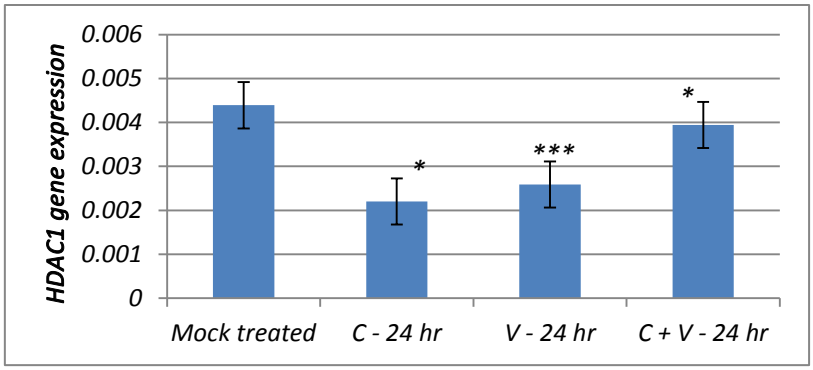
Figure (3): Growth inhibition of HepG2 cells after the treatment with curcumin + valproic acid. Cells were cultured for 24 h before drug treatment in 96 well plates. Cells were treated with curcumin + valproic acid for 24 h and cell viability was measured by MTT assay. Data are expressed as mean ± SD (N=3).



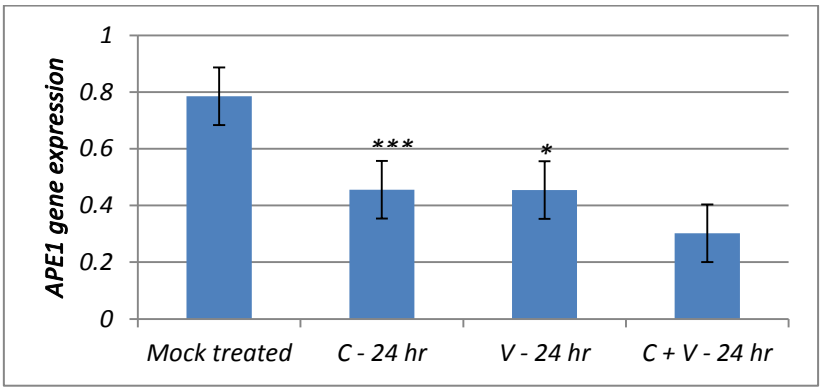
(A) (B) (C) (D)

Figure (4): DNA laddering in HepG2. DNA laddering was visualized in HepG2 after treatment where (A): mock treated, (B): HepG2 cell treated with IC50 of curcumin show DNA laddering as a result of DNA fragmentation, (C): HepG2 cell treated with IC50 of valproic acid show DNA laddering as a result of DNA fragmentation, (D): HepG2 cell treated with IC50 of curcumin + valproic acid show DNA laddering as a result of DNA fragmentation.

Biochemistry letters, 12 (10) 2017, Pages: 110-123



(A)



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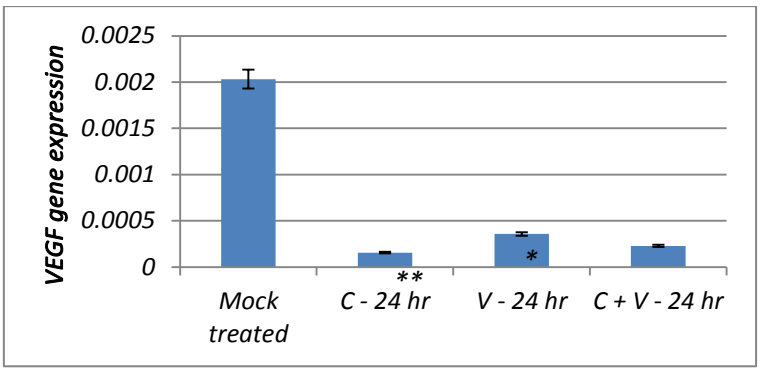

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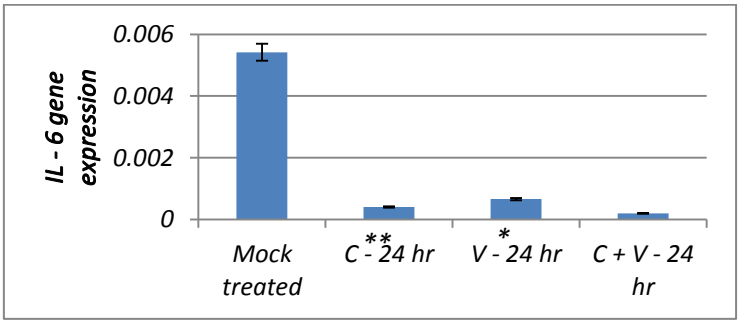

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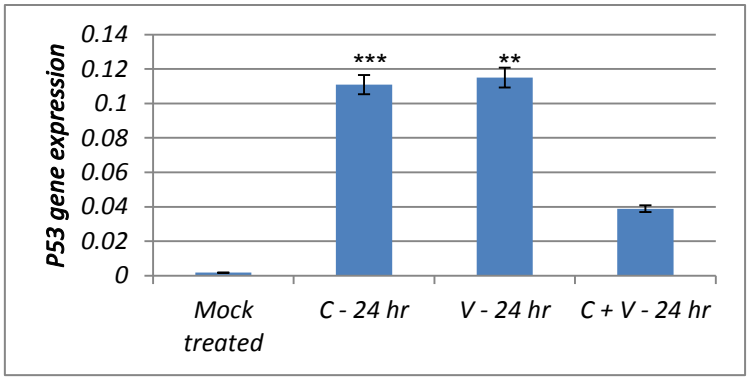
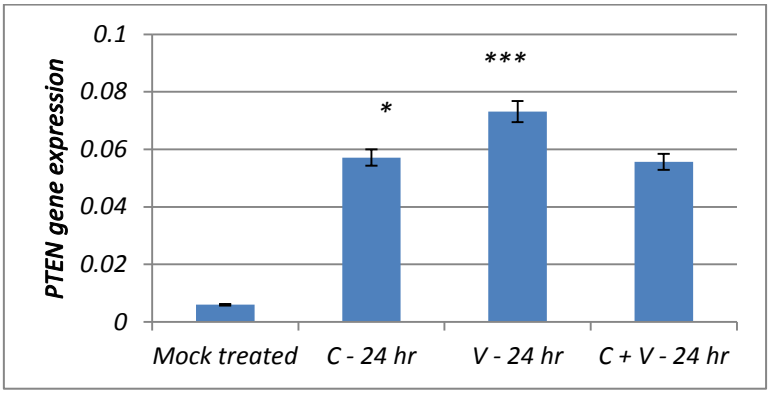


(E)



(F)

Figure (5): Comparison between the (A) HDAC1,(B) APE1, (C) VEGF, (D) IL-6, (E) P53 and (F) PTEN genes expressions in HepG2 cell treated groups. Data are expressed as mean ± SD (N=3). * ($p < 0.01$), ** ($p < 0.001$) *** ($p < 0.0001$).

Table (1): IC_{50} of curcumin, valproic acid and curcumin + valporic acid.

Treatment substance	**IC_{50} (mM)**
Curcumin	0.91
Valporic acid	2.61
Curcumin + Valporic acid	1.12