ORIGINAL ARTICLE

Cinnamolide sesquiterpene lactone suppresses *in vitro* and *in vivo* cancer cell growth in cisplatin-resistant human cervical carcinoma cells by inducing mitochondrial mediated apoptosis, caspase activation, loss of MMP and targeting Akt/β-Catenin signaling pathway

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Summary

Purpose: *This study was designed to examine the in vitro and in vivo antitumor effects of Cinnamolide against cisplatin-resistant human cervical cancer cells (HeLa cells).*

Methods: *Cell viability was examined by WST-1 cell viability assay. Cinnamolide-induced apoptosis was examined by fluorescent microscopy using acridine orange (AO) /ethidium bromide (EB) staining and flow cytometry in combination with annexin-V/propidium iodide (PI) staining. Western blot was used to study the effects of Cinnamolide on apoptosis-related protein expressions including Bax and Bcl-2 as well as to study effects on numerous caspases and Akt/β-Catenin signaling pathway. Effects on mitochondrial membrane potential (MMP) were evaluated by flow cytometry. In vivo studies using xenograft mouse model were carried out to evaluate the efficacy of Cinnamolide under in vivo conditions.*

Results: *Cinnamolide decreased the viability of the HeLa human cervical cancer cells and exhibited an IC$_{50}$ of 16.5 μM. The cytoxicity of Cinnamolide was also investigated on the MDCK normal cervical cells which showed that Cinnamolide exerted very low toxic effects on these cells. Cinnamolide also*

caused remarkable changes in the morphology of the HeLa cancer cells and suppressed their colony forming potential. The AO/EB staining showed that this molecule inhibits the viability of cancer cells via induction of apoptotic cell death which was associated with increase in Bax and decrease in Bcl-2 levels. The apoptotic cells increased from 3.5% in control to around 59% in HeLa cells at 50 μM concentration. Cinnamolide treatment also led to activation of caspase-3 and caspase-9. It was also seen that Cinnamolide treatment led to a significant and dose-dependent loss of MMP in HeLa cancer cells. It also significantly inhibited the Akt/β-catenin signalling pathway by reducing the levels of phosphorylated Akt and GSK-3β. The results also showed that Cinnamolide suppressed the tumor volume and the tumor weight of the xenografted tumors.

Conclusion: *The results of this study indicate that Cinnamolide natural product has the potential to be developed as a promising anticancer agent against human cervical carcinoma.*

Key words: *cinnamolide, cervical cancer, apoptosis, western blot, caspases, fluorescent microscopy*

Introduction

Numerous women are diagnosed with cervical cancer (CC) every year and its incidence is increasing at an alarming rate [1,2]. CC ranks 4th in incidence and 4th in mortality in women worldwide [3]. In 2012 alone, around 0.52 million new cases and 0.26 million CC deaths were recorded, account-

ing for around 8% of total deaths and new cancer cases in a single year [4]. CC shows geographical variations with higher prevalence (about 70%) and deaths (90%) in developing countries [5]. In economically weak countries CC still remains a dominant type of cancer while in developed countries CC rates have recently decreased due to extensive cervical screening programs (CSP) and general awareness [6,7]. There are three major risk factors of developing CC including long term HPV infection, smoking and oral contraceptives, while minor factors include multiple pregnancies and HIV infection [8-11]. CC is radiosensitive and hence radiotherapy can be used in a situation where surgery cannot be performed, while possibly better results are achieved by surgery [12]. Chemotherapy is also used for CC treatment with remarkable efficiency over conventional radiotherapy [13]. Despite the recent advancements in the treatment of cancer, CC treatment demands finding more effective drugs which will diminish disease recurrence [14-16]. Natural products, with their huge diversity and easy availability can offer a large number of potent anticancer drugs that can be used in systemic therapies [17,18]. Naturally occurring sesquiterpene lactones belong to a sub-class of sesquiterpenoids that poses a vast and potent pharmacological and biological activities including anti-feedants, antimicrobial, antiinflammatory, antimigraine, antitumor, treatment of skin infection, stomach-ache, cardiotonic, neurocytotoxic and antiulcer properties [19-24]. Cinnamolide, a naturally available sesquiterpene lactone, mostly isolated from Drimys winteri J.R. Forst, a plant used as folk medicine in many Latino American countries, is a pharmacologically active compound [25,26]. The current study on Cinnamolide was designed to unveil its *in vitro* and *in vivo* cancer suppressive effects on HeLa cisplatin-resistant human cervical carcinoma cells. Its anticancer effects were examined via mitochondrial mediated apoptosis, caspase activation, loss of MMP and targeting Akt/β-Catenin signaling pathway.

Methods

Estimation of cell proliferation rate

Cell viability estimations were performed via WST-1 proliferation assay using cytotoxicity assay kit (Beyotime Institute of Biotechnology, China), following the manufacturer's guidelines. In brief, cervical HeLa carcinoma cells and normal MDCK cells (Madin-Darby canine kidney), procured from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, were cultured onto 96-well plates at 37°C for 24 h in an incubator with 5% CO_2. Both cancer and normal cervical cells were harvested at 80% confluence and treated with Cinnamolide at variant

doses i.e. 0, 20, 40, 80 and 160 μM. Cinnamolide-treated cells were then exposed to WST-1 stock solution for nearly 2 h. Finally, a spectrophotometer (BioTek, United States) at 450 nm was used to obtain the optical density (OD) and hence cell viability was assessed.

Clonogenic assay for cell colony estimation

Human cervical HeLa carcinoma cells were harvested at the exponential phase of growth for clonogenic assay and numbered via hemocytometer. Harvested cells were placed in 24-well plates and exposed to Cinnamolide at different doses i.e. 0, 20, 80 and 160 μM for 48 h and incubated for attachment for 6 h. Cells were then washed with phosphate buffered saline (PBS) and cultured for one week at 37°C. Afterwards, cell colonies were collected and washed with PBS. Finally, cell colonies were fixed with methanol, followed by staining with crystal violet and numbered under light microscope.

AO/EB staining assay for apoptosis determination

Cervical HeLa carcinoma cells were placed in 96-well plates using RMPI-1604 culture medium at a final cell density of 2×10^5 cells per well. Afterwards, HeLa cells were treated with Cinnamolide at varying doses i.e. 0, 20, 80 and 160 μM for around 24 h. Thereafter, trypsin (20 μl) was put to each well and cells were let to cast off. Cell suspensions were loaded on glass slides (25 μl) and each cell suspension was treated with dual fluorescent solution AO and EB, with 100 μg/ml each (AO/EB, Sigma, St. Louis, MO). The glass slides were cover-slipped and apoptotic cell morphology was examined under a fluorescent microscope (Olympus, Japan).

Annexin V-FITC/PI staining to quantify apoptosis

To estimate the number of apoptotic cells, human cervical HeLa carcinoma cells were analyzed using annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech Co. Ltd. Najing, China). After treatment with different Cinnamolide doses i.e. 0, 20, 80 and 160 μM, each sample was stained with annexin V-FITC/PI (5 μl each) in the dark at room temperature for about 5 min. Finally, using Muse flow cytometry (Millipore, Billerica, MA, USA) each sample was analyzed to quantify the extent of apoptosis.

Assessment of mitochondrial membrane potential (MMP)

MMP assessment was made through flow cytometry using Rh-123 dye. Briefly, human cervical HeLa cells were harvested and exposed to varying Cinnamolide doses, i.e. 0, 20, 80 and 160 μM for 24 h. After drug treatment, RMPI-1604 culture medium was discarded and cells were sampled among different groups with 1,000 cells for each sample. Finally, MMP was estimated by supplementation of rhodamine fluorescent dye (Rh-123) to each sample and examined with flow cytometry.

Western blotting analysis

Cinnamolide-treated HeLa cells (0, 20, 80 and 160 μM) were washed with PBS and RIPA cell lysis buffer was used to lyse the cells for about 20 min. Forty μg of cell lysate were centrifuged and cleared for 5 min with sampling buffer 3×SDS through boiling. Afterwards,

7.5% SDS-PAGE protein extracts were resolved followed by transference to PVDF membranes. The membranes were incubated at 4°C overnight with mouse anti-Bax, anti-Bcl-2, anti-caspase-3 and 9, anti- Akt and GSK-3β antibodies at 1:1000 dilution of tris-buffered saline (TBS) comprising 0.1% tween-20 (TBST). Afterwards, the membranes were incubated at room temperature with alkaline phosphate goat anti-mouse antibody for about 2 h at 1:1000 dilution. Next, electroblotting of resolved proteins was performed onto Hybond nitrocellulose ECL membranes. Finally, the membranes were visualized under enhanced chemiluminescence and quantification of protein signals was performed by scanning densitometry via bio-image analysis software (Bio-Rad, USA).

In vivo studies

In vivo studies regarding the effects of Cinnamolide treatment on HeLa cells were carried out on xenografted models of Female BALB/c nude mice. Female BALB/c nude mice (4–6 weeks old, body weight 20–24 g) were obtained from the Experimental Animal Center of Affiliated Hospital of Chengde Medical University, China. The NIH standards for the care and use of laboratory animals were approved by the Ethics committee of the Department of Obstetrics, Affiliated Hospital of Chengde Medical University, Chengde, Hebei, China. The left flank of mice was subcutaneously injected with HeLa cells (5×10^6) and after nearly two weeks the tumour became superficially apparent (about 4 mm) and Cinnamolide treatment to the mice was initiated via intraperitoneal injection of Cinnamolide dissolved in 0.1% DMSO and diluted with 100 μL normal saline at 20 mg/kg, 40 mg/kg and 80 mg/kg per body weight. Administration of Cinnamolide was repeated thrice a week and in case of controls only normal saline with 0.1% DMSO was administered. Finally, to perform *in vivo* studies mice were sacrificed and live tumours were extracted and examined for tumour growth and other studies.

Statistics

The experiments were performed in triplicate and the values taken are mean±SD. P<0.05 was considered statistically significant. Student's t-test using Graphpad prism software was employed for statistical analyses.

Results

Selective cell proliferation inhibition by Cinnamolide

WST-1 cell cytotoxicity assay was performed to study the effects of Cinnamolide treatment on cell viability of normal MDCK and cancer HeLa cells. The results indicated significant suppression of cell viability of cancer HeLa cells. The viabil-
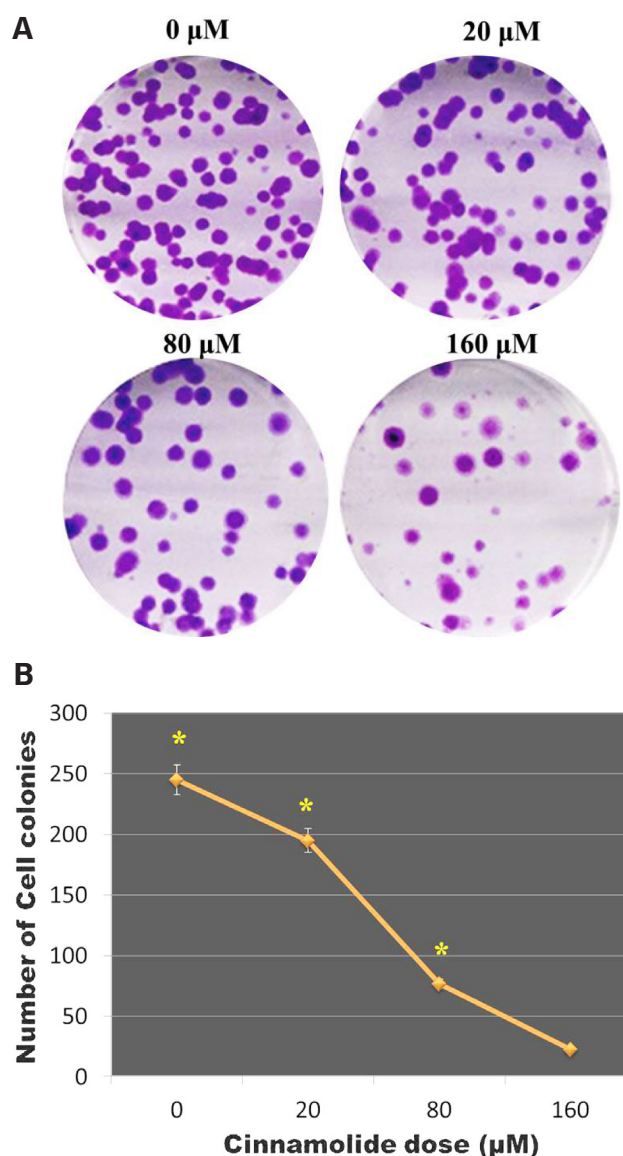
Figure 2. A: Clonogenic assay for determination of anti-colony potential of Cinnamolide in human cervical HeLa cancer cells. Cinnamolide treatment led to a significant and dose-dependent reduction in the number of cancer cell colonies. **B:** Graphical representation of clonogenic assay results at indicated doses of Cinnamolide. The Figure shows the decrease of cancer cell colonies after cinnamolide treatment. The values are the mean±SD of three experiments. *p<0.05.
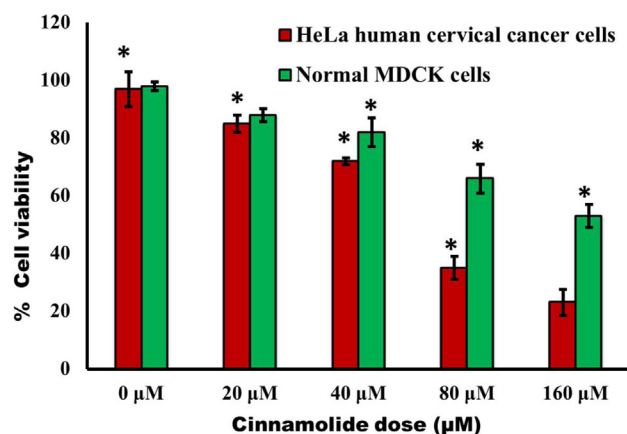

Figure 1. Cell viability of Cinnamolide treated human cervical HeLa cancer and MDCK normal cervical cells via WST-1 viability assay at indicated doses. *p<0.05.

ity of the control was 100% but at 40 µM of Cinnamolide the viability reduced to about 75% and at 160 µM the viability was suppressed to almost 20% (Figure 1). The cell viability of normal MDCK cells after drug exposure was not affected much, even at higher doses. Thus Cinnamolide treatment of HeLa cells resulted in concentration-dependent and selective cell viability suppression.

Effect of colony inhibition in HeLa cells by Cinnamolide

The effect of Cinnamolide treatment on colony formation was assessed via clonogenic assay and the results revealed significant inhibition of HeLa cell colonies (Figure 2A). In control, the number of cells was about 250 but after drug treatment it reduced to about 20 only, even at 160 µM dose (Figure 2B). Thus Cinnamolide treatment led to concentration-dependent suppression of HeLa cell colonies.

Apoptotic studies of Cinnamolide treatment on cervical HeLa cancer cells

Apoptotic studies of HeLa cells after Cinnamolide treatment were carried out through different assays. Firstly, HeLa cells were examined through AO/EB staining assay, which revealed significant rise in the number of apoptotic cells with increasing Cinnamolide doses. Yellow-green, orange and orange-red fluorescence signified early apoptotic, late apoptotic and necrotic cells, respectively (Figure 3). Next, quantification of apoptotic cell percentage was performed via annexin V-FITC/PI assay which showed substantial rise in the per-
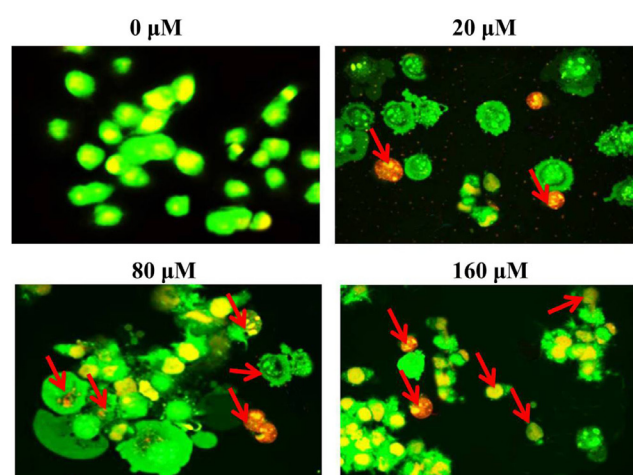
centage of apoptotic cells with increased Cinnamolide concentrations. In control, the number of apoptotic cells was 21%, which increased to 27% at 20 µM, 44% at 80 µM and to 68% at 160 µM of Cinnamolide doses, indicating dose-dependent increase in the number of apoptotic cells (Figure 4). Western blotting analysis was performed to check the levels of apoptosis-related proteins which showed significant suppression in Bcl-2 levels and upsurge in Bax levels after Cinnamolide exposure (Figure 5). Along with the expressions of apoptosis-related genes the expressions of caspase-3, -8 and -9
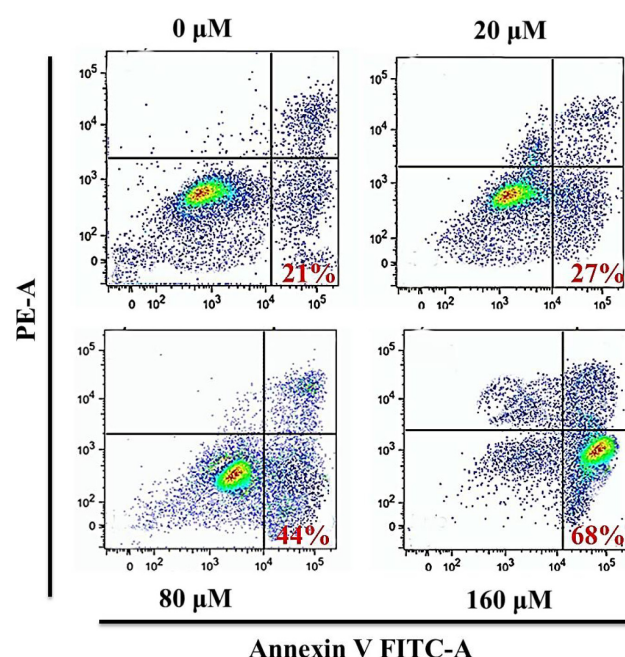

Figure 4. Annexin V-FITC/PI assay for quantification of apoptotic cell percentage after Cinnamolide exposure of human cervical HeLa cancer cells at indicated doses. The Figure shows increased percentage of apoptotic cells dose-dependently.
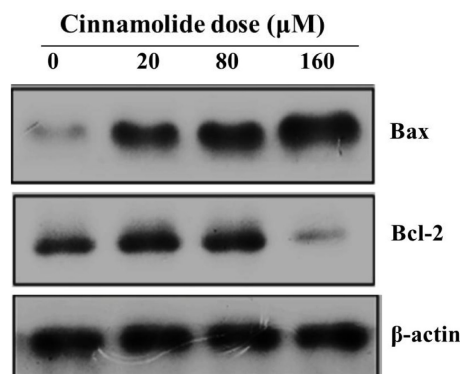

Figure 3. AO/EB dual staining assay for determination of cellular morphology and apoptosis after Cinnamolide treatment to human cervical HeLa cancer cells. The results indicate membrane blebbing, increased number of yellow-green (early apoptotic cells), orange (late apoptotic cells) and orange-red (necrotic cells) fluorescent cells. Arrows indicate apoptotic cells.

Figure 5. Activity of apoptosis-related proteins after Cinnamolide treatment to human cervical HeLa cancer cells via western blotting analysis, using β-actin for normalization. The Figure shows concentration-dependent decrease in the expression of Bcl-2 and increase in the expression of Bax.

were also evaluated through western blotting assay and the results revealed significant dose-dependent rise in the expressions of caspase-3, caspase-8 and caspase-9, further confirming the induction of caspase-dependent apoptosis in HeLa cells (Figure 6). Thus AO/EB dual staining, annexin V-FITC/PI and western blotting analysis together confirmed that Cinnamolide treatment of HeLa cells resulted in dose-dependent induction of caspase-dependent apoptotic cell death.

Effects on MMP of human HeLa cells by Cinnamolide molecule

MMP analysis of Cinnamolide-treated HeLa cells was performed by flow cytometry using Rh-123 dye. The results showed significant loss in MMP after Cinnamolide treatment. In controls, MMP was nearly 100%. Cinnamolide treatment of 20 µM led to MMP decrease to about 80% and at 160 µM the MMP was significantly reduced to about 15%, indicating remarkable decrease in the MMP levels of Cinnamolide-treated HeLa cells with increasing drug doses (Figure 7).

Effect of Cinnamolide on tumor weight and volume

Xenografted models of mice were used to carry out *in vivo* results of Cinnamolide treatment which revealed significant suppression in the growth of mice tumour, which was depicted by decrease in weight and volume of the tumour. In control, the weight of the tumour neared 3 g which then reduced to 2.6 g at 20 mg/kg Cinnamolide concentra-
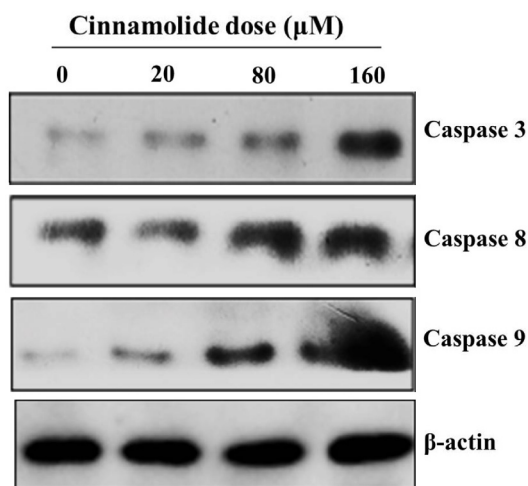

Figure 6. Caspase activity check after Cinnamolide exposure of human cervical HeLa cancer cells at indicated doses. The Figure shows concentration-dependent increase in the expression levels of caspase-3, caspase-8 and caspase 9.
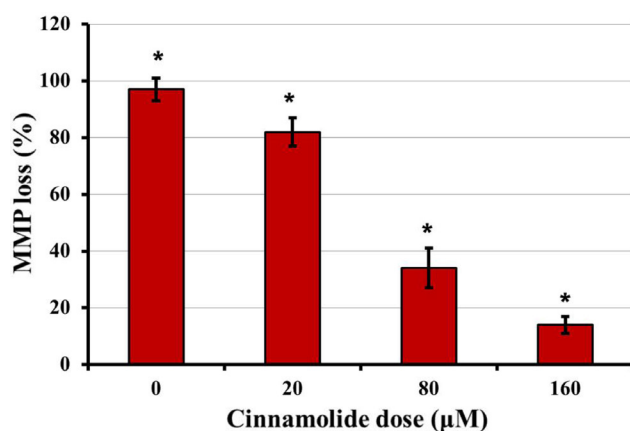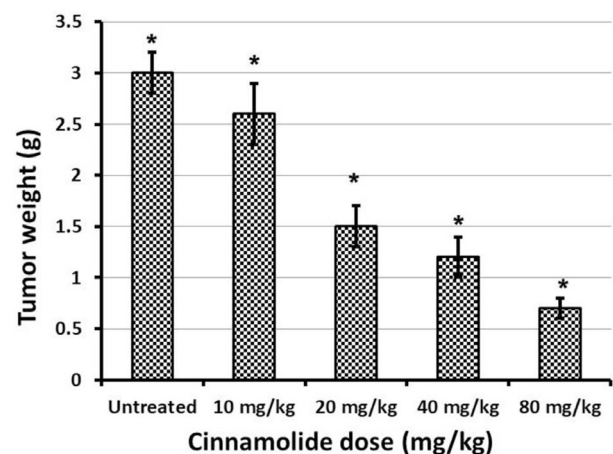

Figure 8. Effect on tumor weight after treatment with indicated Cinnamolide doses for *in vivo* examinations using xenografted models of mice. The Figure shows dose-dependent decrease in tumor weight. The values are the mean±SD of three experiments. *p<0.05.

Figure 7. MMP measurements of human cervical HeLa cancer cells via flow cytometry using Rh-123 after Cinnamolide treatment at indicated doses. The values are the mean±SD of three experiments. The Figure shows dose-dependent decease in MMP. *p<0.05.
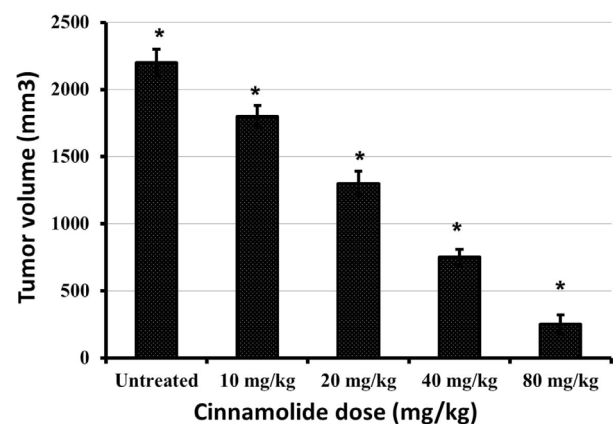

Figure 9. Effect on mice tumor volume after treatment with indicated Cinnamolide doses for *in vivo* examinations using xenografted models of mice. The Figure shows dose-dependent decrease in tumor volume. The values are the mean±SD of three experiments. *p<0.05.
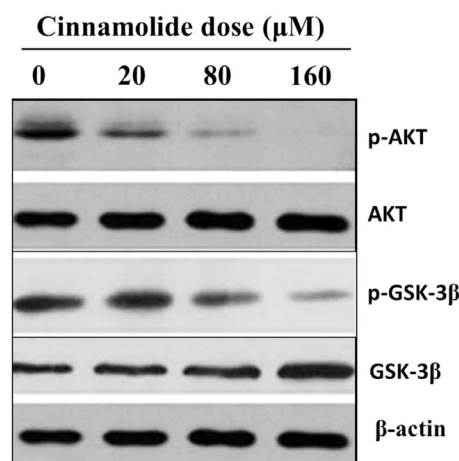
Cinnamolide dose (µM)

Figure 10. Western blotting analysis to determine the effect of Cinnamolide on Akt/β-Catenin signalling pathway by examining the expressions of AKT, p-AKT, GSK-3β and p-GSK-3β. The expression levels of Akt and GSK-3β increased very slightly while the expression levels of p-Akt and p-GSK-3β decreased significantly and in a dose-dependent manner. Actin was used for normalisation control. The experiments were performed in triplicate

tion. Tumour weight reduced to almost 0.7 g at 80 mg/kg (Figure 8) thus demonstrating significant dose-dependent reduction in mice tumour weight after treatment. Next, studies were performed on tumour volume and the results showed reduction in tumour volume after treatment with Cinnamolide, showing that Cinnamolide treatment led to dose-dependent reduction in tumor volume (Figure 9).

Cinnamolide inhibits Akt/β-catenin signalling pathway

Western blot was used to examine the effects of increasing concentrations of the molecule on Akt/β-catenin signalling pathway. The results, which are depicted in Figure 10, indicate that Akt/β-Catenin pathway-associated proteins were significantly affected by the molecule. The expression levels of Akt and GSK-3β increased very slightly while the expression levels of p-Akt and p-GSK-3β decreased significantly and in a dose-dependent manner (Figure 10).

Discussion

Cervical cancer is a malignancy affecting a large number of women globally. Disease relapse, lower survival and higher side-effects of conventional treatments pose a great challenge for clinicians and demand novel therapeutic agents with higher efficiency to overcome these problems [27]. The anomalous activation of AKT/β-catenin signalling pathway is among the major causes of cancer, which is regulated by GSK-3β phosphorylation.

Hence, targeting AKT/β-catenin signalling pathway serves as a novel therapeutic strategy to overcome cancer in addition to apoptosis, autophagy and necrosis. Herein, we aimed at examining the *in vitro* and *in vivo* anticancer effects of Cinnamolide molecule on human cervical HeLa carcinoma cells via suppression of cisplatin-resistant cancer cell growth by inducing mitochondrial mediated apoptosis, caspase activation, loss of MMP and targeting Akt/β-Catenin signalling pathway. The anti-cell proliferation effect of this molecule on HeLa cells was assessed through WST-1 proliferation assay, which revealed significant dose-dependent inhibition of growth with an IC_{50} 16.5 µM. Also, this molecule was able to inhibit colony formation in HeLa cells. Further, apoptotic studies on HeLa cells were carried out after Cinnamolide treatment through AO/EB staining and annexin V-FITC/PI, revealing dose-dependent apoptosis induction as demonstrated from the increased number of yellow-green, orange and orange-red fluorescent cells. Apoptosis-related proteins Bax and Bcl-2 levels revealed increase in Bax and decrease in Bcl-2 levels, evidencing apoptotic cell death of HeLa cells by Cinnamolide sesquiterpene treatment. Next, western blotting analysis also revealed that exposure to this molecule led to activation of caspase 3 -8 and -9 proteins in a dose-dependent manner. The results of MMP analysis revealed substantial loss in MMP of HeLa cells by Cinnamolide exposure. Further, the effect of this molecule on Akt/β-catenin signalling pathway revealed significant reduction of phosphorylated Akt and GSK-3β levels by drug exposure. Along with the *in vitro* studies, *in vivo* examination of xenografted cervical tumours revealed remarkable reduction in tumour volume and tumour weight with increasing molecule's concentrations.

Conclusions

In conclusion, *in vitro* and *in vivo* examinations of Cinnamolide sesquiterpene on HeLa cells revealed significant suppressive effect on tumour cell growth, weight and volume. In addition, induction of mitochondrial mediated apoptosis, caspase activation, loss of MMP and targeting Akt/β-Catenin signaling pathway are responsible for the anticancer effects of Cinnamolide. Hence, based on *in vitro* and *in vivo* analyses, it is clear that Cinnamolide is a potential molecule against cervical cancer.

Conflict of interests

The authors declare no conflict of interests.

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