Pharmacogn. Mag.
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ORIGINAL ARTICLE

Withaferin A Attenuates Epithelial-Mesenchymal Transition and Cancer Stem Cells Properties in Hepatocellular Carcinoma Cells by Inhibiting the PI3K/AKT Pathway through miR-200c

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Submitted: 16-Feb-2022 Revised: 26-Apr-2022 Accepted: 23-Aug-2022 Published: 23-Nov-2022

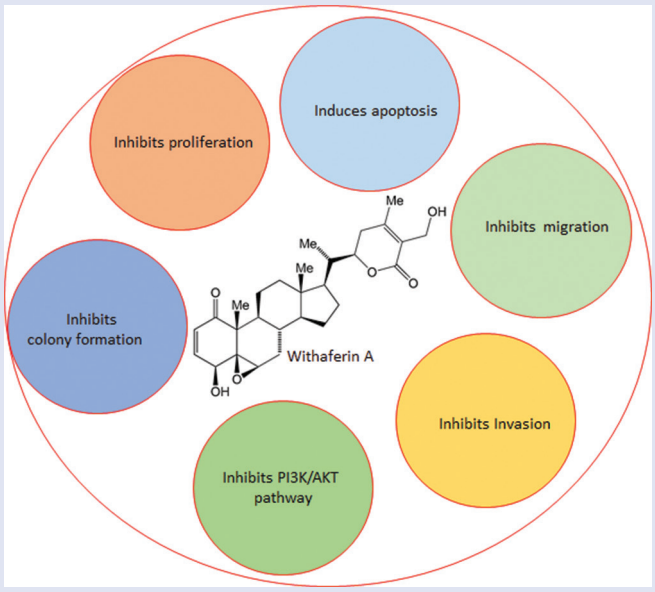
ABSTRACT

Background: Withaferin A is a triterpenoid steroidal lactone and is commonly isolated from *Withania somnifera*. It has been shown to have tremendous pharmacological potential. **Objectives:** The present study was aimed at elucidating the mechanism of action of withaferin A against hepatocellular carcinoma (HCC) cells via the PI3K/AKT signaling pathway. **Materials and Methods:** MTT (tetrazolium dye) Assay was performed for the relative assessment of the viabilities of cell lines. The effect of withaferin A on the proliferative capability of HCC cells was analyzed using the EdU staining assay, and the colony-forming potential was assessed with the help of a clonogenic assay. Cell apoptosis was estimated through (Modified Annexin V/Propidium Iodide Apoptosis Assay) staining followed by flow cytometry. Transwell assays were carried out for estimating the migration and invasion of cancer cells. The qRT-PCR and western blotting, respectively, were used for gene and protein expression studies. **Results:** Withaferin A selectively inhibited the viability of HCC cells although the viability of normal liver cells was minimally affected. The IC_{50} of withaferin A against the HepG2 cells was found to be 12 μM as against 150 μM for normal THLE-2 cells. The treatment with withaferin A significantly minimized the proliferation and colony-forming potential of cancer cells by inducing cell apoptosis. The percentage of apoptosis increased from 3.8% in control to 20.3% at 24 μM withaferin A. The cancer cell migration and invasion were significantly declined by withaferin A together with the inhibition of Epithelial to mesenchymal transition (EMT) of HCC cells. The withaferin A treatment decreased the expression of carcinoma cell markers CD44, CD90, and EpCAM. The expression of miR-200c markedly increased under withaferin A treatment and the latter was shown to exert its anti-cancer effects through miR-200c-mediated inhibition of the PI3K/AKT signaling pathway in HCC cells. **Conclusion:** In conclusion, withaferin A modulated the expression of miR-200c in HCC cells to inhibit the PI3K/AKT pathway and restricted the cancer cell EMT together with the inhibition of *in vitro* cancer cell growth and viability via induction of apoptosis.

Key words: Anticancer, apoptosis, hepatocellular carcinoma, proliferation, withaferin A

SUMMARY

- Withaferin A inhibits the proliferation of HCC cells via induction of apoptosis.
- Withaferin A restricts migratory rate, invasiveness, and epithelial to mesenchymal transition of HCC cells.
- Withaferin A inhibits the expression of hepatocellular cancer stem cell markers.
- Withaferin A exerts its anti-cancer effects against HCC cells via the miR-200c/PI3K/AKT signaling pathway.



Abbreviations used: FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

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DOI: 10.4103/pm.pm_86_22

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most frequent neoplastic disorder and of note the most dominant type of primary liver cancer, which is responsible for a great proportion of cancer-related deaths, worldwide.[1] HCC is the most common form of liver cancer and accounts for ~90% of cases and is the third most lethal type of human cancer. Hepatitis B virus (HBV) infection is the most prominent risk factor for HCC development, accounting for ~50% of cases.[2] At the global level, preventive measures are being conducted with the purpose to reduce the risk of fresh infection together with the screening of people

Cite this article as: Tian H. Withaferin a attenuates epithelial-mesenchymal transition and cancer stem cells properties in hepatocellular carcinoma cells by inhibiting the PI3K/AKT pathway through miR-200c. Phcog Mag 2022;18:1190-5.

at risk using ultrasound half-yearly to ensure the diagnosis at earlier stages.[3] Despite such worldwide surveillance programs, most tumors are diagnosed with intermediate or advanced disease stages where only palliative treatment measures can be applied.[4] In such cases, the patients have a median survival below 10 months without treatment.[5] The treatment procedure currently employed against HCC includes surgical resection, liver transplantation, and ablation followed by the application of radio or chemotherapies.[4] However, the clinical results are not much soothing and thus demand exploration of better therapeutic measures against the HCC. According to recent reports, around 60% of the currently used anticancer medication is supplied by natural resources, and a number of plant-based chemical compounds such as curcumin, resveratrol, lycopene, and caffeine have been shown to be effective in the treatment of HCC.[6] Considering this, the present study was conducted to unravel the effects of a phytochemical, withaferin A against the HCC cells *in vitro*. Withaferin A belongs to a group of 28-carbon triterpenoid steroidal lactones and is commonly isolated from *Withania somnifera*.[7] *W. somnifera*, commonly called Indian Ginseng or Indian winter cherry is highly valued in Indian traditional medicine and is known to exhibit health benefits in terms of its antioxidant, anti-inflammatory, and anti-depressant properties.[8-10] The anti-cancer potency of withaferin A has been established against several types of human cancers through both *in vitro* and *in vivo* experimentation, wherein it was shown to exert pleiotropic therapeutic effects on the growth and proliferation of cancer cells.[11] The efficacy of withaferin A has also been deduced against HCC in one of the recent studies and it was shown to induce apoptosis and autophagy in HCC cells.[12] The current study represents an elaboration of the previously established role of withaferin A against HCC. Herein, the HCC cell growth and viability were significantly declined by withaferin A treatment through induction of apoptotic cell death. The treatment with withaferin A further exhibited anti-metastatic effects against the HCC cells. The results showed that the anti-cancer effects of withaferin A were shown to be exerted via the miR-200c/PI3K/AKT molecular axis.

MATERIALS AND METHODS

In vitro propagation of cell lines and cell transfection

HepG2 HCC cell line and THLE-2 normal hepatocytes were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were mycoplasma infection-free as assessed by the mycoplasma stain assay kit (Beyotime, Haimeng, China). Both types of cells were cultured using Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) and maintained at 37°C with 5% CO_2 using a humidified incubator. For cell propagation, DMEM was incremented with fetal bovine serum (FBS; Gibco, 10%), streptomycin (100 μg/mL), and penicillin (100 U/mL, both from Gibco, Gaithersburg, MD).

For transfection, HepG2 cancer cells were initially cultured in 12-well plates at 37°C for 24 h. With the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and following the manufacturer guidelines, the cells were transfected with miR-200c mimics (RiboBio, Guangzhou, China) or its negative control. The transfection of HepG2 cancer cells with miR-200c mimics was meant for the overexpression of miR-200c.

MTT viability and EdU staining assays

To evaluate the antiproliferative effects of withaferin A treatment and miR-200c overexpression, an MTT assay was performed. Differentially treated withaferin A HepG2 and THLE-2 cells and HepG2 cells overexpressing miR-200c along with the corresponding negative control cells were plated in 96-well plates at a cell density of 5×10^4 cells per well and grown for different periods at 37°C. Afterward, the cells were inoculated with 40 μL of MTT (3 [4,5 dimethyl-2-thiazolyl] 2,5 diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO) and incubated additionally for 4 h at 37°C. Next, 150 μL of DMSO per well was added to dissolve the crystals of formazan. Lastly, the absorbance was read at 570 nm wavelength using a microplate reader to analyze the cell viability.

The EdU staining assay was performed to assess the proliferative viability of HepG2 cancer cells variedly treated with withaferin A (0–18 μM) for 24 h. The assay was carried out with the help of an EdU staining kit (Roche, Mannheim, Germany). HepG2 cancer cells were cultured in the EdU solution for 5 h and fixed using 70% ethanol. This was followed by permeabilization of cancer cells by treating them with PBS (Phosphate buffered saline) containing 0.5% Triton X-100 (PBS-T). Then, the cells were co-incubated with the Apollo staining solution. The nuclei of cancer cells were stained using DAPI (4',6-diamidino-2-phenylindole). A fluorescent microscope was used for the visualization of EdU-positive cells.

Clonogenic assay

Approximately, 3,000 HepG2 cells initially treated with withaferin A (0–18 μM) for 24 h were placed into 6-well plates (SPL Life Sciences). The cells were allowed to form colonies by incubating them at 37°C for 2 weeks. The fixation of the resulting colonies was carried out using 4% paraformaldehyde and then stained with crystal violet (0.25% w/v) for 20 min at 25°C. The wells were photographed, and the colonies were manually counted to analyze the relative percentage of colony formation.

Annexin V-FITC/PI apoptosis assay

The quantitative assessment of HepG2 cancer cell apoptosis was made through annexin V/propidium iodide (PI) assay. Briefly, the HepG2 cells were incubated with 0, 6, 12, or 18 μM withaferin A and cultured for 24 h at 37°C in 12-well plates with an inoculum density of 2.5×10^4 cells per well. The samples were then centrifuged for 5 min at 1500 rpm and the cells were stained using an Annexin V–FITC/PI staining assay kit (Roche) following the manufacturer's instructions. Post-staining, the cells were incubated for 20 min in dark at room temperature. The cell samples were analyzed by flow cytometry (MACS Quant 10; Miltenyi Biotec, GmbH, Germany) and the percent cell apoptosis was determined using the FlowJo software (Tree Star, San Carlos, CA).

Analysis of migration and invasion

The transwell chambers (Corning) without or with Matrigel coating (BD Biosciences) were used to respectively determine the *in vitro* migration and invasion of withaferin A-treated (0-18 μM) HepG2 cancer cells. For the analysis of cell migration, 2×10^5 cells were seeded into the upper chambers of the transwell inserts without Matrigel coating, whereas for invasion analysis, 4×10^5 cells were plated in the upper chambers of transwell inserts with Matrigel coating. After 24 h incubation at 37°C, the cancer cells unable to cross the basement membrane were swabbed away with cotton, whereas those migrating/invading the membrane were ethanol fixed, stained with 0.2% crystal violet, visualized, and manually counted under a light microscope (Olympus, Tokyo, Japan).

Western blotting

Total protein extraction was performed from HepG2 cancer cells differentially treated with withaferin A or transfected with miR-200c along with the corresponding negative control HepG2 cells, after their digestion with radioimmunoprecipitation assay buffer (RIPA, Sigma-Aldrich). Extracted proteins were subjected to electrophoresis on 8–10% sodium

dodecyl sulfate-polyacrylamide gels. Following their resolution, the gel contents were transferred to nitrocellulose filter membranes. Then, the membranes were exposed overnight to specific primary antibodies (1:1000; Cell Signaling Technology) at 4°C. Afterward, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology) at room temperature for 3 h. Finally, the specific protein bands were detected using a FluroChem E system (Protein Simple, Silicon Valley) and their intensities were quantified. β-actin was used as a loading control.

Total RNA isolation and gene expression analysis

The extraction of total RNA from HepG2 and THLE-2 cells was isolated with the help of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Nanodrop (Thermo Scientific Fisher, Waltham, MA, USA) was used to quantify the RNA extracted. Afterward, the RNA was passed through miRNEasy spin columns (Qiagen, Hilden, Germany) for its purification. Following its purification, the RNA was used to synthesize cDNA with the help of SuperScript Master Mix (Invitrogen) following the manufacturer's protocol. The qRT-PCR was performed using the SYBR Green qPCR Master Mix (Thermo Fisher Scientific) on QuantStudio 3.0 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The estimation of relative gene expression was made using the 2^{-ddCt} method. GAPDH was used as a reference gene in the expression studies. Three replicates were used for each experiment. The sequences of RT-PCR primers were GADPH: forward 5'-CACCGTCAAGGCTGAGAA-3'; reverse 5'–CCTTCTCCATGGTGGTGA-3', CD44: forward 5'-GAGC ATCGGATTTGAGAC- 3'; reverse 2 5'-CATACTGGGAGGTGTTGG-3', CD90: forward 5'-AGGAGAAACAGGAAACCT-3'; reverse 2 5'-CA GACACAGTCCAACTTC-3', EpCAM: forward 5'-AGGAGAAACA GGAAACCT-3'; reverse 2 5'- CAGACACAGTCCAACTTC-3' and miR-200c: forward 5'–TGTTGGTTGTTTGGTAGG-3'; reverse 2 5'-ACAACCTTTCCCAACCCA -3'.

Statistical analysis

The final values obtained from three independent experimental replicates were given as the mean ± standard deviation (SD). Student's *t*-test was performed using the GraphPad Prism 7.0 software to analyze the significance level of the differences between treatment groups. The difference between the two values was considered to be statistically significant at $P < 0.05$.

RESULTS

Withaferin A inhibits the proliferation of hepatocellular carcinoma cells via induction of apoptosis

The HepG2 HCC cells and THLE-2 normal hepatocytes were incubated with different concentrations (0–160 μM) of withaferin A at 37°C for 24 h. The MTT assay was used to analyze the effect of withaferin A treatment on the viability of HepG2 and THLE-2 cells. Withaferin A inhibited the viability of HepG2 cancer cells in a dose-dependent fashion with an estimated IC_{50} of 12 μM [Figure 1a]. Although the viability of THLE-2 cells was also negatively affected by withaferin A, the decline in cell proliferation was insignificant compared to that of HCC cells with a much higher IC_{50} value of 150 μM. The antiproliferative effects of withaferin A against the HepG2 cancer cells were also observed in terms of a dose-dependent decline in the percentage of EdU-positive cells and a reduction of their colony-forming potential [Figure 1b and c]. To study whether the loss of HCC cell viability resulted because of induction of apoptosis, the HepG2 cancer cells were treated with 0, 6, 12, or 18 μM of withaferin A for 24 h, and their relative apoptotic levels were determined. The level of cancer cells apoptosis was shown to be increasing proportionally with the withaferin A dose applied and reached 18.80% at 18 μM concentration with 21.60% of necrotic cells, which were significantly higher than those of untreated HepG2 cells [Figure 1d]. Thus, it is evident that withaferin A induced apoptosis in HCC cells to inhibit their proliferation.

Withaferin A restricts migratory rate, invasiveness, and epithelial to mesenchymal transition of hepatocellular carcinoma cells

Transwell assays were performed to investigate the effect of the withaferin A treatment (0–18 μM) on the migration of HepG2 cancer cells *in vitro*. With the increasing doses of withaferin A, the migration of cancer cells decreased and was less than one-third of the untreated/control cancer cells [Figure 2a]. The effect of the withaferin A *in vitro* treatment on the invasion of HepG2 cells was very much similar to that on their migration and inhibited the cancer cell invasiveness in a dose-dependent manner [Figure 2b]. Moreover, the western blot analysis was used for assessing the effect of withaferin A on epithelial (E-cadherin) and mesenchymal (N-cadherin, Vimentin, and Snail) marker proteins. The withaferin A treatment was shown to significantly increase the expression of E-cadherin, whereas the expression of mesenchymal marker proteins decreased in a dose-dependent manner by withaferin A [Figure 2c]. Withaferin A, therefore, exhibits anti-metastatic potential against HCC cells in terms of inhibition of migration, invasion, and epithelial to mesenchymal transition *in vitro*.

Withaferin A inhibits the expression of hepatocellular cancer stem cell markers

The anti-cancer effects of Withaferin A on HCC cells were also analyzed by performing the expression analysis of carcinogenic or cancer stem cell markers, CD44, CD90, and EpCAM from differentially treated (0–18 μM of Withaferin A) HepG2 cancer cells. Expression of all these marker genes was found to decrease with increasing withaferin A doses [Figure 3a-3c]. This suggests that treatment with withaferin A might restrict the tumorigenesis of HCC by restricting the proliferative capability of HCC stem cells but this needs to be confirmed using *in vivo* animal systems.

Withaferin A exerts its anti-cancer effects against hepatocellular carcinoma cells via miR-200c/PI3K/AKT signaling pathway

MicroRNA-200c (miR-200c) acts as a tumor suppressor in HCC. As expected, the HepG2 cancer cells were shown to exhibit significantly lower miR-200c expression levels in comparison to normal hepatocytes [Figure 4a]. Surprisingly, the treatment of HepG2 cancer cells with 12 μM of withaferin A significantly enhanced miR-200c expression [Figure 4b]. To confirm whether withaferin A inhibited HCC via up-regulation of miR-200c, miR-200c was overexpressed in HepG2 cancer cells [Figure 4c]. The MTT assay showed that miR-200c overexpression significantly inhibited the proliferation of HepG2 cancer cells when analyzed at different culture durations [Figure 4d]. The results of western blot analyses revealed that the PI3K/AKT signaling pathway was inhibited both under miR-200c overexpression [Figure 4e] and withaferin A treatment [Figure 4f]. Together, the results signify that withaferin A enhances the expression of miR-200c expression in HCC cells and inhibits the PI3K/AKT signaling pathway via miR-200c upregulation and thereby the *in vitro* HCC cell growth and viability.

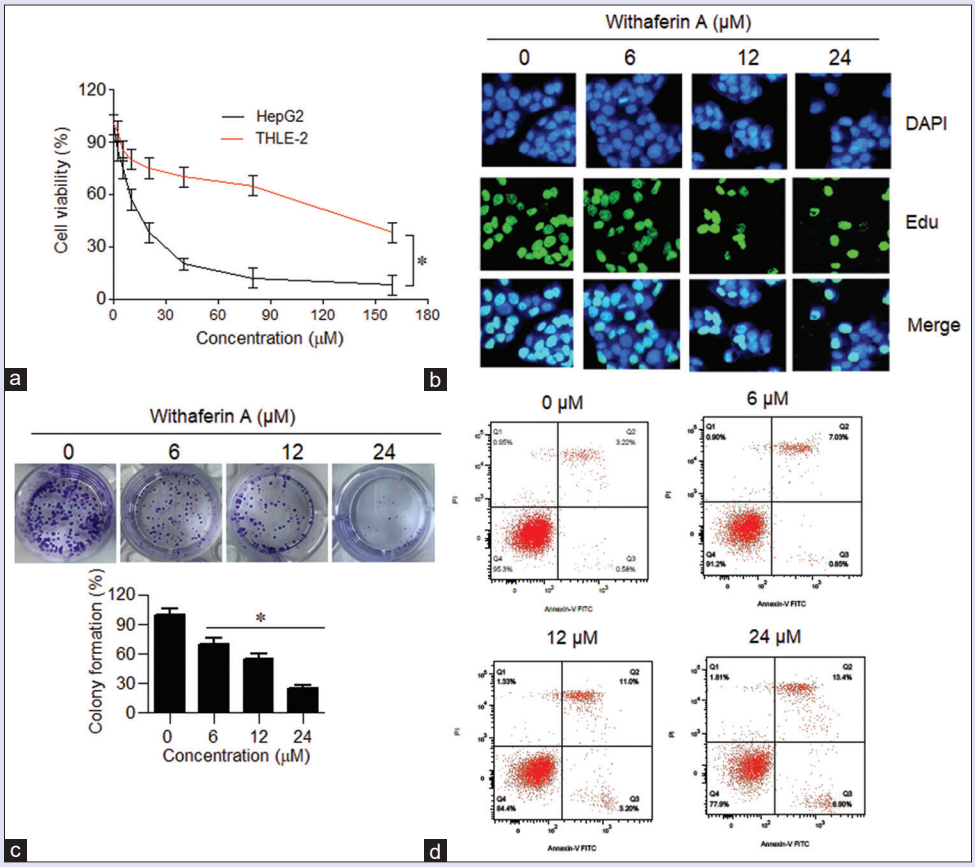

Figure 1: Withaferin A inhibits hepatocellular carcinoma cell proliferation via apoptosis. (a) Analysis of the effect of withaferin A treatment on HepG2 hepatocellular carcinoma and THLE-2 normal liver cells by MTT assay (b) EdU staining assay of HepG2 cancer cells treated with different doses of withaferin A (c) Assessment of the effect of withaferin A treatment (0–18 μM) on the colony-forming potential of HepG2 cells (d) Analysis of the relative percentage of apoptosis of HepG2 cells under varied doses of withaferin A. Experiments were performed using at least three replicates (*$P < 0.05$)

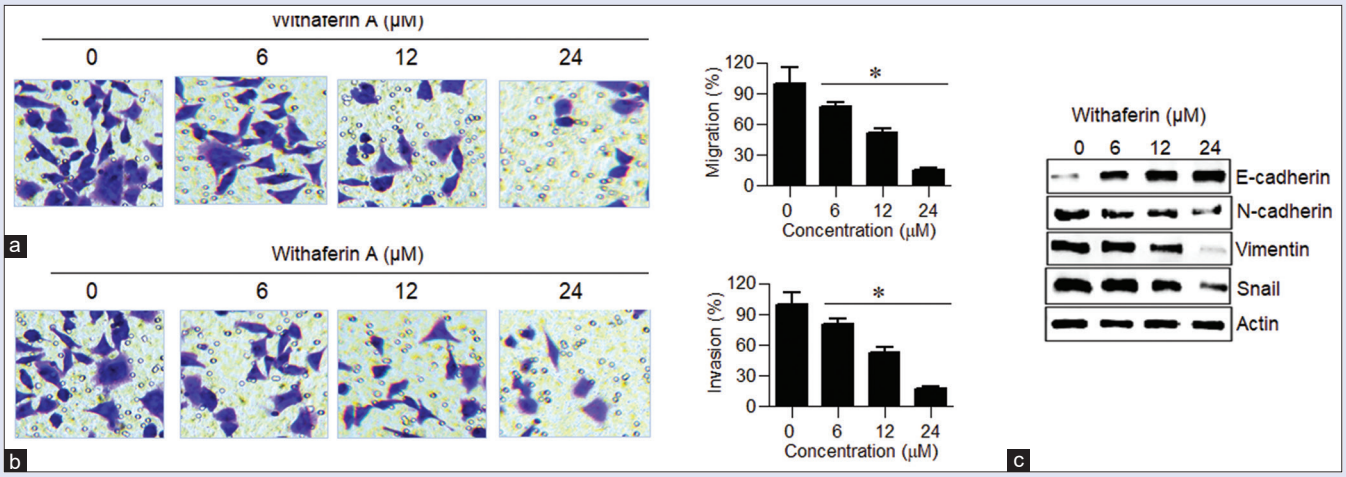

Figure 2: Withaferin A reduces hepatocellular carcinoma cell motility and inhibits EMT. (a) Transwell migration assay of HepG2 cancer cells variably treated with withaferin A (b) Transwell invasion assay of HepG2 cancer cells variably treated with withaferin A (c) Western blot analysis of EMT marker proteins from HepG2 cancer cells variably treated with withaferin A. Experiments were performed using at least three replicates (*$P < 0.05$)

DISCUSSION

The plant-based natural compounds represent an ideal source of disease preventive and curative therapeutic agents and serve in anti-cancer drug discovery.[13] The therapeutic applicability of phyto-bioactive compounds needs assessment of treatment effects and a precise understanding of the mechanism of their action.[14] Cancer cells evade the pathways of programmed cell death or apoptosis, and the restoration of the latter is regarded as one of the major therapeutic approaches against cancer and is an area of highly active research in the present

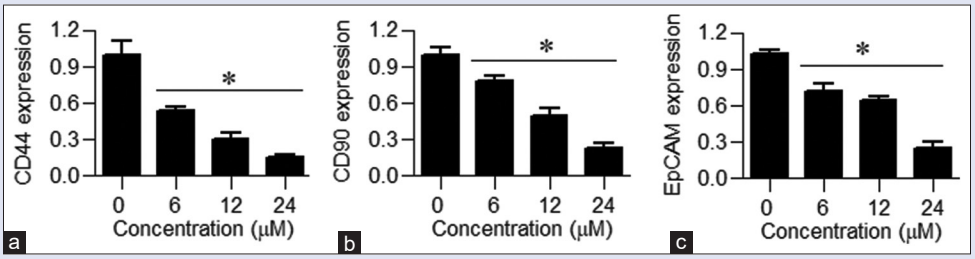

Figure 3: Withaferin A represses the expression of hepatocellular carcinoma stem cell markers. qRT-PCR expression analysis of relative expression levels of (a) CD44 (b) CD90, and (c) EpCAM from HepG2 cancer cells treated with different concentrations of withaferin A. The expression analyses were carried out using at least three replicates (*$P < 0.05$)

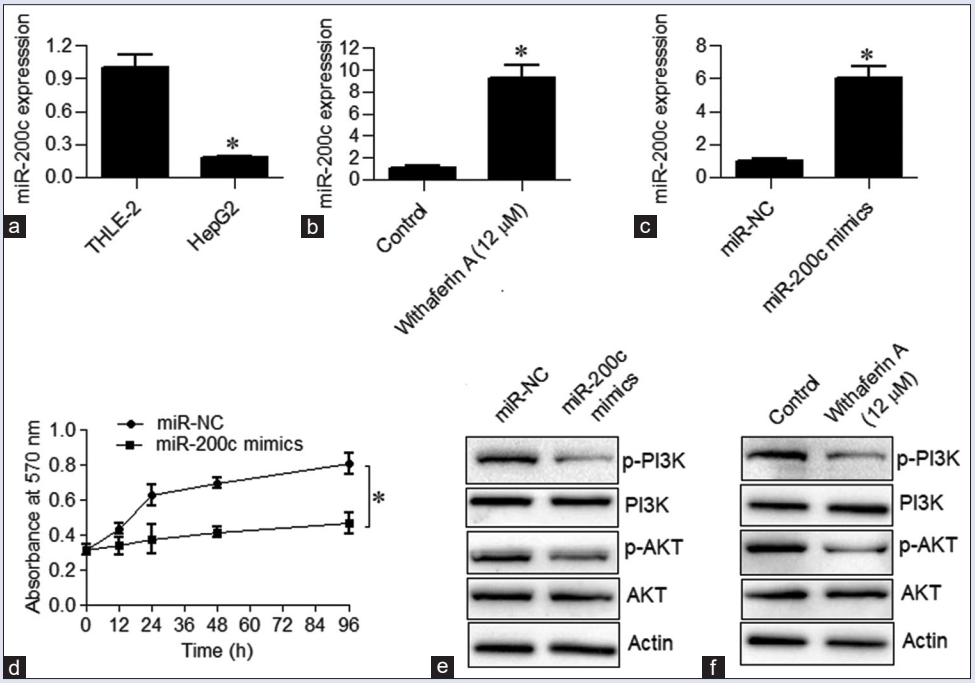

Figure 4: Withaferin A targets the PI3K/AKT signaling pathway in hepatocellular carcinoma cells via miR-200c up-regulation. (a) Analysis of expression of miR-200c from HepG2 hepatocellular carcinoma and THLE-2 normal liver cells by qRT-PCR (b) Estimation of relative expression of miR-200c from HepG2 cancer cells treated with 12 μM withaferin A with reference to untreated HepG2 cells (c) Confirmation of miR-200c overexpression in miR-200c mimics transfected cells by qRT-PCR (d) MTT proliferation assay of HepG2 cancer cells transfected with miR-200c mimics or negative control miR-NC (e) Western blot analysis of signaling components of the PI3K/AKT pathway from HepG2 cancer cells transfected with miR-200c mimics or negative control miR-NC (f) Western blot analysis of signaling components of the PI3K/AKT pathway from HepG2 cancer cells administered with 0 or 12 μM withaferin A. Each experiment was performed using at least three replicates (*$P < 0.05$)

times. The plant-derived natural products have shown great potential to aid in cancer treatment in terms of their pro-apoptotic effects on cancer cells.[15] A number of research studies have disclosed that withaferin A exhibits cytotoxic properties against a variety of cancer cells and promotes cancer cell apoptosis.[16-18] The results of the present study were indicative of the antiproliferative action of withaferin A against the HCC cells. However, it showed little effect when administered against the normal liver cells. Previous studies have rather revealed withaferin A to exhibit hepatoprotective potential, which suggests its selective cytotoxic action against malignant liver cells.[19,20] In the present study, withaferin A-induced apoptosis in HCC cells limits their *in vitro* growth. Induction of apoptosis in HCC cells by withaferin A has also been reported earlier.[12] The migration and invasion of HCC cells were significantly restrained under withaferin A treatment. Withaferin A also inhibited the epithelial to mesenchymal transition (EMT) of cancer cells as evidenced by the expression of markers EMT. The findings are indicative of the anti-metastatic potential of withaferin A as has been already confirmed by contemporary researchers.[21,22] The attenuation of carcinogenic marker protein expression levels by withaferin A also provides insight into the decline in the proliferative and metastatic potential of HCC cells under withaferin A treatment. This also highlights the effectiveness of withaferin A to help in preventing the recurrence of HCC.[23] The *in vitro* treatment of HCC cells augmented the expression of miR-200c. MicroRNA-200c has been previously shown to exert tumor-suppressive effects in HCC.[24] Additionally, miR-200c has been shown to modulate the expression of the PI3/AKT signaling pathway to exert its growth inhibitory effects in non-small cell lung cancer.[25] The PI3K/AKT signaling pathway was significantly inhibited by both miR-200c overexpression and withaferin A treatment in HCC cells. The PI3K/AKT pathway is aberrantly activated in cancer cells and regulates their proliferation and survival and affects the tumor progression and response to cancer therapies.[26] The study through *in vitro* experimentation thus precisely established

that withaferin A targets the PI3K/AKT signaling pathway in HCC cells via miR-200c upregulation to exert its anti-cancer effects. However, the mechanism needs to be validated *in vivo* systems also.

CONCLUSION

Collectively, withaferin A significantly inhibited the proliferation of HCC via induction of apoptosis. Withaferin A also exhibited anti-metastatic potential against HCC cells and attenuated the expression of carcinogenic marker proteins. At the molecular level, withaferin A was found to exert its tumor-suppressive effects via the miR-200c/PI3K/AKT signaling pathway indicative of its therapeutic potential.

Acknowledgements

I acknowledge the funding support from the Youth Foundation of Zhongshan Hospital (No: 2018ZSQN33).

Financial support and sponsorship

This project is supported by Youth Foundation of Zhongshan Hospital (No: 2018ZSQN33).

Conflicts of interest

There are no conflicts of interest.

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