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Anticancer activity of *Aloe vera* and *Calligonum comosum* extracts separetely on hepatocellular carcinoma cells

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PEER REVIEW

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Comments

Up-regulation of expression of p53 and down-regulation of Bcl2 in a time and dose dependent manner were evident in the human hepatocellular carcinoma cell line which is a major pathway for regulation of programmed cell death. Both extracts could have cytotoxic and genotoxic activity. *C. comosum* showed a higher level in inducing morphological changes associated apoptosis, DNA damage, gene and protein expressions. In conclusion, the present study demonstrated that the herbal extracts of *A. vera* and *C. comosum* could induce cytotoxic and genotoxic activites on human hepatocellular carcinoma (HepG2) cells through induction of apoptotic pathway.
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ABSTRACT

Objective: To investigate the in *vitro* anticancer effect of *Aloe vera* (*A. vera*) and *Calligonum comosum* (*C. comosum*) extracts against hepatocellular carcinoma (HepG2) cells.
Methods: HepG2 cells were tested against different doses of *A. vera* and *C. comosum*. Viability of the cells was assessed by MTT assay. Evaluation of apoptosis and DNA damage in HepG2 cells were performed using annexin V apoptosis detection kit. The expression of p53 and anti-apoptotic (Bcl-2) were tested by real time-PCR and flow cytometer analyser. Hematoxylin and eosin stained sections from untreated and treated HepG2 cells were observed using light microscopy.
Results: The IC_{50} values of *A. vera* and *C. comosum* extracts were (10.45 ± 0.31) and (9.60 ± 0.01) μg/mL respectively. The extracts separately increased cytotoxicity against HepG2 cells in a time and dose dependent manners. Also, it apparently induced apoptosis through increase P53 and decrease Bcl-2 genes expressions.
Conclusions: The results indicated that the extracts could have anti-hepatocarcinogenic effect, at least in part, through modulation of apoptosis.

KEYWORDS
HepG2, Hepatocellular carcinoma, *Aloe vera*, *Calligonum comosum*, Apoptosis

1. Introduction

Hepatocellular carcinoma (HCC) is among the most lethal and common malignancies in the human population, with approximately 550 000 new cases and almost as many deaths per year[1,2]. For instance, a highly rate of HCC is observed in Egypt, where there is high prevalence of hepatitis B and C viral infections[3]. It is noteworthy that it is too hardly to eliminate hepatitis viruses infection, therefore chemoprevention could be the more suitable challenge with the HCC, especially in carriers of hepatitis B and C viruses[4]. Locally available plants could provide accessible and economically feasible sources for herbal HCC preventives. *Aloe*

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vera (*A. vera*) and *Calligonum comosum* (*C. comosum*) "arta" are Egyptian plants that are used as source of medicine in rural areas. Apoptosis is a genetically controlled process of selective cell deletion involved in normal cell development and turnover[5]. Apoptosis is controlled by the expression of several regulatory genes, including *c-myc*, *p53* and *apo-1/fas*[6]. *P53* is a tumor suppressor gene acting as a guardian of the genome through conserving its stability and initiating apoptosis of DNA damaged cells. Also, it is the most commonly mutated gene in human cancers[7,8]. However, one of the most important advances in our understanding of apoptotic cell death has come from studies of the oncogenic Bcl-2 family. Bcl-2 promotes cell survival by blocking apoptosis induced by a range of stimuli including growth factor withdrawal[9]. Therefore, the aim of the present study was to evaluate the anti-tumor effect of different doses of *A. vera* and *C. comosum* extracts on HCC through assessment in *vitro* cytotoxicity induced apoptosis in HepG2 cell line.

2. Materials and methods

2.1. Drugs and reagents

Lyophilized *A. vera* extract (Coral Vegetable, Miyakojima, Japan) and methanolic extract of *C. comosum* were dissolved to a concentration of 0.1 mol/L in dimethyl sulfoxide (DMSO) as a stock solution and stored at -20 ℃. The working concentrations used in this study were from 10 μmol/L to 2 000 μmol/L and were freshly diluted with medium before each experiment with a final DMSO concentration of less than 0.1%. Then the extracts were diluted with Dulbecco's modified Eagle's medium to desired concentrations of *A. vera* (2.50, 7.50 and 10.45 μg/mL) and *C. comosum* (2.5, 7.5 and 9.6 μg/mL) for 48 h, and control with 1% DMSO. Cells were washed with phosphate buffer saline (PBS) and trypsinized for collecting different aliquots for studying DNA damage, apoptosis markers and cell cycle by flow cytometry.

2.2. Cell culture

Human hepatocellular carcinoma (HepG2) cell lines were maintained at laboratories of Medical Experimental Research Center, Faculty of Medicine, Mansoura University. The cell culture medium was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 37 ℃ under a humidified atmosphere containing 5% CO_2. Cells were placed in a 75 cm^2 tissue culture flask.

2.3. Evaluation of HepG2 cell viability by MTT assay

Effect on overall cell activity was determined by performing the MTT assay based on the method of Oka *et al*.,[10] using MTT assay kit.

IC_{50} (%) = [100 × (Absorbance of control group - Absorbance of treated group] / Absorbance of control group.

2.4. Assay for apoptosis and DNA damage

The concentrations of *A. vera* used were 2.50, 7.50, and 10.45 μg/mL. The concentrations of *C. comosum* used were 2.5, 7.5, and 9.6 μg/mL, and triplicate cell cultures were exposed to each concentration. Treated and control cell cultures were harvested and stained by hematoxylin and eosin (H&E)[11]. Apoptosis and DNA damage were detected by using an Annexin V-FITC apoptosis detection kit (Cat. no. APOAF, Sigma, USA). Treated and control cells were washed twice with PBS and resuspended in 1-fold binding buffer at a concentration of 1×10^6 cells/mL. Five microliter of Annexin V FITC Conjugate and 10 μL of propidium iodide solution were added to 500 μL of each cell suspension, incubated at room temperature for 10 min, and then analyzed by flow cytometry instrument (FACStar caliber, Becton Dickinson, USA) using 488 nm excitation and a 515 nm band pass filter for FITC detection and a filter over 600 nm for PI detection. The percentage of each phase was calculated with the software Cell-Quest[12].

2.5. Detection of Bcl2 and P53 genes expression (real time –PCR)

Isolation of total RNA and real time-PCR analysis of *p53* and *Bcl2* genes were applied in HepG2 cells as mentioned in Shak *et al*.[13]. HepG2 cells were cultured for 24 h then incubated for 48 h with fresh medium containing different concentrations of the extracts (test cells) or 1% DMSO (control cells). The concentrations of *A. vera* used were 2.50, 7.50 and 10.45 μg/mL. The concentrations of *C. comosum* used were 2.5, 7.5 and 9.6 μg/mL, and triplicate cell cultures were exposed to each concentration. Treated and control cell cultures were harvested and used for total RNA extraction. Total RNA was isolated from the HepG2 cells using RNA extraction kit (GF-1 total RNA extraction, Vivantis, Malasyia) according to the manufacturer's specifications. The quantity and quality of the extracted RNA were determined by measuring the absorbance at 230, 260 and 280 nm of the spectrophotometer (Nano drop 2000, Thermo Scientific, USA) and 2 characteristic bands of RNA were appeared by gel electrophoresis. One microgram of total RNA was synthesized cDNA by using Thermo Scientific kit (Maxima First Strand cDNA kit, Thermo Scientific, USA). Primers were designed online in NCBI site and real-time PCR was performed by using Syber Green dye (QuantiTect Sybr Green PCR kit, Qiagen, USA) according to manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was preserved as a reference gene. Table 1 shows the primer sequences which were used in the amplification.

Table 1

Primer sequences for real time-PCR.

Name of gene	Primer forward	Primer reverse
p53	TGAGGTGCGTGTTTGTGCCTGT	TCGGAACATCTCGAAGCGCTCA
Bcl-2	ATCGCCCTGTGGATGACTGAG	CAGCCAGGAGAAATCAAACAGAGG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

2.6. Determination of p53 and Bcl2 proteins by flow cytometry

2.6.1. Detection of p53

The tested and control cells were fixed with 4% paraformaldehyde

(10 min) and then permeabilized with 0.1% PBS-Tween for 20 min. The cells were incubated in 1-fold PBS/ 10% normal goat serum/ 0.3 mol/L glycine to block non-specific protein-protein interactions followed by the Anti-p53 antibody [DO-1] (ab1101, 1:50 dilution, abcam, San Francisco, USA) for 30 min at 22 ℃ and then analyzed using FACStar caliber (Becton Dickinson).

2.6.2. Detection of Bcl2

The tested and control cells were fixed with 80% methanol for 5 min. Then permeabilized with 0.1% PBS-Tween for 20 min. The cells were then incubated in 1-fold PBS/ 10% normal goat serum/ 0.3 mol/L glycine to block non-specific protein-protein interactions followed by Anti-Bcl2A1 antibody ((ab33862) (1/100 dilution, abcam, San Francisco, USA) for 30 min at 22 ℃.

2.7. Statistical analysis

The parameters in this study were subjected to statistical analysis using Microsoft excel spread sheet. For analysis of the data SPSS version 15 and EPIINFO 2006 software's were used.

3. Results

3.1. Effect of A. vera and C. comosum on cell viability

MTT reduction assay was performed to study mitochondrial/ non mitochondrial dehydrogenase activity as a cytotoxic test for a variety of chemical compounds. The effect of extracts on the growth of HepG2 cells was assessed by the MTT assay. The IC_{50} value was calculated as 10.45 ± 0.31 μg/mL for *A. vera* extract and 9.60 ± 0.01 μg/mL for *C. comosum* extract (Figure 1).

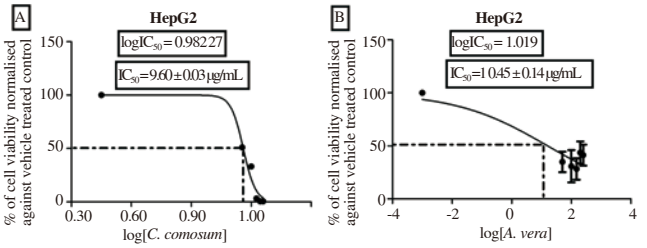

Figure 1. A: Evaluation of IC_{50} for different concentrations of *C. comosum* on HepG2 cells, IC_{50} = 9.60 ±0.01 μg/mL derived from at least n = 4 replicates ± SEM. B. Evaluation of IC_{50} for different concentrations of *A. vera* on HepG2 cells, IC_{50} = 10.45 ±0.31 μg/mL derived from at least n = 4 replicates ± SEM.

3.2. Assay for apoptosis and DNA damage by direct staining methods

Annexin V staining for detection of apoptosis and DNA damage using Annexin V-FITC apoptosis kit showed considerable increased DNA damage in HepG2 cells (2×10^5 cells/mL) exposed to the extracts for 48 h in compared with control cells (Figures 2-3). It was detected that a high percentage of apoptosis with increasing the dose of extracts and the percentage of dead cells were increased (Figure 2). We also have found a positive co-relation between dead cells stained with FITC annexin and propidum iodide of each concentration and IC_{50} of each extract which is highly significant (Table 2). Microscopically, sections stained with H & E showed marked reduction in number of HepG2 cells at 10.45 μg/mL for *A. vera* extract and 9.60 μg/mL for *C. comosum* extract (Figures 4-5). Moreover, it was noticed most of morphological changes associated with apoptosis like nuclear condensation, membrane blobbing, nuclear fragmentation and apoptotic bodies were observed in the treated cells by *C. comosum* extract specially. Interestingly, *C.*

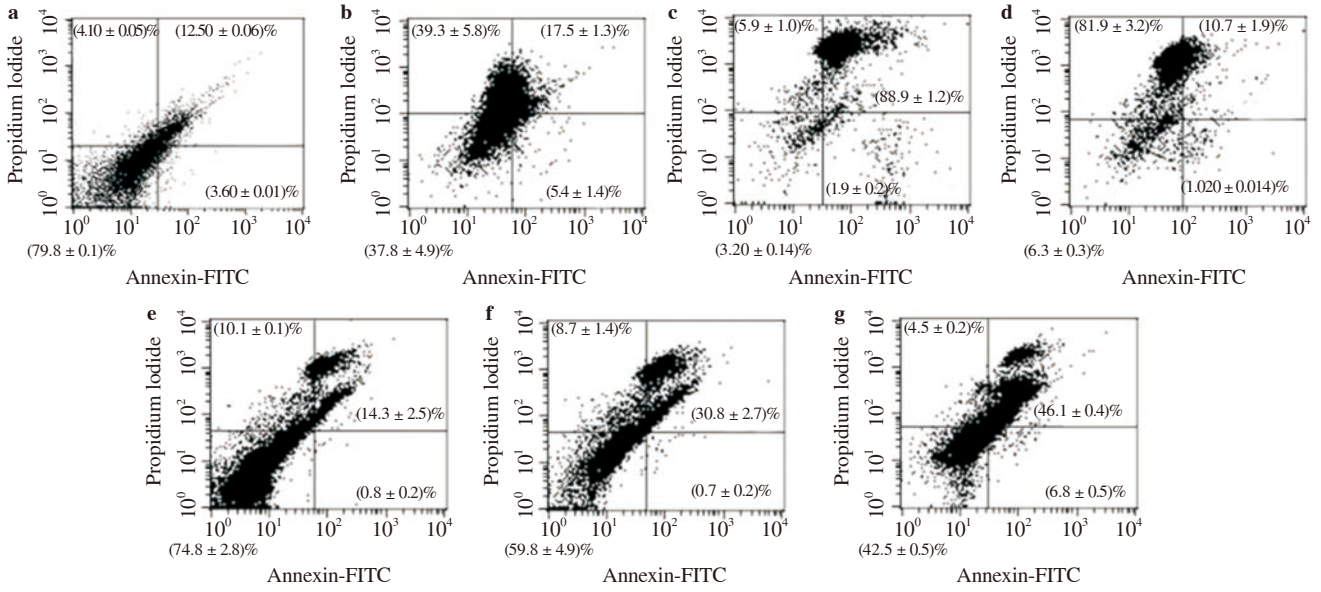

Figure 2. Quadrant statistic analysis of flow cytometer shows the percentages of apoptosis in which upper left means necrotic cells, lower left means viable cells, upper right means late apoptotic cells and lower right means early apoptotic cells. a: control HepG2 cell line; b: HepG2 cell line treated with 2.50 μg/mL of *A. vera*; c: HepG2 cell line treated 7.50 μg/mL of *A. vera*; d: HCC cell line treated with 10.45 μg/mL of *A. vera*; e: HepG2 cell line treated with 2.5 μg/mL of *C. comosum*; f: HepG2 cell line treated with 7.5 μg/mL of *C. comosum*; g: HepG2 cell line treated with 9.6 μg/mL of *C. comosum*.
These percentages were the mean of 3 replicates.

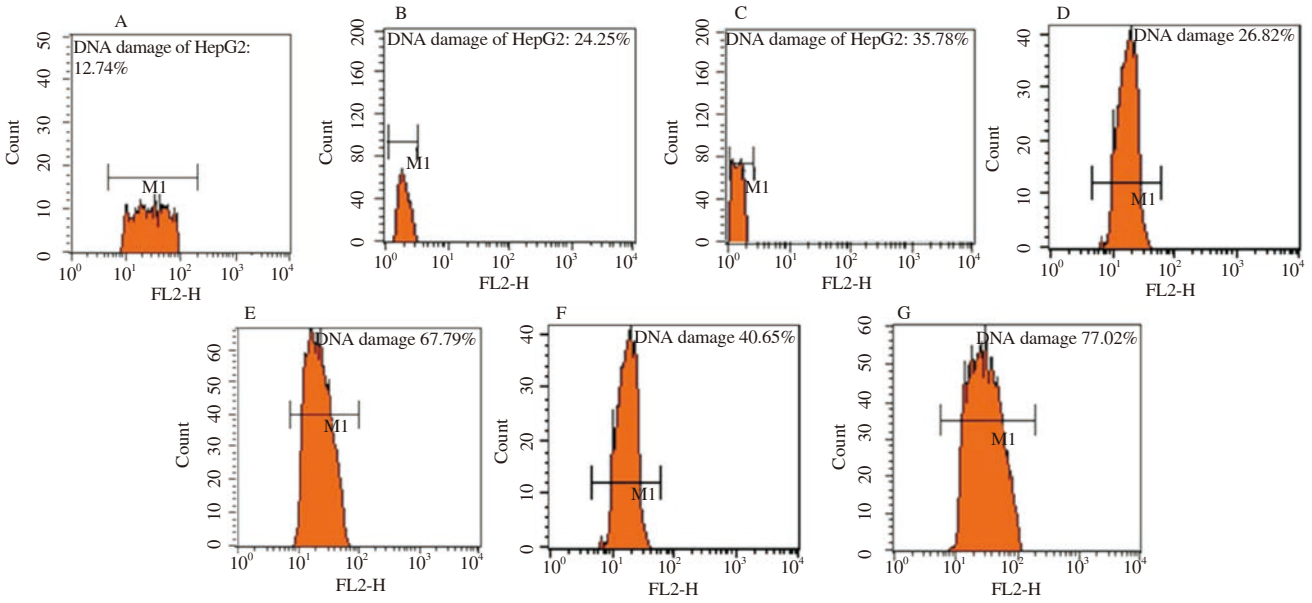

Figure 3. Detection of DNA damage. Increase in DNA damage as evident for apoptosis with increasing the dose of extracts A: Control cells; B: *A. vera* 2.50 μg; C: *C. comosum* 2.5 μg; D: *A. vera* 7.50 μg; E: *C. comosum* 7.5 μg; F: *A. vera* 10.45 μg; G: *C. comosum* 9.6 μg.

comosum revealed dose dependant effect (Figure 4).

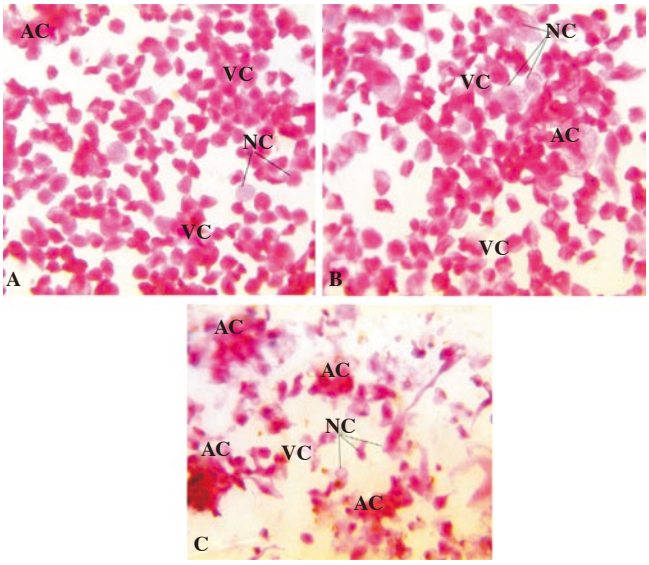

Figure 4. A: Control HepG2 cell line (HE × 200); B: HepG2 cell line treated with 10.45 μg/mL of *A. vera* (HE × 200); C: HepG2 cell line treated with 9.6 μg/mL of *C. comosum* shows the presence of marked morphological changes associated with apoptotic cells (HE × 200).
VC: viable cells, NC: necrotic cells and AC: apoptotic cells.

Table 2
There is a highly significant positive correlation between necrotic cells for each concentration and IC_{50}.

IC_{50} correlations	R (Co-relation coefficient)	*P* value
IC_{50} and necrotic cells	0.548	0.01
IC_{50} and necrotic cells in *A. vera*	0.713	0.03
IC_{50} and necrotic cells in *C. comosum*	0.909	0.01

3.3. P53 and Bcl-2 gene expression in HepG2 cells

P53 and *Bcl-2* gene expression were normalized to the house keeping *GAPDH* gene. As evident from charts (Figure 6) real time PCR evaluation of HepG2 cells treated with different concentrations of the extracts showed in a significant dose and time dependent increase in p53 mRNA expression along with a significant decrease in Bcl-2 mRNA expression.

3.4. Analysis of p53 and Bcl-2 proteins expression

As showed in Figures 7 and 8, flow cytometer analysis of p53 and Bcl-2 protein expression in HepG2 cells (2×10^5 cells/mL) treated

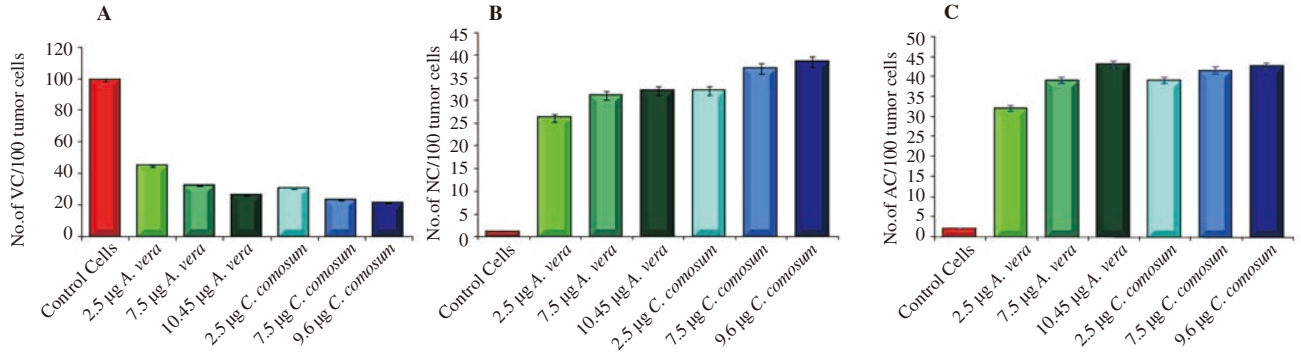

Figure 5. Statistical analysis of number of A: Vaible cells; B: Necrotic cells; C: Apoptotic cells per 100 tumor cells, respectively.

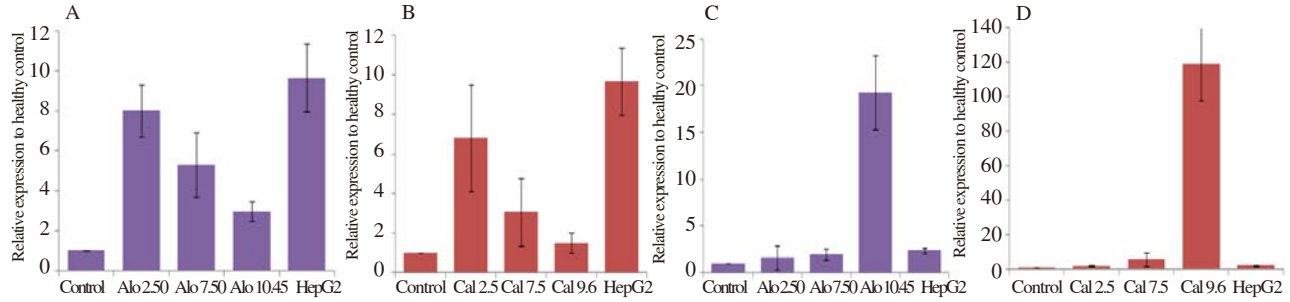

Figure 6. Real time-PCR analysis of Bcl-2 gene in control and tested cells with *A. vera* (A) and *C. comosum* (B) shows down regulation of Bcl-2 gene expression; Real time-PCR analysis of p53 gene in control and tested cells with *A. vera* (C) and *C. comosum* (D) shows up regulation of p53 gene expression.

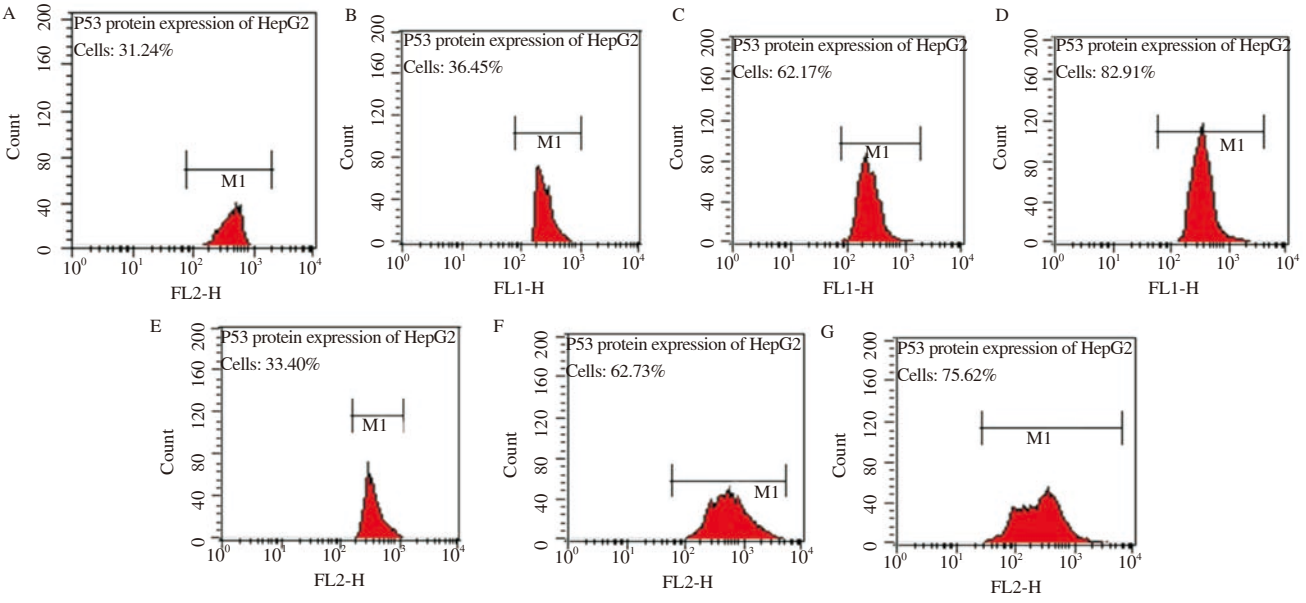

Figure 6. Detection of P53 protein percentage by flow cytometry shows up regulation of P53 protein in a dose and time dependent manner. A: Control HepG2 cell line: (31.24 ± 0.80)%; B: HepG2 cell line treated with 2.50 μg/mL of *A. vera*: (36.45 ± 0.79)%; C: HepG2 cell line treated 7.50 μg/mL of *A. vera*: (62.17 ± 0.58)%; D: HCC cell line treated with 10.45 μg/mL of *A. vera*: (82.91 ± 0.76) %; E: HepG2 cell line treated with 2.5 μg/mL of *C. comosum*: (33.40±1.07)%; F: HepG2 cell line treated with 7.5 μg/mL of *C. comosum*: (62.73 ± 1.96)%; G: HepG2 cell line treated with 9.6 μg/mL of *C. comosum*: (75.62 ± 1.04)%.
Results were derived from at least $n = 3$ replicates ± SE.

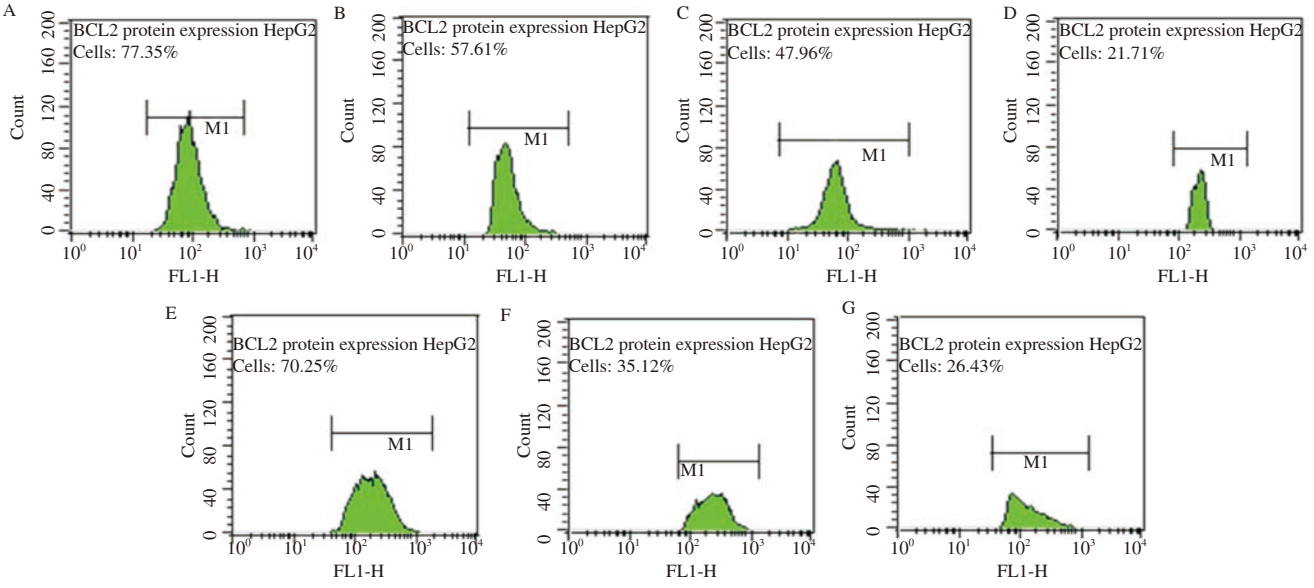

Figure 7. Detection of Bcl-2 protein by flow cytometry shows down regulation of Bcl-2 protein in a dose and time dependent manner. A: Control HepG2 cell line: (57.51 ± 3.45)%; B: HepG2 cell line treated with 2.50 μg/mL of *A. vera*: (57.61 ± 3.45)%; C: HepG2 cell line treated 7.50 μg/mL of *A. vera*: (47.96 ± 0.85)%; D: HCC cell line treated with 10.45 μg/mL of *A. vera*: (21.71± 0.95)%; E: HepG2 cell line treated with 2.5 μg/mL of *C. comosum*: (70.25 ± 0.59)%; F: HepG2 cell line treated with 7.5 μg/mL of *C. comosum*: (35.12 ±2.51)%; G: HepG2 cell line treated with 9.6 μg/mL of *C. comosum*: (26.43±1.64)%.
Results were derived from at least $n = 3$ replicates ± SE.

with different concentrations of the standardized extracts for 48 h also resulted in a time and dose dependent up regulation of p53 along with a down regulation of Bcl-2 when compared to control samples which confirmed the results from q-PCR analysis of p53 and Bcl-2 mRNA expression in HepG2 cells.

4. Discussion

Recently, many of publications showed the effect of many herbal plants in treatment of wide range of illnesses. Also several naturally produced herbal formulations are currently available for cancer patients. As most of chemotherapeutic agents were cytotoxic to normal cells and developed drug resistance[14]. Therefore scientific consideration and test of traditionally used herbs for the treatment of different malignancies could be also considered as a very valuable source for new chemotherapeutic drugs[15]. A number of studies carried out over the last few decades on prevention and treatment of HCC have led to the identification of several herbal compounds and formulations that can affect the initiation, promotion as well as the progression processes of HCC[16]. The most important active constituents of the Aloe plants were anthraquinones like aloin, barbalion, anthranol, cinnamic acid, aloetic acid, emodin, chrysophanic acid, resistanol, and enzymes (including cyclooxygenase and bradykininase), together with Other compounds such as vitamins, saccharides, and amino acids[17]. It was reported that the other anthraquinones of Aloe plants had mutagenic and genotoxic effects in bacterial and mammalian test systems[18-19], the genotoxic effects were illustrated in present research by DNA damage assay and Real Time-PCR. The antitumor activity of 50% ethanol extract (100 mg/kg) of *A. vera* was evaluated by Bharath[20]against Ehrlich ascites carcinoma tumor in mice. Ethanol extract of *A. vera* exhibited antitumor effect by modulating lipid peroxidation and augmenting antioxidant defense system in Ehrlich ascites carcinoma bearing mice[20]. Also, Aloe-emodin is one of the active components in the leaves of *A. vera*[21] which revealed anticancer and cytotoxic activities against neuroectodermal tumors, lung squamous cell carcinoma and hepatoma cells[22-24]. *C. comosum* also, has been exhibited anti- inflammatory, anti-ulcer and anti-cancer activities in rat and shrimp animals model[25], Dehydrodicatechin A is an active component of *C. comosum* which inhibits the growth of Ehrlich ascites[26]. Abdel-Sattar *et al.*[27] showed that *C. comosum* methanolic and aqueous extracts ameliorated haloperidol induced neuro- and hepatotoxicities in male Albino rat. In our study, we have noticed that the cytotoxic activity of *A. vera* and *C. comosum* might be through modulation of apoptosis, therefor both extracts demonstrated antitumor effects against HepG2 cells. Gene and protein expressions of both p53 and Bcl2 were significantly altered in response to extracts. Up-regulation of expression of p53 and down-regulation of Bcl2 in a time and dose dependent manner were evident in the human HCC cell line which is a major pathway for regulation of programmed cell death. Both extracts could have cytotoxic and genotoxic activity. *C. comosum* showed a higher level in inducing morphological changes associated apoptosis, DNA damage, gene and protein expressions.

In conclusion, the present study demonstrated that the herbal extracts of *A. vera* and *C. comosum* could induce cytotoxic and genotoxic activites on human hepatocellular carcinoma (HepG2) cells through induction of apoptotic pathway.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We thank Dr. Safaa A. Derbala for her excellent works in scientific statistical analysis and flow cytometer.

Comments

Background

HCC is among the most lethal and common malignancies in the human population, with approximately 550 000 new cases and almost as many deaths per year. For instance, a highly rate of HCC is observed in Egypt, where there is a high prevalence of hepatitis B and C viral infections.

Research frontiers

The present research work depicts to evaluate the anti-tumor effect of different doses of *A. vera* and *C. comosum* extracts on HCC through assessment *in vitro* cytotoxicity induced apoptosis in HepG2 cell line.

Related reports

The most important active constituents of the *Aloe* plants were anthraquinones like aloin, barbalion, anthranol, cinnamic acid, aloetic acid, emodin, chrysophanic acid, resistanol, and enzymes (including cyclooxygenase and bradykininase), together with other compounds such as vitamins, saccharides, and amino acids.

Innovations and breakthroughs

The present study demonstrated that the herbal extracts of *A. vera* and *C. comosum* could induce cytotoxic and genotoxic activites on human hepatocellular carcinoma (HepG2) cells through induction of apoptotic pathway.

Applications

Recently, many of publications showed the effect of many herbal plants in treatment of wide range of illnesses. Also several naturally produced herbal formulations are currently available for cancer patients.

Peer review

Up-regulation of expression of p53 and down-regulation of Bcl2 in a time and dose dependent manner were evident in the human HCC cell line which is a major pathway for regulation of programmed cell death. Both extracts could have cytotoxic and genotoxic activity. *C. comosum* showed a higher level in inducing morphological changes associated apoptosis, DNA damage, gene and protein expressions. In conclusion, the present study demonstrated that the herbal extracts of *A. vera* and *C. comosum* could induce cytotoxic and genotoxic activites on human hepatocellular carcinoma (HepG2) cells through induction of apoptotic pathway.

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