

ORIGINAL ARTICLE

## 4-Terpineol exhibits potent in vitro and in vivo anticancer effects in Hep-G2 hepatocellular carcinoma cells by suppressing cell migration and inducing apoptosis and sub-G1 cell cycle arrest

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### Summary

**Purpose:** The main purpose of this study was to demonstrate the anticancer effects of 4-terpineol against Hep-G2 hepatocellular carcinoma (HCC) cells by evaluating its effect on apoptosis induction, cell migration, DNA fragmentation and cell cycle phase distribution.

**Methods:** MTT assay was used to evaluate the cytotoxic effect of 4-terpineol on Hep-G2 cells, while fluorescence microscopy and flow cytometry were used to study apoptosis induction. Wound healing assay was used to study the effects of 4-terpineol on cell migration, while gel electrophoresis was performed to evaluate the effects on DNA fragmentation. Flow cytometry using propidium iodide (PI) as a probe was used to evaluate the effects on cell cycle arrest. Cells treated with dimethylsulfoxide (DMSO) only served as controls. BALB/c nude mice weighing about 35 g each were used for in vivo studies using 10 and 20 mg/kg of 4-terpineol dose.

**Results:** 4-terpineol induced dose-dependent cytotoxicity in Hep-G2 hepatocellular carcinoma cells. Gel electrophoresis indicated that DNA fragmentation was associated with

increasing dose of 4-terpineol. It was also observed that a wound scratch in the vehicle-treated control cells was practically entirely closed after 48 hrs of incubation. However, treatment with 0, 25, 50 and 100  $\mu$ M dose of 4-terpineol resulted in inhibition of wound healing in a dose-dependent manner. The percentage of apoptotic cells increased from 2.5% in the control cells to 10.3, 64.6 and 78.9% in cells treated with 25, 50 and 100  $\mu$ M of 4-terpineol respectively. 4-terpineol-treated cells exhibited increased percentage of cells in sub-G1 phase of the cell cycle. The in vivo mouse results indicated that 10 and 20 mg/kg of 4-terpineol decreased the tumor weight and tumor volume in a dose-dependent manner.

**Conclusion:** The results of this study showed that 4-terpineol exhibits anticancer effects in Hep-G2 cells by inducing apoptosis, DNA fragmentation, inhibition of cell migration and sub-G1 cell cycle arrest.

**Key words:** apoptosis, cell migration, flow cytometry, hepatocellular carcinoma, 4-terpineol

### Introduction

HCC or liver cancer is a lethal malignancy and the fifth leading cause of cancer-related mortality. Liver cirrhosis due to hepatitis B (HBV) or C virus (HCV) has been reported to be associated with HCC. The prevalence of liver cancer increases continuously globally with a dis-

tinctive sex, age and geographical distribution [1,2]. Presently, non-alcoholic fatty liver disease is emerging as the main cause of liver cancer. HBV vaccination has been reported to decrease the HBV-related HCC globally. One of the main problems with HCC is the absence of symp-

toms which could be attributed to this cancer. As a result, most of the HCC patients are diagnosed only in advanced stages often impeding effective treatments. This has led to an overall survival rate of 12% and a median survival of 1-2 years [3,4]. As far as treatment of HCC is concerned, orthotopic liver transplantation has been found to be an effective treatment option. However, as most HCC patients are diagnosed in an advanced stage makes them unfit for orthotopic liver transplantation [5]. It has been reported that various signalling pathways like vascular endothelial growth factor (VEGF), Ras mitogen-activated protein kinase (MAPK), PI3K/PDEN/Akt/mammalian target of rapamycin (mTOR) and Wnt/ $\beta$ -Catenin pathways are associated with HCC [6-8]. Systemic therapy of HCC involves the use of sorafenib. Other agents which are under investigation include sunitinib, linafanib, brivanib, tivantinib and everolimus. Since sorafenib is the only approved systemic therapy for advanced HCC, there is an urgent need for novel, effective and cheap anticancer agents against HCC. As such, the main objective of the present study was to investigate the *in vitro* and *in vivo* antitumor and apoptotic effects of 4-terpineol against Hep-G2 cells. Effects on cell cycle arrest, and cell migration were also investigated.

## Methods

### Drugs and other reagents

4-terpineol (purity >98% as determined by high performance liquid chromatography (HPLC), and 3-(4,5-dimethyl-2-thiazolyl) 2, 5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). 4-terpineol was dissolved to a desired concentration in DMSO (Sigma Aldrich, St Louis, MO, USA) and stored at -20 °C. Dulbecco's modified Eagle's medium (DMEM) (GibcoLife Technologies, Grand Island, NY, USA) and RPMI-1640 medium and Hoechst 33342 were purchased from Wuhan Boster Biological Technology Ltd (Wuhan, China). Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Tianjin HaoYang Biological Manufacture Co., Ltd. (Tianjin, China). Annexin-V and PI were obtained from Becton Dickinson, USA.

### Cell line and cell culture conditions

The Hep-G2 cell line was procured from the Cancer Research Institute of Beijing, China, and maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100  $\mu$ g/ml streptomycin) at 37°C in a humidified incubator (BD Biosciences, San Diego, CA, USA) containing 5% CO<sub>2</sub> and 95% air.

### Cell proliferation assay

The cytotoxic effects of 4-terpineol on Hep-G2 cell proliferation were evaluated by MTT assay. Briefly, Hep-G2 cells (2x10<sup>5</sup> cells/well) were seeded and cultured with varying doses of 4-terpineol (0, 5, 10, 25, 50 and 100  $\mu$ M) for 24 hrs. Following 4-terpineol treatment, the medium was changed and MTT 5 mg/ml was added for 2 hrs. The number of viable cells was proportional to the formation of formazan crystals which were dissolved in ethanol and the optical density was measured on a microplate reader (ELX 800; Bio-tek Instruments, Winooski, VT, USA) at a wavelength of 490 nm. The effects of 4-terpineol on cell viability were presented as an inhibition ratio (I %) using the following equation:

$$I\% = \frac{[OD_{490}(\text{Control}) - OD_{490}(\text{Treated})]}{OD_{490}(\text{Control})} \times 100\%$$

### DNA fragmentation analysis

Hep-G2 cells were seeded in a 100-mm cell culture dish for 48 hrs, and treated with 0, 25, 50 and 100  $\mu$ M 4-terpineol for 48 hrs. The cells were harvested and washed with PBS, and the pellets were lysed with a 400  $\mu$ l DNA lysis buffer for 30 min. After centrifugation, the supernatants were prepared in an equal volume of 1.5 % sodium-dodecyl sulphate, incubated with 5 mg/ml RNase A at 55 °C for 3 hrs, followed by digestion with 5 mg/ml proteinase. Subsequent to the addition of 5 M ammonium acetate, the DNA was precipitated with cold ethanol and collected by centrifugation at 12,000 rpm for 20 min. DNA was then dissolved in gel loading buffer, separated by electrophoresis in 1.5% agarose gel and visualized under UV light, following ethidium bromide staining.

### Wound healing assay for cell migration

Hep-G2 cells were put in a sterile 12-well plate and horizontal lines were drawn on the base of the plate by keeping it upside down. Then 2 ml of cell culture containing media was transferred into each well. The plate was covered by the lid and then kept in the incubator for 48 hrs at 37 °C. Then, the plate was taken out from the incubator and a scratch in each well was made using a 100  $\mu$ l micropipette tip. Following this the cells in the plate were subjected to different concentrations of 4-terpineol (0, 25, 50 and 100  $\mu$ M) and then incubated for 48 hrs, fixed and stained with 3.5% ethanol containing 1.5% crystal violet powder for 30 min. Then, using a phase contrast microscope (Olympus, Tokyo, Japan), randomly selected fields were chosen and photographed.

### Fluorescence microscopic study

Hep-G2 cells were treated with several concentrations (0, 25, 50 and 100  $\mu$ M) of 4-terpineol and then

were placed in the incubator for 48 hrs at 37 °C. After incubation, the cells were fixed with 3.0 % formaldehyde for 30 min and washed with PBS twice. Hoechst 33342 solution was added to the cells and after 15 min of staining the cells were observed under a fluorescence microscope at 200x magnification (Nikon, Tokyo, Japan).

#### Annexin V-FITC assay

Annexin V-FITC assay was used to measure the extent of apoptosis induced by 4-terpineol in Hep-G2 cells. In brief, Hep-G2 cells were seeded at a density of  $2 \times 10^5$  cells/ml and then treated with different doses of 4-terpineol (0, 25, 50 and 100  $\mu$ M). Subsequently, the cells were incubated for 48 hrs, washed with PBS and then stained with PI and Annexin V-FITC as per the manufacturer's instructions. The cells were analyzed by flow cytometry using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software.

#### Cell cycle analysis

Hep-G2 cells ( $2 \times 10^5$  cells/ml) were seeded in 60-mm plates and treated with 0, 25, 50 and 100  $\mu$ M of 4-terpineol for 48 hrs. Subsequent to drug treatment, the cells were trypsinized and washed twice with PBS. The cells were fixed with 70% cold ethanol overnight and treated with 50  $\mu$ g/mL RNase A, and then stained with 5  $\mu$ g/mL of PI. Finally, the DNA content and cell cycle distribution was analysed by flow cytometry. The cell cycle analysis was performed by FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software with DNA PI staining.

#### Tumor xenograft mouse model of Hep-G2 cells

Six-week-old female BALB/c nude mice weighing

around 35 g each (National Rodent Laboratory Animal Resources, Shanghai, China) were maintained in a laminar airflow cabinet under pathogen-free conditions and a 12-hr light-dark cycle. The mice were maintained according to NIH guidelines for Animal Research and Care and all of the experimental protocols were approved by the Animal Investigation Committee of the Chongqing Medical University, Jieqing Road, Huxi University City, Chongqing 401331, China. The mice were randomly divided into two groups of 6 each. Hep-G2 cells were injected subcutaneously into the mice ( $2 \times 10^6$  cells per mouse). The mice were injected with either vehicle (control) or 4-terpineol (10 and 20 mg/kg) for 26 days after tumors grew to about 100 mm<sup>3</sup>. Tumor measurements were obtained in the cranial/caudal (length), superior/inferior (height) and medial/lateral (width) directions using a Vernier caliper (Fisher, Pittsburgh, PA, USA) and the tumor volume was calculated using the formula: tumor volume=length x height x 0.5 width.

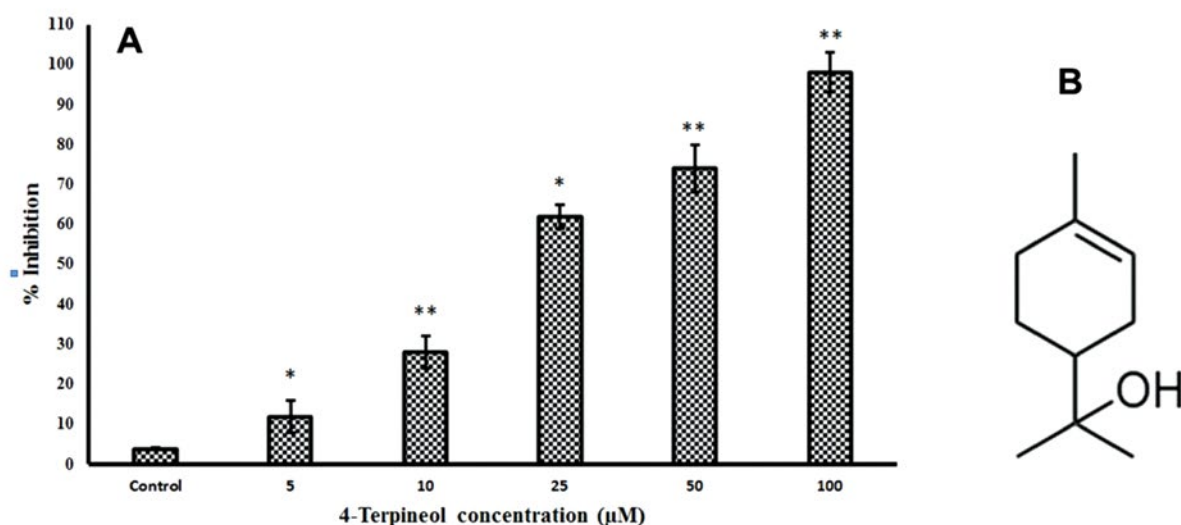
#### Statistics

All the results were presented as mean  $\pm$  standard error of the mean (SEM) from at least three independent experiments. The differences between groups were analyzed by one-way analysis of variance (ANOVA), and the significance of difference was designated as \* $p < 0.05$  or \*\* $p < 0.01$ .

## Results

#### Cytotoxic activity of 4-terpineol in Hep-G2 cells

The cytotoxic activity and chemical structure of 4-terpineol are shown in Figure 1 A and 1 B, respectively. MTT cell viability assay indicated that 4-terpineol induced dose-dependent cytotoxicity



**Figure 1. A:** Dose-dependent cytotoxic effect of 4-terpineol on the Hep-G2 hepatocellular carcinoma cells at various doses. \* $p < 0.05$  vs control group. \*\* $p < 0.01$  vs control group. **B:** Chemical structure of 4-terpineol.

in Hep-G2 cells. The potency of the compound was determined by calculating the  $IC_{50}$  value which is the concentration required to cause cell growth inhibition by 50%.  $IC_{50}$  value was found to be 19.5  $\mu$ M.

#### 4-terpineol induced DNA fragmentation in Hep-G2 cells

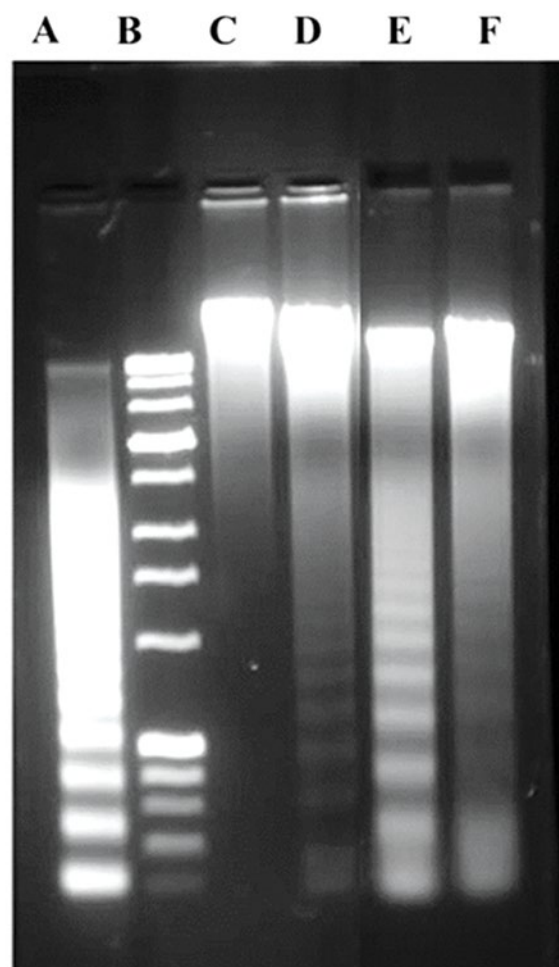
The fact that 4-terpineol induced DNA fragmentation in Hep-G2 cells was observed by the formation of DNA ladder using agarose gel electrophoresis. The results of this assay are depicted in Figure 2 and indicate that DNA fragmentation process was associated with increasing dose of 4-terpineol. It was also observed that the positive control also induced significant DNA ladder formation (Lane-A). DNA fragmentation is a marker of apoptotic process which begins within the cell, further proving that the 4-terpineol induced cell death via apoptosis.

#### 4-terpineol inhibited cell migration in Hep-G2 cells

In this assay, the effect of 4-terpineol on cell migration in Hep-G2 cells was evaluated by the *in vitro* wound healing assay. Cell migration along with cell invasion are the two key features of cancer metastases and agents which inhibit such processes are thought to be promising anticancer agents. The effects of 4-terpineol on Hep-G2 cell migration are shown in Figure 3 A and indicate that a wound scratch in vehicle-treated control cells was practically entirely closed after 48 hrs of incubation. Nonetheless, treatment with 0, 25, 50 and 100  $\mu$ M dose of 4-terpineol (Figure 3 B-E) resulted in inhibition of wound healing in a dose-dependent manner.

#### 4-terpineol induced apoptotic morphological features in Hep-G2 cells

Fluorescence microscopy using Hoechst 33342 as a staining agent was used to study the apoptotic effects of 4-terpineol on Hep-G2 cells. The results which are shown in Figure 4 A-D indicate that as compared to untreated control cells which showed regular morphology and homogeneous growth and confluence (Figure 4 A), Hep-G2 cells treated with 25, 50 and 100  $\mu$ M of 4-terpineol exhibited significant apoptotic morphological features including cell shrinkage, chromatin condensation, nuclear fragmentation and membrane blebbing (Figure 4 B-D). This apoptotic effect was found to be proportional to the dose of 4-terpineol.



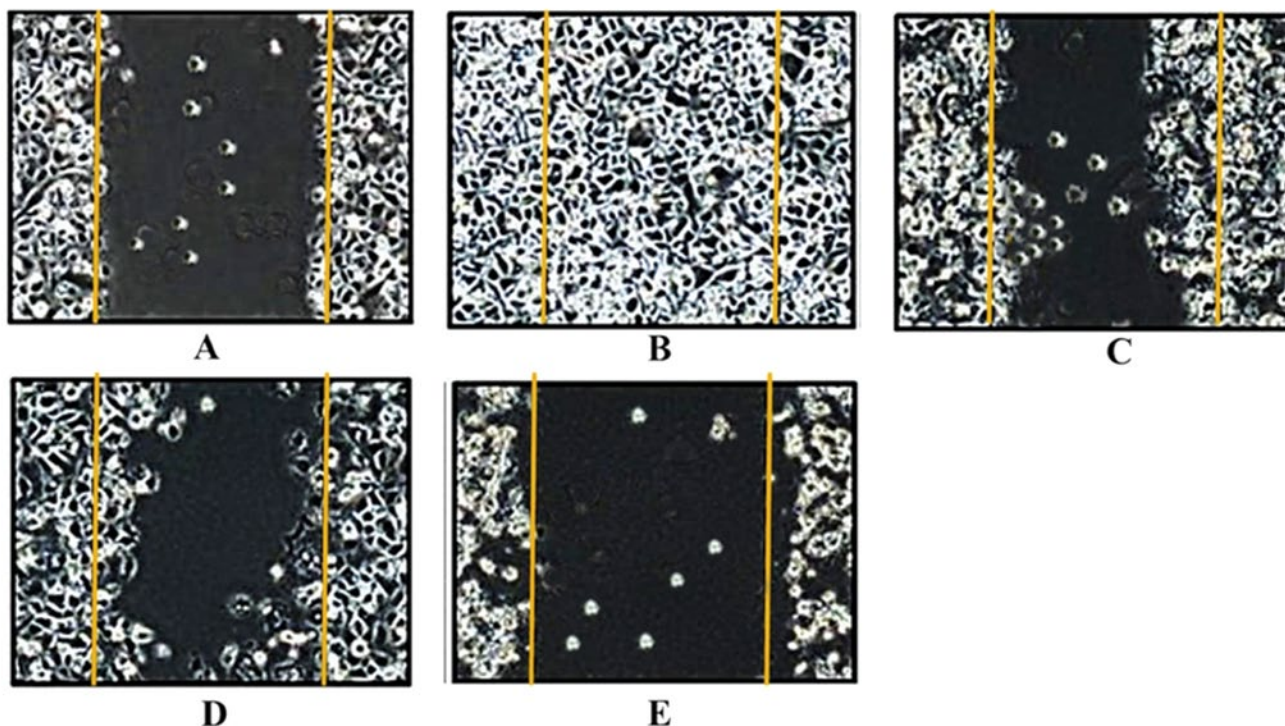
**Figure 2.** DNA fragmentation induced by 4-terpineol in Hep-G2 hepatocellular carcinoma cells after 12-h treatment. Lane A: positive control; Lane B: marker; Lane C-F: cells treated with 5, 25, 50 and 100  $\mu$ M of 4-terpineol for 12 hrs.

#### 4-terpineol induced both early and late apoptosis in Hep-G2 cells

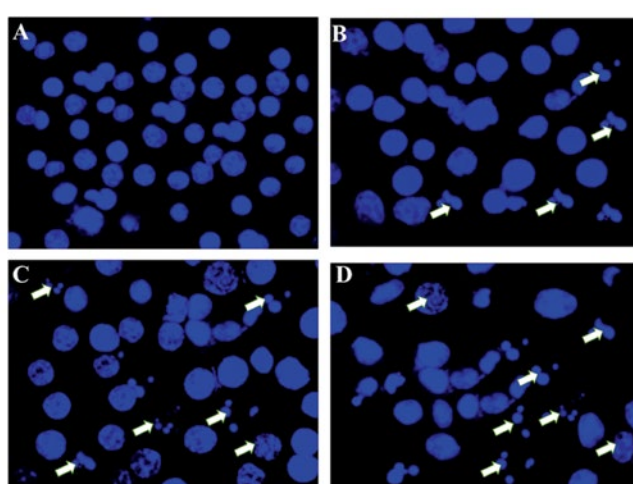
The 4-terpineol induced apoptosis in Hep-G2 cells was quantified by flow cytometry using annexin V-FITC and PI. Annexin V staining can detect phosphatidyl serine and as such can be used for its analysis. The results indicated that 4-terpineol treated cells exhibited higher proportion of apoptotic cells as compared to control cells. 4-terpineol induced both early and late apoptosis in Hep-G2 cells. The percentage of apoptotic cells increased from 2.5% in the control cells to 10.3, 64.6 and 78.9% in cells treated with 25, 50 and 100  $\mu$ M of 4-terpineol, respectively (Figure 5).

#### 4-terpineol induced sub-G1 cell cycle arrest

The fact that 4-terpineol induced sub-G1 cell cycle arrest in Hep-G2 cells was evaluated by flow cytometry using PI. The results are shown

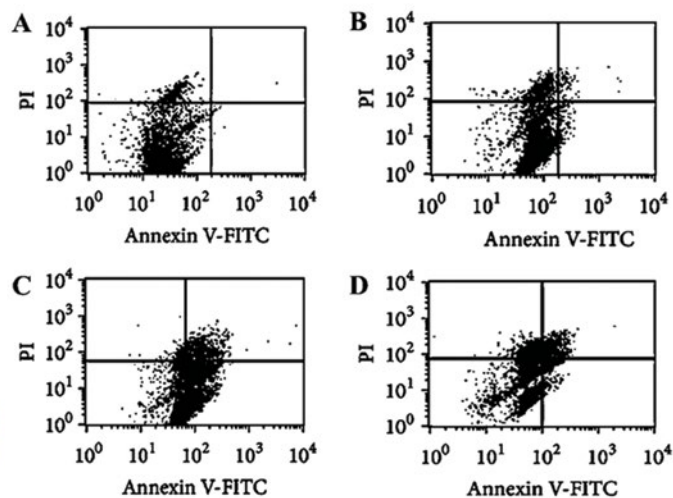


**Figure 3.** 4-terpineol inhibited cell migration in Hep-G2 hepatocellular carcinoma cells. In vitro wound healing assay was used to analyze the effect of 4-terpineol on cell migration. Cells were treated with 0 (B), 25 (C), 50 (D) and 100 (E)  $\mu\text{M}$  of 4-terpineol. A shows an artificial wound made by scratching with a micropipette tip.



**Figure 4.** Apoptotic effect of 4-terpineol in Hep-G2 human hepatocellular carcinoma cells using fluorescence microscopy and Hoechst 33342. A shows untreated cells (control), while B, C and D show groups treated with 25, 50 and 100  $\mu\text{M}$  of 4-terpineol, respectively. Arrows indicate apoptotic cells.

in Figure 6 A-D, indicating that as compared to the control, 4-terpineol-treated cells exhibited increased percentage of cells in sub-G1 phase of the cell cycle. Sub-G1 phase represents the apoptotic cells and as such this experiment in turn gives an idea about the percentage of apoptotic cells. The percentage of these sub-G1 cells

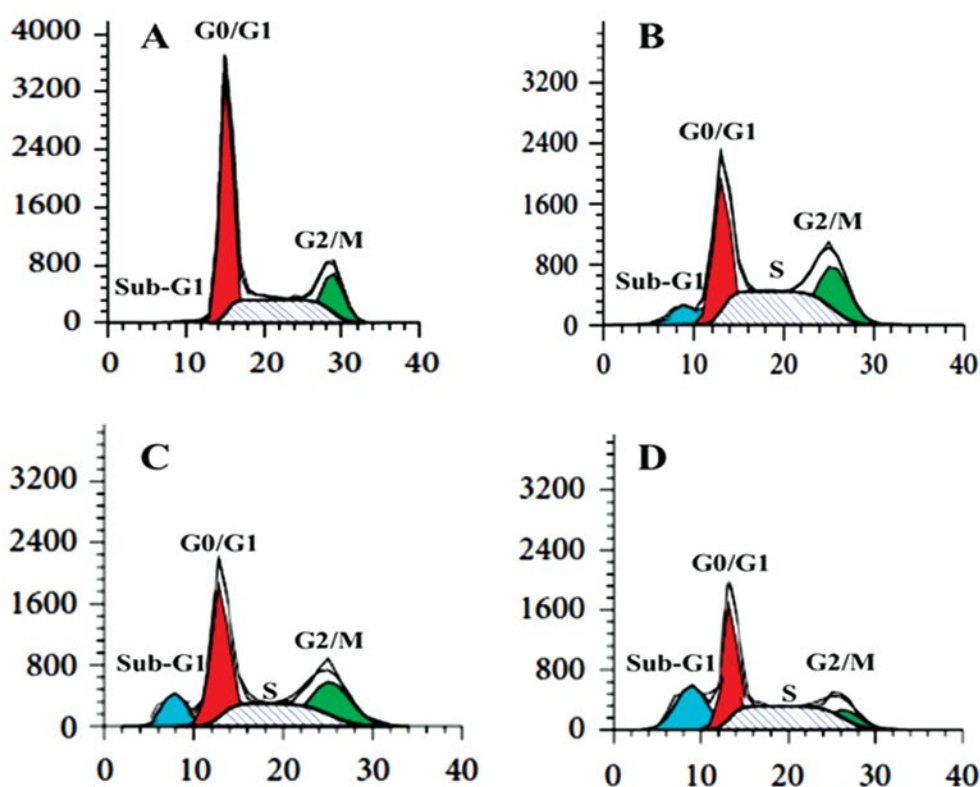


**Figure 5.** 4-terpineol induced early and late apoptosis in Hep-G2 hepatocellular carcinoma cells. Hep-G2 cells were treated with 0 (A), 25 (B), 50 (C) and 100 (D)  $\mu\text{M}$  of 4-terpineol and then analyzed by flow cytometry stained with annexin V-FITC/PI. With increasing dose of 4-terpineol, the percentage of early and late apoptotic cells increased.

increased with increasing doses of 4-terpineol. This was accompanied with a corresponding decrease in the G2/M phase cells.

*4-terpineol inhibited in vivo tumor growth in xenograft mouse model*

After observing that 4-terpineol inhibits *in*

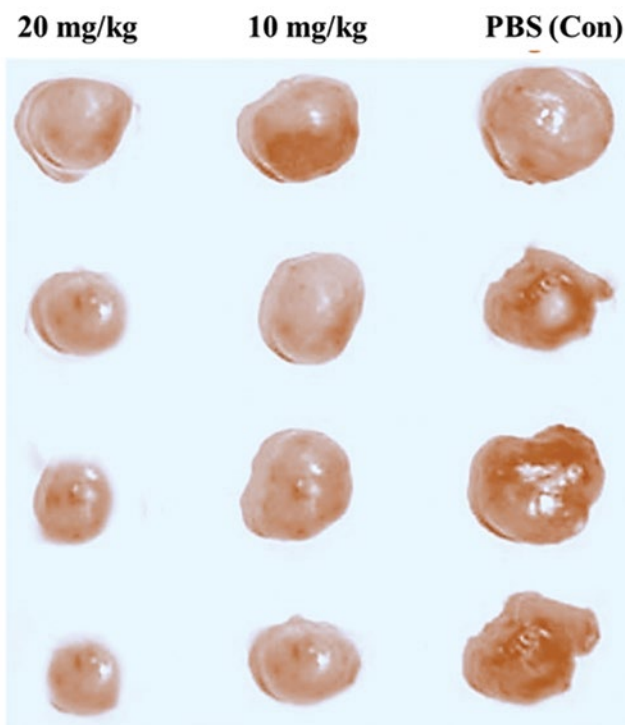


**Figure 6.** 4-terpineol induced sub-G1 cell cycle arrest in Hep-G2 hepatocellular carcinoma cells. The cells were treated with increasing doses of 4-terpineol [0 (A), 25 (B), 50 (C) and 100 (D)  $\mu$ M] and then analyzed by flow cytometry using PI. With increasing the dose of 4-terpineol, the percentage of sub-G1 phase cells increased.

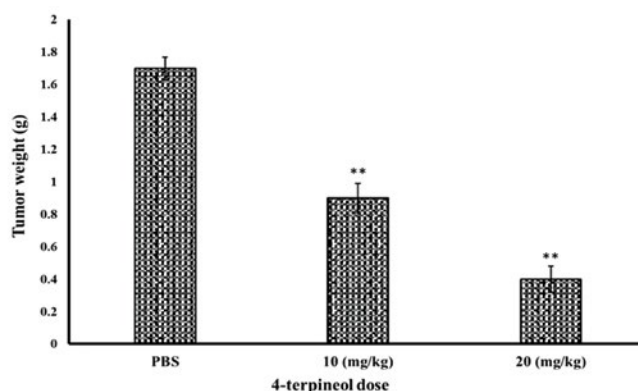
*in vitro* tumor growth of Hep-G2 cells, the antitumor effects of the compound were then evaluated in a xenograft mouse model. The *in vivo* results indicated that 10 mg/kg and 20 mg/kg of 4-terpineol decreased the tumor weight in a dose-dependent manner (Figure 7 and Figure 8). Doses of 10 mg/kg and 20 mg/kg of 4-terpineol decreased the tumor volume dose-dependently (Figure 9). The findings revealed that 10 mg/kg and 20 mg/kg 4-terpineol injection reduced the tumor weight from 1.7 g in PBS-treated group (control) to 0.91 g and 0.42 g, respectively. Similarly, 10 mg/kg and 20 mg/kg 4-terpineol injection reduced the tumor volume from 2.1  $\text{cm}^3$  in PBS-treated group (control) to 0.75  $\text{cm}^3$  and 0.26  $\text{cm}^3$ , respectively. This suggests that 4-terpineol considerably inhibited tumor growth in the xenograft mouse hepatocellular cancer model.

## Discussion

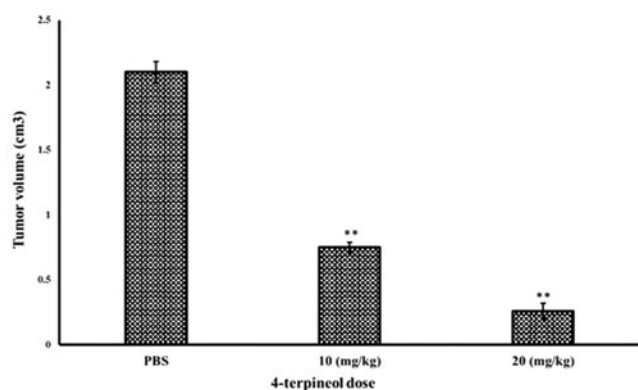
Apoptosis induction in tumor cells is one of the principal characteristics of various antitumor drugs which ultimately leads to cancer prevention by controlling cell death. The process of apoptosis can be characterized by various morphological features as well as biochemical signalling pathways. Morphological features of apoptosis include cellular shrinkage, chromatin condensation, nuclear



**Figure 7.** 4-terpineol inhibited tumor growth in a mouse xenograft model. Images of cancer tissues removed from the mice in control and drug-treated groups. 4-terpineol 10 and 20 mg/kg injection reduced the tumor weight from 1.7 g in PBS-treated group (control) to 0.91 and 0.42 g, respectively.



**Figure 8.** 4-terpineol reduced tumor weight in the treated mice. The values are means $\pm$ SEM (N=6). \*\*p<0.01.



**Figure 9.** 4-terpineol reduced tumor volume in the treated mice. The values are means  $\pm$  SEM. \*\*p<0.01.

fragmentation, membrane blebbing and apoptotic body formation [9-11]. Apoptosis is a highly specialized biochemical process which eradicates redundant cells from the body and as such is the key for maintaining tissue homeostasis. Any disturbance in this process eventually results in numerous diseases including cancer. Both intrinsic as well as extrinsic stimuli can trigger the process of apoptosis which finally lead to the activation of proteases (caspases) and nucleases, resulting in the destruction of the cell [12,13]. Cell migration

and invasion of malignant tumors are the two important features resulting in high morbidity as a result of the tumor being more deadly. These are the indications of cancer movement and metastasis. It has earlier been reported that cell migration is regulated by several signalling pathways including PI3K, p38MAPK, pJNK and FAK [14,15]. Accordingly, research work in this field can result in therapeutic methods for preventing cancer invasion, migration and metastasis, eventually resulting in improvement of survival of cancer patients. The results of this study indicated that 4-terpineol, which is a monoterpene, exhibits potent cytotoxic effects in Hep-G2 cells via induction of early and late apoptosis, DNA fragmentation, sub-G1 cell cycle arrest and inhibition of cell migration. Published reports indicate that 4-terpineol and its other isomers have been reported to exhibit anticancer activity against a range of cancer cells [16], but the mechanism of action of 4-terpineol including effects on cell cycle, cell migration and apoptosis have not been reported so far. As such the current study is the first attempt in this direction.

## Conclusion

The results of this study indicate in detail that 4-terpineol is a potent cytotoxic agent against Hep-G2 hepatocellular carcinoma cells and these effects are mediated via induction of apoptosis, inhibition of cell migration DNA fragmentation and cell cycle arrest in sub-G1 phase. If further *in vivo* studies are done on this compound, they could possibly result to a new anticancer drug in the future.

## Conflict of interests

The authors declare no conflict of interests.

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