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Irigenin exerts anticancer effects on human liver cancer cells via induction of mitochondrial apoptosis and cell cycle arrest

Wanggang Xu, Yingmin Kuang, Dan Wang, Zhen Li and Renpin Xia[*]

Abstract

Irigenin has been reported to exhibit remarkable anticancer effects against several human cancers. Nonetheless, the anticancer effects of irigenin against the human liver cancer cells are still largely unexplored. Consistently, this study was designed to evaluate the anticancer effects of irigenin against human liver cancer cells and to unveil the underlying molecular mechanisms. The results showed that irigenin significantly ($p < 0.05$) inhibited the growth of the human HepG2 and SNU-182 liver cancer cells with an IC_{50} value of 14 μM. Nonetheless, the cytotoxic effects of irigenin against the normal THLE-2 cells were comparatively lower as evident from the IC_{50} of 120 μM. The AO/EB and DAPI staining showed that irigenin induces apoptosis in the human liver cancer cells. Annexin V/PI staining assay revealed a significant ($p < 0.05$) increase in the percentage of apoptotic HepG2 and SNU-182 liver cancer cells upon treatment with irigenin. It was found that the number of apoptotic HepG2 and SNU-182 cells enhanced from 2.3 to 41.75% and 1.16 to 51.9% at IC50, respectively. Western blot showed a considerable increase in Bax and decrease in the Bcl-2 expression upon irigenin treatment further confirming the induction of apoptosis. Flow cytometric analysis revealed that irigenin also induces G2/M cell cycle arrest of HepG2 and SNU-182 cells. The percentage of G2/M phase HepG2 and SNU-182 cells increased from 17.92 to 34.35% and 23.97 to 38.23% at IC_{50}, respectively This was also accompanied by decrease in the expression of CDK1 and Cyclin-B in HepG2 and SNU-182 cells. Taken together, the results of the present study suggest that irigenin inhibits the growth of the human liver cancer cells via induction of apoptosis and cell cycle arrest. These results point towards the potential of irigenin as a lead for the development of chemotherapy for liver cancer.

Keywords: Liver cancer, Isoflavonoids, Irigenin, Cell cycle, Apoptosis

Introduction

Liver cancer is a disastrous human malignancy and a leading cause of mortality in several regions of the world [10, 11]. World has seen huge upsurge in liver cancer incidences from past two decades. It has been ranked fourth among highest mortality causing cancers with more than 750,000 cases diagnosed annually [19]. Over 80% of liver cancer cases befall in middle and low-resource countries, predominantly in sub-Saharan Africa and Eastern Asia, where social and health care systems are mostly constrained. Major risk factors that contribute to liver cancer development includes chronic hepatitis B and C, metabolic liver disease (MLD, non-alcoholic fatty liver disease, alcohol consumption and exposure to dietary toxins like aristolochic acid and aflatoxins [7, 22]. Liver cancer surveillance is imperative for high risk patients which permits early disease detection and boosts the efficacy of curative methodologies. Curative treatments involved in early-stage liver cancer include surgical resection, local ablation, chemotherapy, and organ transplantation. Regardless of substantial advancements made in liver cancer diagnosis and treatment, the overall survival remains extremely

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poor with high frequency of disease recurrence [18]. Therefore, to address the rising global liver cancer burden, potential treatment methodologies and chemopreventive drugs are need of the hour. Natural products have impressed the researchers with their enormous structural diversity and bioactivity profiles [3]. More than 70% of chemopreventives for cancer belong to either natural products or compounds derived from them [6]. Isoflavonoids are a major class of natural products and exhibit several bioactivities such as antiproliferative, anticarcinogenic, antimutagenic and antioxidant activities [4, 15, 17]. Irigenin is one of the active isoflavonoids present in several plant species [9]. Irigenin has been shown to exert anticancer effects on breast MBD-MA-231 cancer cells by inducing apoptosis. These proapoptotic effects of irigenin were reported to interceded via reactive-oxygen species (ROS) mediated mitochondrial dysfunction [24]. Irigenin has been reported to suppress prostaglandin (PG) E2 production, lipopolysaccharide (LPS)-induced nitric oxide (NO) production, NF-κB activity and expression levels of p-65 protein, in murine RAW 264.7 macrophages [1]. Furthermore, it has been shown to exhibit substantial inhibitory effects on lung cancer metastasis induced via Fibronectin-Extra Domain A [2]. In yet another study irigenin has been reported to possesses promising TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) based proapoptotic effects against gastric cancer [20]. However, the anticancer effects of irigenin against liver cancer and its underlying mechanism is still largely unknown. Against this backdrop, the anticancer effects of irigenin were examined against liver cancer HepG2 and SNU-182 cells in vitro and the potential underlying mechanisms were explored.

Materials and methods
Reagents, chemicals, and cell cultures
Irigenin (98% purity, CAS Number: 548-76-5) was obtained from Sigma-Aldrich (St. Louis, Missouri, United States). Other chemicals involved in this research were bought from Merck KGaA (Darmstadt, Germany), unless otherwise mentioned. The HepG2 and SNU-182 liver cancer and THLE-2 normal liver cells were bought from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. RPMI-1640 media (Hyclone, Logan, UT) was used for seeding all the cell culture maintaining fetal bovine serum (FBS; 10%) (Thermo Fisher Scientific, Inc., Waltham, United States), penicillin G (100 U/ml) and streptomycin (100 μg/ml) as potential antibiotics. Humid environment of 5% CO_2 concentration and 37 °C was used to maintain and culture all-inclusive cell lines.

Proliferation assay
The effects of irigenin treatment on proliferation rate of HepG2 and SNU-182 liver cancer and THLE-2 normal liver cells were monitored by WST-1 viability assay as described previously [10, 11]. Briefly, HepG2, SNU-182 and THLE-2 cell lines were precultured for 24 h followed by transference to 96-well plates at a concentration of 1.5×10^5 cells per well. Afterwards, irigenin was added to each well plate at altering concentrations ranging from 0 to 640 μM. After 48 h of treatment, each cell line was washed using phosphate buffered saline (Sigma) and then subjected to 3 h of incubation with WST-1 (10 μL) at 37 °C. DMSO (Sigma) was used as vehicle control. Finally, viability was determined by taking optical density (OD) at 450 nm using ELISA microplate reader (BioTek, China). The absorbance of untreated HepG2, SNU-182 and THLE-2 cells (control) were considered as 100% viable and absorbance was also considered as 100%. The experiments were performed in triplicate and the percentage inhibition was calculated by using the given formula:

$$\text{Inhibition (\%)} = 100 - \frac{\text{OD of treated cells}}{\text{OD of untreated control cells}} \times 100$$

Apoptosis assays
To determine apoptotic effects of irigenin against HepG2 and SNU-182 liver cancer cells Acridine Orange/Ethidium Bromide (AO/EB) staining, 4,6-diamidino-2-phenylindole staining (DAPI) staining were performed as described previously [23]. For AO/EB staining, both the cancer cell lines were seeded in 6-well plates containing RPMI1604 culture medium (Hyclone, Logan, UT) at the density of 0.4×10^6 cells each well. Afterwards, cells were precultured for 12 h prior to irigenin exposure at doses of 0 and 14 μM at 37 °C for 24 h. Post treatment, as the cells start to slough off, 25 μl of cell culture was loaded to glass slides. Thereafter, 1 μl of AO (100 μg/ml) and EB (100 μg/ml) staining solution was added to each slide followed by covering with coverslips. Finally, these slides were placed under a fluorescence microscope (Nikon Inc., Japan) for investigation of apoptotic effects. For DAPI staining assay, cells were cultured and treated with irigenin same as for AO/EB staining assay. Following irigenin treatment, cells were washed with PBS and then fixed within 10% of formaldehyde. Post fixation cells were again washed with PBS and then stained with DAPI (1 μg/ml) for 25 min in dark. Finally, DAPI stained cells were loaded to a fluorescence microscope (Nikon Inc., Japan) for apoptosis assessment. Annexin V/PI staining assays were performed in

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accordance with previously described procedures [12]. The experiments were performed in triplicate.

Flow cytometry

Flow cytometry was performed to check the effects of irigenin on different cell cycle check points in HepG2 and SNU-182 liver cancer cells [8]. Both the cancer cell lines at a density of 1×10^4 cells/well were incubated for 12 h prior to their exposure with variant irigenin doses (0–320 μM) for a time period of 24 h. Post irigenin treatment, cells were subjected to washing with PBS three times followed by PI staining. Finally, the distribution of irigenin treated cells in different cell cycle phase was monitored by flow cytometry using a FACS flow cytometer (BD FACSCalibur, BD Biosciences, USA). The experiments were performed in triplicate.

Western blotting

Western blotting was performed to examine the expression of proteins in in irigenin treated HepG2 and SNU-182 liver cancer cells as described previously [8]. In brief, both the cell lines were treated with different irigenin doses (0 and 14 μM) for 48 h at a concentration of 1.5×10^4 cells/well of 24-well plate. Treated HepG2 and SNU-182 cells were then lysed incubating the cells with RIPA lysis buffer (Sigma) on ice for 20 min. Each lysate was subjected to BCA assay for protein quantification. About 40 μg of proteins from each lysate were separated using 10% SDS-PAGE separation followed by electrophoretic transference to nitrocellulose membranes (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). These membranes were blocked by non-fat milk followed by subjecting to primary antibody treatment overnight at 4 °C using antibodies against Bax (Cat# sc-20067), Bcl-2 (Cat# sc-517390), CDK1 (Cat# sc-53217), Cyclin-B (Cat# sc-166210), Cyclin-A (Cat# sc-239), Cyclin-D (Cat# sc-53271), Cyclin-E (Cat# sc-377100), Actin (Cat# sc-58673) (all 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, United States). Afterwards, membranes were then exposed to 1 mg/mL horse radish peroxidase-conjugated anti-rabbit secondary antibody (Cat# sc-2372) (1:10,000 dilutions, Santa Cruz Biotechnology, Inc., Dallas, United States). Finally, the protein signals were recorded with the help of ECL Detection kit (GE Healthcare Life Sciences, Sweden).

Statistical analysis

One-Way Analysis of Variance was used to carry out statistical analysis and the significance level was tested at $p < 0.05$. All the data from independent triplicate experiments was expressed as mean ± SD. GraphPad Prism software (version 7; GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis.
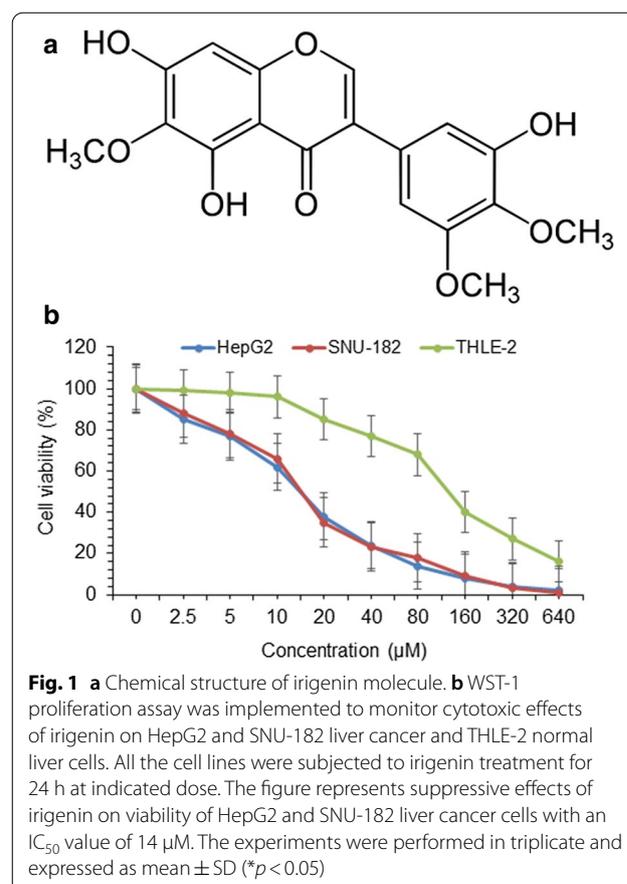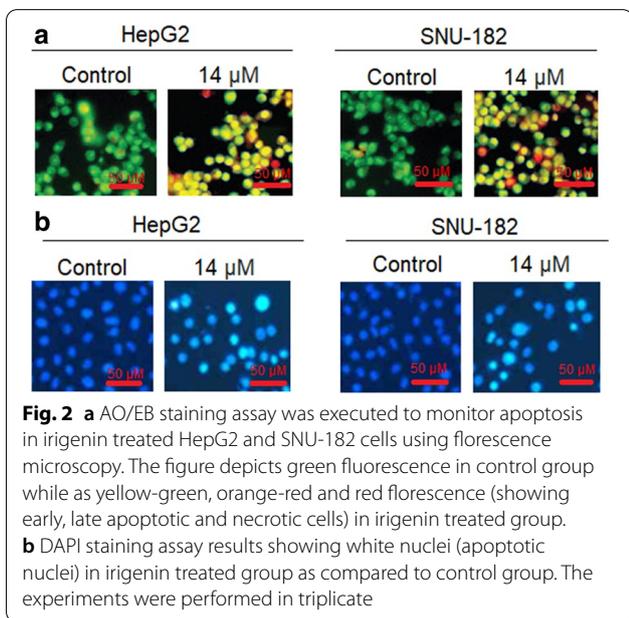
Results

Irigenin inhibited proliferation of HepG2 and SNU-182 liver cancer cells

The cytotoxic effects of irigenin (Fig. 1a) were investigated against HepG2 and SNU-182 liver cancer and THLE-2 normal liver cells using WST-1 assay. The results showed a significant ($p < 0.05$) decrease of HepG2 and SNU-182 cell proliferation upon treatment with different concentrations of irigenin (Fig. 1b). Irigenin induced dose-dependent cytotoxicity against HepG2 and SNU-182 cells and exhibited an IC_{50} of 14 μM. Nonetheless, the cytotoxic effects of irigenin against the normal THLE-2 were found to be comparatively less as evident from an IC_{50} of 120 μM.

Irigenin induced apoptotic cell death in HepG2 and SNU-182 liver cancer cells

Herein, we investigated the proapoptotic related antiproliferative effects of irigenin against HepG2 and SNU-182
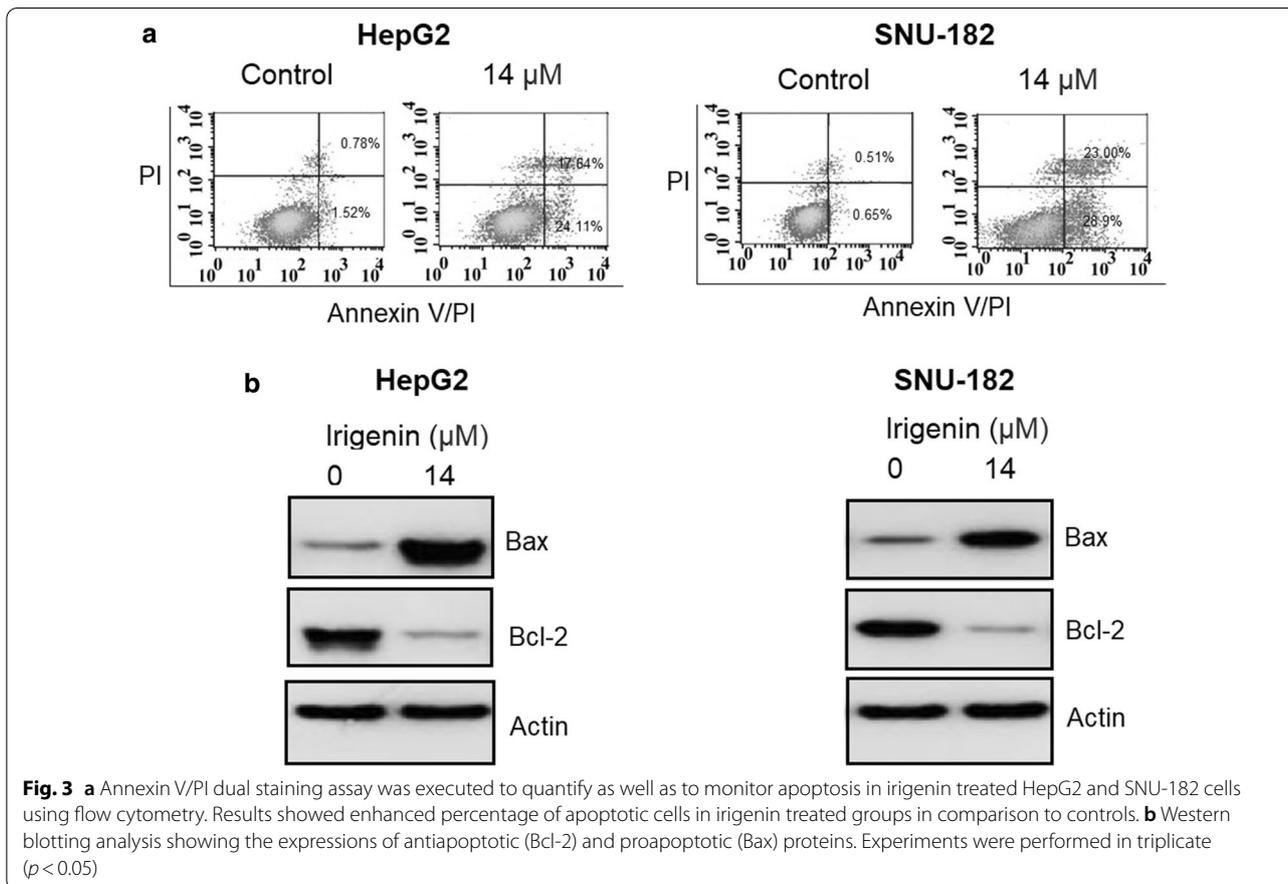

Fig. 1 **a** Chemical structure of irigenin molecule. **b** WST-1 proliferation assay was implemented to monitor cytotoxic effects of irigenin on HepG2 and SNU-182 liver cancer and THLE-2 normal liver cells. All the cell lines were subjected to irigenin treatment for 24 h at indicated dose. The figure represents suppressive effects of irigenin on viability of HepG2 and SNU-182 liver cancer cells with an IC_{50} value of 14 μM. The experiments were performed in triplicate and expressed as mean ± SD (*$p < 0.05$)

Fig. 2 a AO/EB staining assay was executed to monitor apoptosis in irigenin treated HepG2 and SNU-182 cells using florescence microscopy. The figure depicts green fluorescence in control group while as yellow-green, orange-red and red florescence (showing early, late apoptotic and necrotic cells) in irigenin treated group. **b** DAPI staining assay results showing white nuclei (apoptotic nuclei) in irigenin treated group as compared to control group. The experiments were performed in triplicate

cells. AO/EB staining results showed that irigenin induced apoptosis in HepG2 and SNU-182 cell lines in a dose-reliant manner. In control group only green fluorescence could be seen, marking normal cells, while in treated group yellow-green, orange-red, and red florescence was observed, marking apoptotic (early and late stage) and necrotic cells (Fig. 2a). Further, DAPI staining showed white colored nuclei in irigenin treated group as compared to control group indicative of apoptotic cell death in HepG2 and SNU-182 cells (Fig. 2b). Furthermore, annexin V/PI dual staining revealed a significant ($p < 0.05$) increase in the percentage of the apoptotic HepG2 and SNU-182 cells. It was found that the number of apoptotic HepG2 and SNU-182 cells enhanced from 2.3 to 41.75% and 1.16 to 51.9% at IC_{50}, respectively (Fig. 3a). The antiproliferative effects of irigenin were further supported by western blotting. Results detected remarkable increase in the expression of Bax proteins and decrease in expression of Bcl-2 (Fig. 3b). Therefore, it may be concluded that antiproliferative effects of irigenin are mediated via mitochondrial apoptosis in HepG2 and SNU-182 cancerous liver cells.

Irigenin induced cell cycle arrest in HepG2 and SNU-182 liver cancer cells

The effects of irigenin on cell cycle progression were assessed by flow cytometric analysis. After exposure of HepG2 and SNU-182 cells to different irigenin doses

Fig. 3 a Annexin V/PI dual staining assay was executed to quantify as well as to monitor apoptosis in irigenin treated HepG2 and SNU-182 cells using flow cytometry. Results showed enhanced percentage of apoptotic cells in irigenin treated groups in comparison to controls. **b** Western blotting analysis showing the expressions of antiapoptotic (Bcl-2) and proapoptotic (Bax) proteins. Experiments were performed in triplicate ($p < 0.05$)

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(0–320 μM), staining with PI was performed prior to flow cytometric assessment. Results showed that the percentage of G2/M phase HepG2 and SNU-182 cells increased from 17.92 to 34.35% and 23.97 to 38.23% at IC$_{50}$, respectively (Fig. 4a). These results indicated that irigenin inhibited the cell cycle progression at G2/M phase. Moreover, western blotting showed that irigenin could potentially downregulate the expressions of important cell cycle associated proteins including CDK1 and Cyclin-B in HepG2 and SNU-182 cells (Fig. 4b). The expression of Cyclin A, D and E remained apparently unchanged.

Discussion

Unfortunately, there is an alarming increase in the number of liver cancer incidences and deaths, globally. Despite of improvements have been made in diagnosis, monitoring and treatment, it remains one of the biggest challenges for researchers. Growing alcohol abuse and unprecedented upsurge of obesity in developing as well as developed countries is an issue of major concern in growing global liver cancer burden [21]. The presently accessible treatment options of liver cancer possess potential shortcoming like disease relapse, lethal side effects and poor survival rates, except being opted at early stages. Therefore, there is a scarcity of standard and capable chemopreventives which can produce efficient results against liver cancer. The current investigation was instigated to unveil the anticancer effects of irigenin isoflavonoid against liver cancer. Irigenin molecule has been reported of remarkable biological and pharmacological potential [2]. Irigenin has been earlier reported of strong antiproliferative effects against several human cancer cell including prostate cancer, gastric cancer and breast cancer [1, 16, 24]. It was reported that irigenin has a remarkable tendency to target key survival signalling pathways in cancer cells like apoptosis. Herein, results showed that irigenin could potentially inhibit the proliferation of both HepG2 and SNU-182 cell lines in a concentration-reliant manner. It showed an IC$_{50}$ value of 14 μM against both the target cancer cell lines. The similar trend of cell viability reduction in both HepG2 and SNU-182 cells could be due to their similar origin (hepatocellular carcinoma
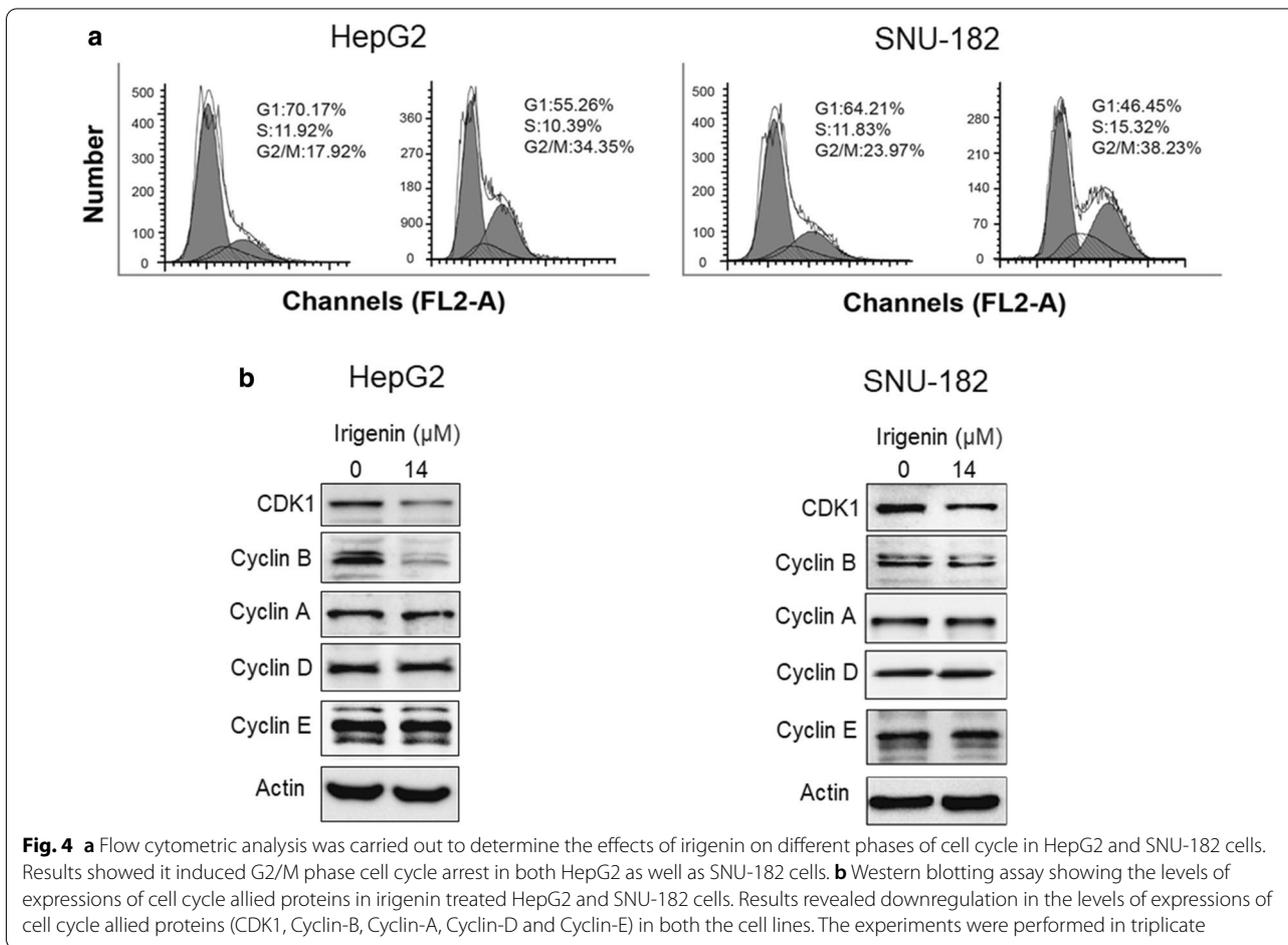

Fig. 4 **a** Flow cytometric analysis was carried out to determine the effects of irigenin on different phases of cell cycle in HepG2 and SNU-182 cells. Results showed it induced G2/M phase cell cycle arrest in both HepG2 as well as SNU-182 cells. **b** Western blotting assay showing the levels of expressions of cell cycle allied proteins in irigenin treated HepG2 and SNU-182 cells. Results revealed downregulation in the levels of expressions of cell cycle allied proteins (CDK1, Cyclin-B, Cyclin-A, Cyclin-D and Cyclin-E) in both the cell lines. The experiments were performed in triplicate

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cell lines). Moreover, the antiproliferative effects of irigenin on the normal THLE-2 cells was comparatively lower as evident from a very high IC_{50} (120 µM). Nonetheless, irigenin at higher doses causes drastic reduction in the viability of the normal cells as well. This could be due to several reasons, for instances, at higher concentrations irigenin might interfere with several cellular enzymes leading to increased cytotoxicity. It is noteworthy, that the IC_{50} of irigenin against the liver is around 9 times lower than the normal cells and as such irigenin should be considered as an important lead molecule against liver cancer. Next, efforts were made to unveil the underlying mechanism of action of anti-liver cancer effects of irigenin. Apoptosis is one of the leading targets of anticancer drugs and occurs naturally in multicellular organisms to discard damaged, aged, and malfunctioning cells [5]. Apoptosis gets stimulated by intrinsic (mitochondrial mediated) and extrinsic (death receptors) factors. Irigenin has been previously reported of substantial proapoptotic effects against gastric cancer cells through upregulation of PARP, Caspase-3, -8 and -9, and Bax activities [20]. Herein, the apoptotic effects of irigenin against HepG2 and SNU-182 cells were modulated by increase in Bax expression levels and decrease in Bcl-2 expression levels. These results were similar to previously reported for irigenin against gastric cancer cells and breast cancer cells [20, 24]. Therefore, irigenin induced cytotoxic effects in HepG2 and SNU-182 cell lines may be attributed to its proapoptotic effects.

Further, uncontrolled, random and frequent cell division is one of the primary features of malignant cancers [13]. Cell division commences through different check points (S, G0/G1 and G2/M) during its course of completion [14]. Chemopreventive drugs target these check points and block the progression of cell cycle at any of the stages. Irigenin has been reported of remarkable potential to inhibit the cell cycle progression in different human cancer cell lines including PC3, RWPE-1 and LNCaP prostate cancer cells at G1-phase [16]. Herein, irigenin induced G_2/M phase cell cycle arrest of both HepG2 and SNU-182 cell lines via downregulation of CDK1 and Cyclin-B. The CDK1 and cyclin B are the main regulates of G_2/M check point [14]. Consistently, irigenin inhibits the expression of CDK1 and cyclin B to trigger G_2/M cell cycle arrest. In contrary, Cyclin-A, Cyclin-D and Cyclin-E play roles in other phases of the cell lines and as such their expression remains more or less unaltered. Therefore, the property of inducing cell cycle arrest by irigenin is parallel to previously reported data where irigenin drug induced cell cycle arrest by modulating the expressions of key proteins in prostate cancer cells [16]. Taken together, the results

of this investigation indicate that irigenin could potentially suppress human liver cancer. The anti-liver cancer potency of irigenin was observed to mediate via induction of mitochondrial apoptosis and cell cycle arrest. Therefore, irigenin may prove a lead drug in liver cancer management and research.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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