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Original article

Anticancer properties of *Semen Euphorbiae* towards ACHN human renal adenocarcinoma cells by inducing apoptosis

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ABSTRACT

The main objective of the present research work was to evaluate the antitumor effects of ethanol extract of *Semen Euphorbiae* (EESE) in ACHN human renal carcinoma cells. The effects on apoptosis induction, cell cycle phase distribution and livin protein expression were also evaluated. MTT assay was used to assess cytotoxic effects of the extract while as clonogenic assay was used to evaluate effects on colony formation tendency. Inverted phase contrast and fluorescence microscopic techniques were used to evaluate effects of EESE on cellular morphology and apoptosis. Flow cytometry using annexin V-FITC and propidium iodide were used to quantify the extent of apoptosis and also to evaluate effects on cell cycle. Results indicate that ethanol extract of *Semen Euphorbiae* exhibited potent cytotoxic effects in ACHN human renal cancer cells. These effects were found to be dose-dependent as well as time dependent. Clonogenic assay revealed that EESE led to dose-dependent inhibition of colony formation in these cells. EESE-treated cells also showed evident signs of alterations and deformations in cell morphology including detachment of cells from one another forming small cluster of cells. In contrast to untreated control cells, EESE-treated cells with 10, 100 and 200 μg/ml dose showed an increase in the number of cells emitting red/orange fluorescence indicating onset and execution of apoptosis. EESE extract also led to G2/M cell cycle arrest in these cells.

1. Introduction

Renal cell carcinoma (RCC) or kidney cancer has become a major health problem worldwide and is the third most frequent malignancy affecting genitourinary organs. In USA alone during 2009, around 57,000 patients were diagnosed with renal cell carcinoma and it claimed the lives of as many as 13,000 persons. According to a report, the national incidence of RCC in USA is increasing by 3% per year. RCC is mostly diagnosed at later stages and one-third of all patients with this malignancy are already in the metastatic stage (Chow et al., 1999; Hollingsworth et al., 2006; Jemal et al., 2009). In males, it was the seventh most common cancer in the People's Republic of China in 2012.The majority of kidney cancers in adults are adenocarcinomas which usually arise in renal parenchyma, while as in children mostly nephroblastoma occur. The highest incidence of RCC is reported in Czech Republic with around 22 per 100,000 in men and 11 per 100,000 in women. High rates of rcc are also reported in many Asian and African countries and some American populations. In men, the incidence of rcc is around double than in females and the incidence of rcc also increases after the age of 30 years (Sene et al., 1992). Epidemiologically, the occurrence of kidney cancer is considerably associated with region, race, gender, and age. RCC is lower in developing countries than in developed countries. RCC is also lower in rural areas than in urban areas. However, in China, the incidence of renal cell carcinoma remains low (Zhang et al., 2015). As far as treatment of rcc is concerned, surgical resection remains a major treatment option of localized RCC. As compared to other cancers, rcc usually has a better prognosis after curative surgical resection. About 25–30% of RCC patients with metastatic stage have an average survival of up to 12 months (Xiaojie et al., 2015). RCC is usually resistant to radiotherapy and chemotherapy and the tumor does

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not respond well to these treatments. However, recently targeted therapeutic approach has been in use which uses certain drugs like sorafenib, sunitinib, everolimus, etc. to downsize the tumor size and then remove it using surgery. These agents target tumor angiogenesis and intracellular pathways and as such greatly affect the tumor growth leading to increased survival rates in RCC patients (Escudier et al., 2007, 2012). However, these targeted agents are also not without some serious limitations including development of drug resistance. As such there is a pressing need for design and development of novel chemotherapeutic agents which can target renal cell carcinoma with lesser chances of acquiring drug resistance. The main purpose of the current study was to evaluate the anticancer effects of ethanol extract of *Semen Euphorbiae* in ACHN human renal adenocarcinoma along with assessing its effects on apoptosis induction, cell cycle phase distribution and expression of livin protein.

2. Materials and methods

2.1. Chemicals and other reagents

In the current study, the following drugs and chemical reagents were used. Annexin V-FITC, Hoechst 33258, acridine orange and propidium iodide were obtained from Sigma–Aldrich, St. Louis, MO, USA. MTT kit was purchased from Roche (USA). RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco-BRL, Carlsbad, CA, USA. The various antibodies were purchased from Cell Signaling Technology, USA. Fetal calf serum, trypsin, penicillin, streptomycin, DMSO, RNase, RIPA Buffer were obtained from Hangzhou Sijiqing Biological Products Co. Ltd, China.

2.2. Collection of Semen Euphorbiae preparation of extract

The dry and mature seeds of *Euphorbia lathyris* (*Semen Euphorbiae*) were collected from a local region near Hangzhou City, Zhejiang Province, China. The dried seeds were finely powdered using an electric grinder. The powder was packed in Soxhlet apparatus and extracted with ethanol at desirable temperatures. The extract was then filtered through filter paper, the supernatant was collected and concentrated under reduced pressure at required temperatures using rotavapor. The extract was dried, and stored at 4 °C until used for experimental analysis.

2.3. Cell line and cell culture conditions

ACHN human renal adenocarcinoma cell line was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. The cell culture conditions comprised of Dulbecco's modified Eagle's medium (DMEM) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) supplemented with 10% (v/v) fetal bovine serum (FBS) under humidified atmosphere of 5% CO_2 at 37 °C.

2.4. MTT assay for cell proliferation evaluation

MTT which is a colorimetric cell viability assay was used to evaluate effects of the ethanol extract of *Semen Euphorbiae* (EESE) against ACHN human renal cancer cells. ACHN cells at a density of 2×10^6 cells/well were seeded in a 96-well plate and then incubated for 24 h. The cells were then treated with increasing doses (0, 10, 50, 100, 200 and 400 μg/ml) of EESE for 24, 48 and 72 h time intervals. In the control group, the cells without extract treatment were kept. After different time incubations, the cells were washed with PBS two times before 200 μl of MTT solution was added and the whole cell culture. The cells were again incubated for one hour. Eventually, the absorbance was measured at 490 nm with the use of ELISA plate reader.

2.5. Colony formation assay

Clonogenic assay was used to assess the effects of EESE on the number of colonies formed by ACHN human renal cancer cells. In brief, ACHN cells were initially harvested and counted using hemocytometer. The cells were seeded at a density of 500 cells/well and then incubated for 24 h to form a complete monolayer of cells. Subsequently, various doses of EESE were added to the cell culture and the cells were further incubated for 48 h. The cells were then washed with PBS and the cell colonies were fixed using methanol. Finally, the cells were stained with crystal violet for 30 min and counted using light microscope.

2.6. Inverted phase contrast microscopy

ACHN human renal cancer cells were seeded at a density of 2×10^6 cells/well into six-well plate 48 h before drug treatment. The cells were treated with varying doses of EESE and further incubated for 48 h. After drug treatment, culture plates were examined using an inverted light microscope (Nikon Corp., Tokyo, Japan) and images were captured. DMSO was used as a vehicle control. The morphological changes were monitored and the same spot of cells was photographed. The images were captured at a magnification of ×200.0, 10, 50, 100, 200 and 400 μg/ml.

2.7. Fluorescence microscopy evaluation

The apoptotic effects of EESE on the ACHN human renal carcinoma cells were evaluated by fluorescence microscopy using acridine orange/propidium iodide double staining. The cells were seeded at a density of 2×10^6 cells/well in a 6-well plate. The cells were treated with 0, 10, 100 and 200 μg/ml of ethanol extract of *Semen Euphorbiae* (EESE) for 48 h. Both treated and untreated (control cells) were incubated with acridine orange/propidium iodide (20 μg/ml each) for 2 h before being examined by fluorescent microscope (Nikon, Tokyo, Japan) at a magnification of ×200.

For Hoechst 33258 procedure, ACHN human renal carcinoma cells were plated in 6-well plates at a density of $2 \times 10^6 \times$ cells/well and then cultured for 24 h to allow complete attachment of cells to the surface of the plates. The cells were treated with 0, 10, 100 and 200 μg/ml of EESE for 48 h and then stained with Hoechst 33258 (5 μg/ml) at 37 °C for 20 min. Cell morphological changes was examined using a fluorescence microscope (Olympus, Tokyo, Japan) to identify cells affected by apoptosis.

2.8. Annexin V-FITC assay for apoptosis quantification

For this assay, the ACHN human renal cancer cells were seeded at a density of 2×10^6 cells per ml and then incubated for 24 h before being treated with different doses (0, 10, 100 and 200 μg/ml) of EESE for 48 h. The cells were harvested using trypsinization and then washed with PBS two times. Subsequently, 200 μl binding buffer comprising of 25 μl each of Annexin V-FITC and propidium iodide was added to the cells and incubated for 20 min in the dark before the samples were analyzed by flow cytometer.

2.9. Cell cycle analysis

In this experiment, effects of EESE on the cell cycle phase distribution in ACHN human renal carcinoma cells were studied involving flow cytometry. ACHN human renal cancer cells were seeded at

a density of 2×10^6 cells/ml in 60-mm dishes. The cells were treated with 0, 10, 100 and 200 μg/ml dose of EESE for 48 h. Subsequent to drug treatment, the cells were harvested using trypsinization and washed three times with PBS and then fixed with 70% cold ethanol for 12 h. After this the cells were treated with 20 μg/ml RNase A, then stained with 10 μg/ml of propidium iodide. The cell contents were finally analyzed by FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA, equipped with Cell Quest 3.3 software).

2.10. Western blot assay

The effect of EESE on the expression of livin and other Inhibitor of Apoptosis Proteins (IAPs) including c-IAP1 and c-IAP2 proteins was evaluated by western blot assay. Human renal carcinoma cells ACHN were treated with increasing doses of EESE for 48 h. The drug treated cells were then harvested via trypsinization and washed twice with PBS and then lysed with RIPA buffer and protease inhibitor for 30 min. The contents were then centrifuged and the protein content was estimated by BCA method. The protein lysates (10 μg/lane) were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Millipore, MA, USA). Each membrane was blocked with 5% skim milk, and then incubated with the designated primary antibodies overnight at 4 °C, then washed with buffered saline and incubated with the appropriate secondary antibody (horseradish peroxidase-conjugated, anti-rabbit) for 1 h. The bands were visualized using an ECL chemiluminescent detection kit (Perkin Elmer Cetus, Foster City, CA, USA).

2.11. Statistical analysis

All results were depicted as mean ± standard error (S.E.) from at least three independent experiments. The differences between groups were analyzed by one-way ANOVA, significance of difference was designated as $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Semen Euphorbiae extract exhibits potent antiproliferative effects

The cell proliferation results obtained using MTT cell viability assay are shown in Fig. 1 and indicate that ethanol extract of *Semen Euphorbiae* exhibited potent cytotoxic effects in ACHN human renal cancer cells. The cytotoxic effects were found to be dose-dependent as well as time dependent and it was seen that the cytotoxicity effects increased at higher incubation time intervals and higher extract doses. The IC_{50} values of the extract were calculated and were found to be 185.2, 151.4 and 131.7 μg/ml at 24 h, 48 h and 72 h time incubations respectively.

3.2. Ethanol extract of Semen Euphorbiae (EESE) led to inhibition of colony formation in ACHN cells

The effects of EESE on the colony formation tendency in ACHN human renal carcinoma cells are shown in Fig. 2 and suggest that EESE could inhibit colony formation capacity in these cells. Further, these inhibitory effects of the extract were examined at various doses and it was shown that EESE led to dose-dependent inhibition of colony formation in these cells.

3.3. Effect of EESE on the alterations in cellular morphology

Inverted phase contrast microscopy was used to study effects of EESE on the cellular morphology in ACHN human renal cancer cells. Results which are shown in Fig. 3 indicate that in contrast to untreated control cells, EESE-treated cells showed evident signs of alterations and deformations in cell morphology. Increasing doses of EESE led to significant detachment of cells from one

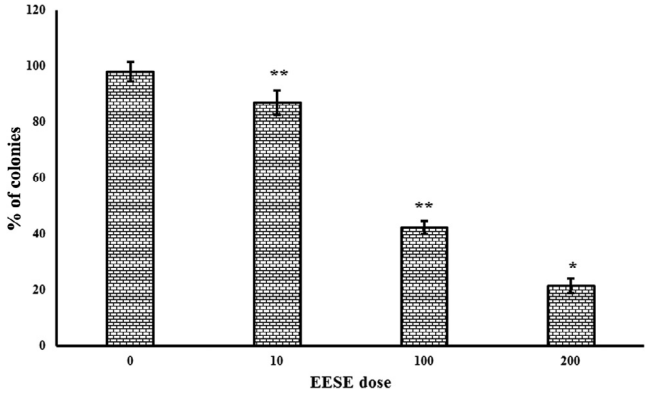

Fig. 2. Inhibitory Effect of ethanol extract of *Semen Euphorbiae* on the colony formation in ACHN human renal carcinoma cells. Data are shown as the mean ± SD of three independent experiments. *, $P < 0.05$, **, $P < 0.01$, vs 0 μM (control).

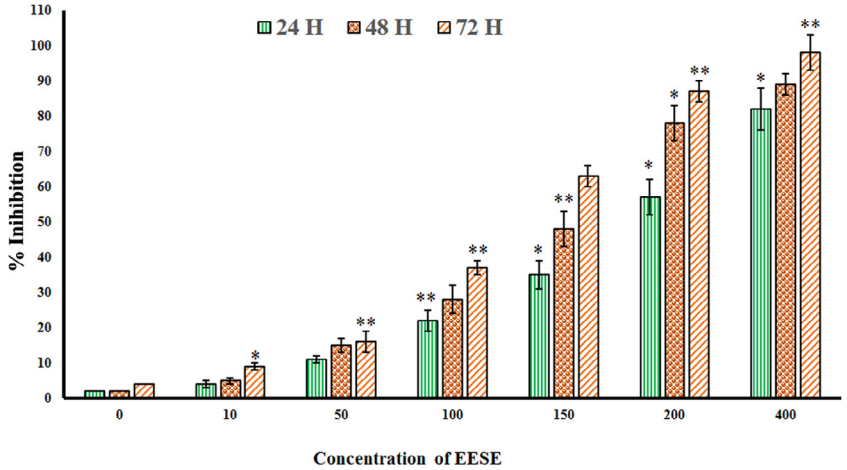

Fig. 1. Cytotoxic effects of ethanol extract of *Semen Euphorbiae* in human renal cancer cells (ACHN) using MTT cell viability assay. Data are shown as the mean ± SD of three independent experiments. *, $P < 0.05$, **, $P < 0.01$, vs 0 μM (control).

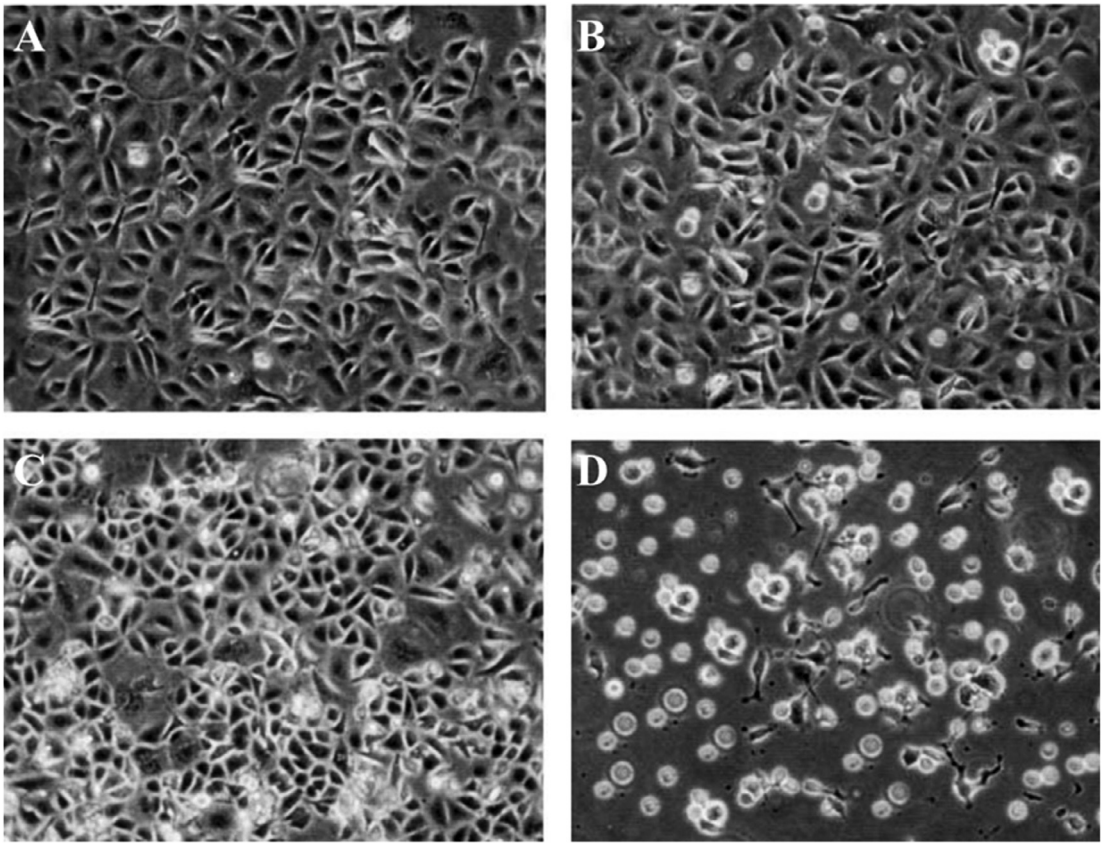

Fig. 3. Cellular morphological evaluation of ACHN human renal carcinoma cells after treatment with 0 (A), 10 (B), 100 (C) and 200 (D) μg/ml of EESE for 48 h. The cells were then analyzed by inverted phase contrast microscope at 200× magnification.

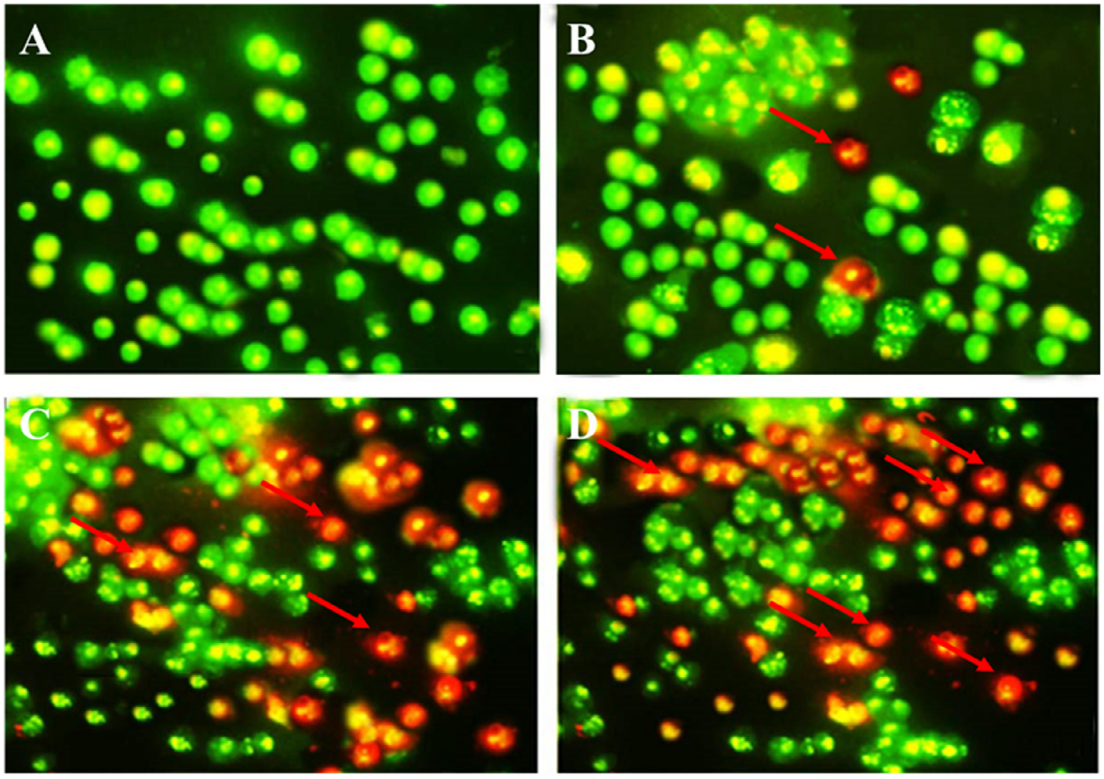

Fig. 4. Effect of EESE on the apoptotic cell death in ACHN human renal carcinoma cells was evaluated by fluorescence microscopy using acridine orange/propidium iodide double staining. The cells were treated with 0 (A), 10 (B), 100 (C) and 200 (D) μg/ml of EESE for 48 h. Apoptotic cells emit red fluorescence while as viable cells emit green fluorescence. An increase in the number of apoptotic cells was observed as the EESE dose increased.

another forming small cluster of cells. The cells also became uneven unable to sustain their intact membranes.

3.4. Apoptotic cell death evaluation by fluorescence microscopy using acridine orange/propidium iodide and hoechst 33258

The fact that ethanol extract of *Semen Euphorbiae* induced apoptotic cell death in ACHN human renal carcinoma cells was demonstrated by using fluorescence microscope using acridine orange/propidium iodide double stain. The findings of the assay are depicted in Fig. 4 and reveal that in contrast to untreated control cells which mostly emitted green fluorescence (Fig. 4A) indicating viable cells with no signs of apoptosis. However, on treating cells with 10, 100 and 200 μg/ml of the EESE extract, it was observed that the number of cells emitting red fluorescence increased with increasing EESE dose (Fig. 4B–D). Red or orange fluorescence is mostly emitted by apoptotic cells. Similar results were obtained when we used hoechst 33258 staining which revealed that on treating cells with increasing doses of EESE, chromatin condensation and DNA fragmentation occurred which are the clear indications of apoptotic process. Further, it was seen that the apoptotic effect enhanced at higher doses of ethanol extract of *Semen Euphorbiae* (Fig. 5A–D).

3.5. Quantitative estimation of EESE-induced apoptosis in ACHN human renal carcinoma cells

In this experiment, apoptotic effects of ethanol extract of *Semen Euphorbiae* in ACHN human renal carcinoma cells were quantifies using annexin V-FITC assay and flow cytometry. As compared to the untreated cells, EESE-treated cells showed a dose-dependent increase in the number of apoptotic cells. The percentage of apoptotic cells increased from 2.3% in untreated control cells to 19.7%, 38.9% and 66.6% in cells treated with 10, 100 and 200 μg/ml of the EESE extract. Necrotic cells, late apoptotic cells, early apoptotic cells and viable cells are depicted by R1, R2, R3 and R4 respectively in Fig. 6.

3.6. Semen Euphorbiae induces G2/M phase cell cycle arrest

Further experiments with flow cytometry indicated that ethanol extract of *Semen Euphorbiae* (EESE) induces G2/M phase cell cycle arrest in ACHN human renal carcinoma cells. The cells were treated with 0, 10, 100 and 200 μg/ml of the EESE extract, stained with propidium iodide and then analyzed by flow cytometry. As the concentration of EESE extract increased from 0 to 10, 100 and 200 μM, the percentage of G2/M phase cells increased from 13.2% to 24.7%, 42.1% and 63.2% respectively (Fig. 7). In addition,

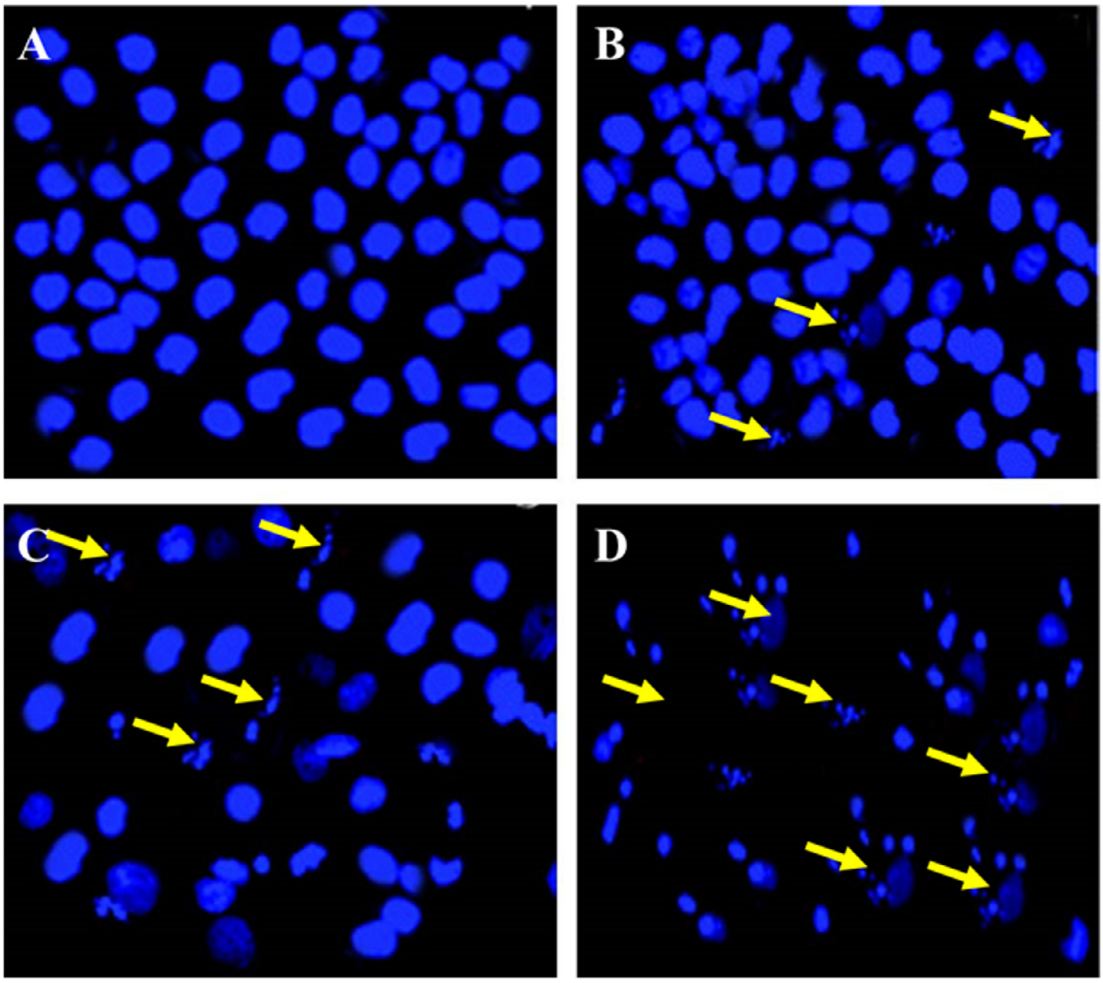

Fig. 5. Fluorescence microscopic evaluation of the effects of EESE on the apoptosis induction in ACHN human renal carcinoma cells. The cells were treated with 0 (A), 10 (B), 100 (C) and 200 (D) μg/ml of EESE for 48 h and then stained with hoechst 33258 before final analysis. Yellow arrows represent apoptotic cells with altered cell morphology, chromatin condensation and apoptotic body formation.

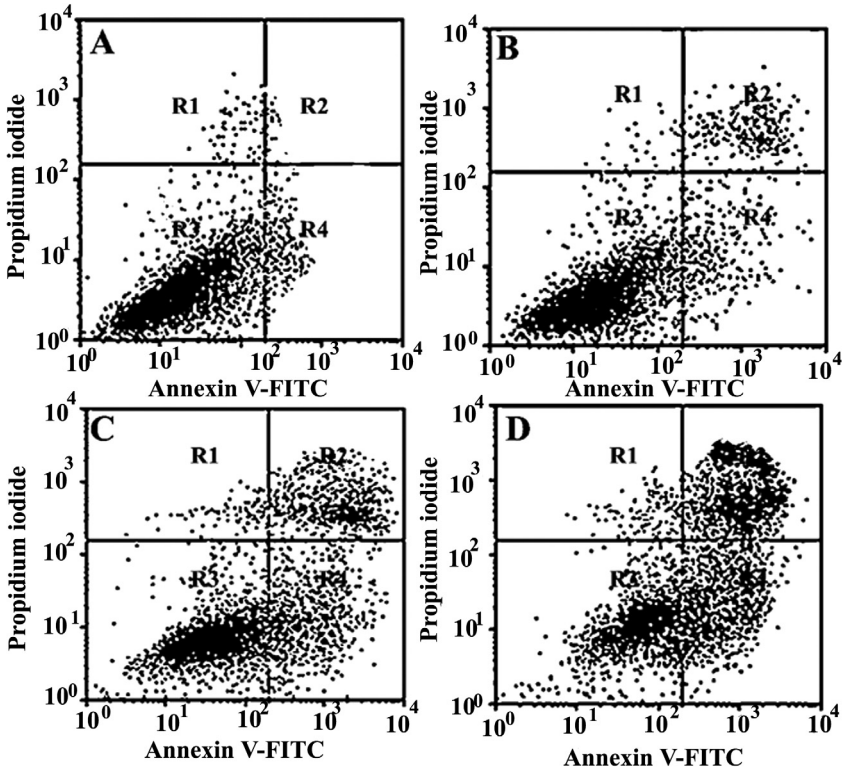

Fig. 6. Apoptosis quantification using Annexin V-FITC assay. The cells were treated with 0 (A), 10 (B), 100 (C) and 200 (D) μg/ml of EESE for 48 h and then stained with annexin V-FITC/propidium iodide before final analysis by flow cytometry. Necrotic cells, late apoptotic cells, early apoptotic cells and viable cells are depicted by R1, R2, R3 and R4 respectively.

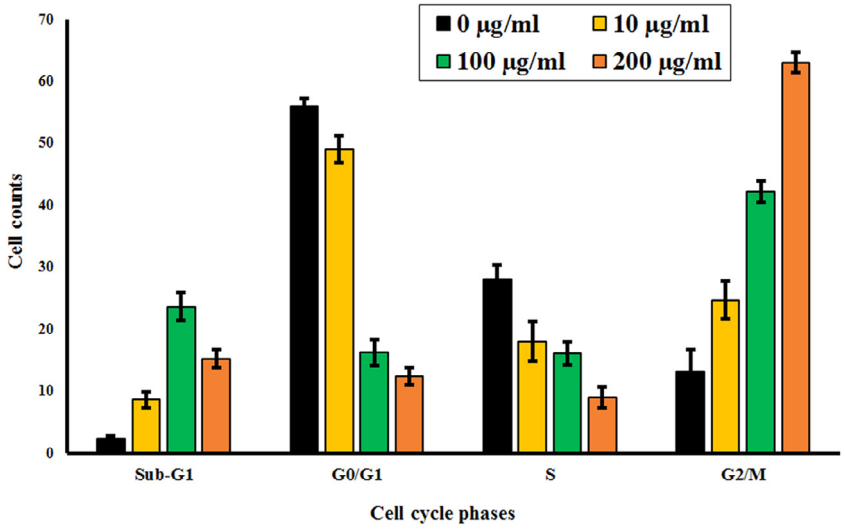

Fig. 7. EESE induced G2/M cell cycle arrest in ACHN human renal carcinoma cells. The cells were treated with 0, 10, 100 and 200 μM dose of EESE and incubated for 48 h, after which cells were analyzed using flow cytometry.

a slight increase in the sub-G1 cells was also seen as the dose of EESE increased.

3.7. Semen Euphorbiae led to inhibition of livin and other inhibitor of apoptosis proteins (IAPs) including c-IAP1 and c-IAP2

In the current study, we also evaluated effects of EESE on the expression levels of livin and other inhibitor of apoptosis proteins (IAPs). These IAPs are very essential in regulating the apoptosis process in cells. Apoptosis inhibition leads to development of various malignant cancers including renal cell carcinoma. Therefore, inhibition of the expression levels of livin and other IAPs is crucial in curbing cancer cell growth. As shown in Fig. 8, as the dose of EESE extract increased from 0 to 10, 100 and 200 μg/ml, the expression levels of livin and other IAPs including c-IAP1 and c-IAP2 decreased significantly. Thus it can be concluded that EESE extract encourages apoptosis induction by suppressing the expression levels of livin and other IAPs factors.

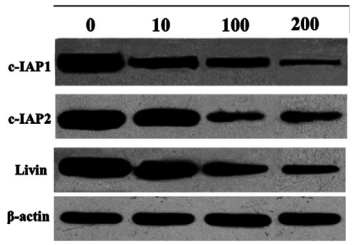

Fig. 8. Western blots showing ethanol extract of *Semen Euphorbiae* (EESE) led to downregulation of livin protein along with other Inhibitor of Apoptosis Proteins (IAPs) including c-IAP1 and c-IAP2. The cells were treated with 0, 10, 100 and 200 μg/ml of EESE for 48 h, β-actin served as positive control.

4. Discussion

Apoptosis is a programmed cell death which is a highly organized biochemical process crucial for the tissue homeostasis and any disruption in it leads to cancer (Serasanambati and Chilakapati, 2016; Neelamkavil and Thoppil, 2016). Two major apoptotic pathways have been identified, one is the extrinsic pathway and the other one is the mitochondrial mediated intrinsic apoptotic pathway. The homeostasis in multicellular organisms is subjected to a subtle balance between cell survival and cell death signals initiated from internal and external domain (Thompson, 1995; Schafer and Kornbluth, 2006). Apoptosis is characterized by distinctive morphological and biochemical features including cell shrinkage, nuclear shrinkage, chromatin condensation, membrane blebbing and DNA fragmentation. Apoptosis is an essential homeostatic machinery that maintains balance between cell division and cell death and maintains the proper number of cells in the body. Various anticancer chemotherapeutic agents used in conventional anticancer treatment regime are known to induce apoptosis in a variety of tumors. The induction of apoptosis in cancerous cells is believed to be a promising method of curbing different forms of cancer (Dixon et al., 1997). A large number of naturally occurring compounds and extracts have been reported to induce apoptosis in a variety of cancer cells. Therefore the focus of the current research work is mostly on screening agents which can induce apoptosis in cancer cells (Taraphdar et al., 2001). The plant-derived chemotherapeutic or chemopreventive compounds are efficient alternative sources of anticancer drugs with the capability to target cancer cells. Several bioactive natural product based compounds inhibit cancer cell growth by targeting the cell cycle, which is in turn regulated and controlled by a series of cell cycle regulators and check points (Mann, 2002; Cragg and Newman, 2005).

Livin, which is a member of the Inhibitor of Apoptosis Proteins (IAPs) family has been reported to be manifested in various malignant tumors along with transformed cells. The cancers in which livin has been reported to express include lung, breast, bladder, prostate and renal carcinomas. Livin is made of a single Baculovirus-Repeat (BIR) domain and a zinc-binding RING-domain. In majority of cancers, the expression of livin protein has been correlated with advanced cancer stage, multidrug resistance and poor outcome (Khursheed et al., 2016). It has been reported that the anti-apoptotic effects of livin are carried out via suppression of the mitochondrial apoptotic pathway enzymes, caspases and degradation of Smac/DIABLO (Yagihashi et al., 2003; Tanabe et al., 2004; Kempkensteffen et al., 2007). In the present study, it was observed that ethanol extract of *Semen Euphorbiae* exhibited potent cytotoxic effects in ACHN human renal cancer cells. The cytotoxic effects were found to be dose-dependent as well as time dependent. EESE also led to inhibition of colony formation in these cells and these effects were also related to the drug dose. Phase contrast microscopy revealed that in contrast to untreated control cells, EESE-treated cells showed evident signs of alterations and deformations in cell morphology. Fluorescence microscopy using acridine orange/propidium iodide revealed that on treating cells with 10, 100 and 200 μg/ml of the EESE extract, it was observed that the number of cells emitting red fluorescence (apoptosis) increased with increasing EESE dose. Similar results were obtained using hoechst 33258 staining which revealed that increasing doses of EESE led to chromatin condensation and DNA fragmentation which are the clear indications of apoptotic process. The annexin v assay indicated that the percentage of apoptotic cells increased from 2.3% in untreated control cells to 19.7%, 38.9% and 66.6% in cells treated with 10, 100 and 200 μg/ml of the EESE extract. EESE extract also led to G2/M phase cell cycle arrest in these cells. *Semen Euphorbiae* led to inhibition of livin and other Inhibitor of Apoptosis Proteins (IAPs) including c-IAP1 and c-IAP2.As the dose of EESE extract increased from 0 to 10, 100 and 200 μg/ml, the expression levels of livin and other IAPs including c-IAP1 and c-IAP2 decreased significantly. Thus it can be concluded that EESE extract encourages apoptosis induction by suppressing the expression levels of livin and other IAPs factors.

In conclusion, the current results reveal that EESE extract exhibits potent antitumor effects in ACHN human renal cancer cells and these antitumor effects are mediated via apoptosis induction, G2/M phase cell cycle arrest and suppression of livin and other Inhibitor of Apoptosis Proteins (IAPs) including c-IAP1 and c-IAP2.

Conflict of interest

The authors declare no conflict of interest.

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