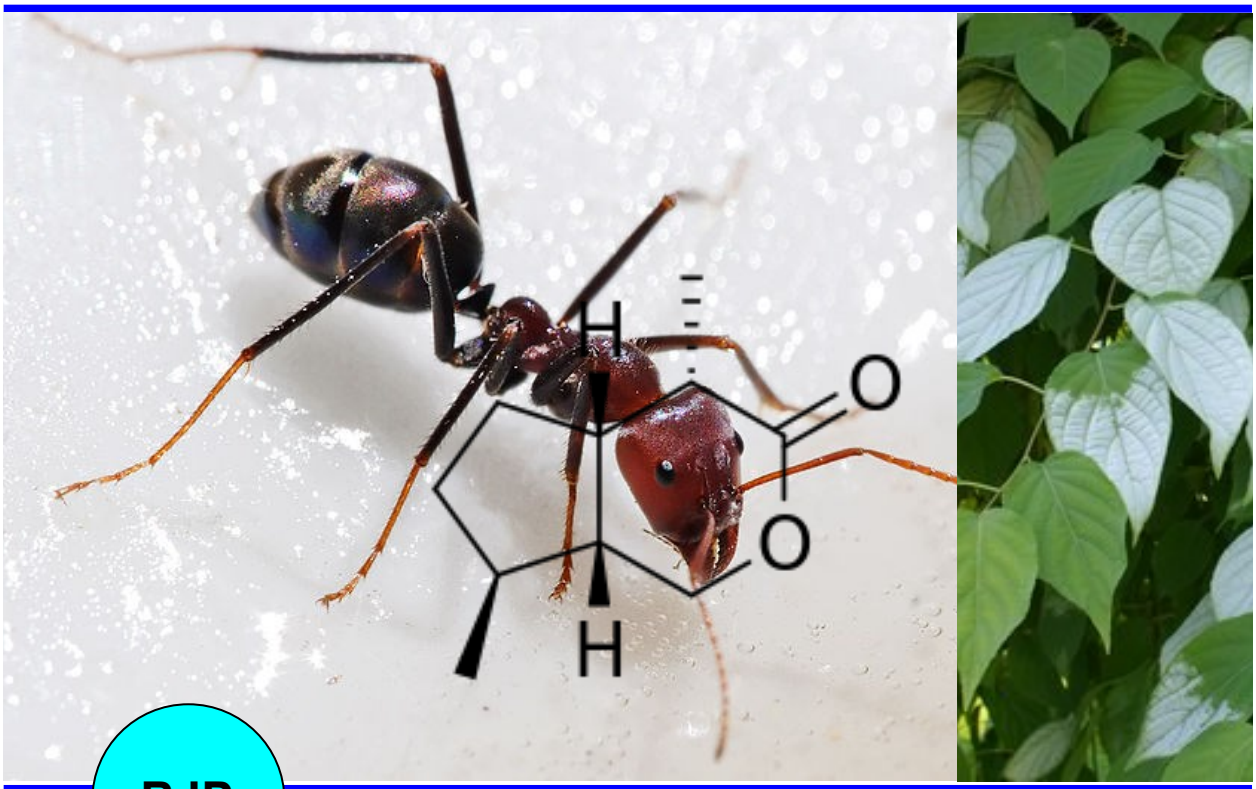


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Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index;
**Bangladesh J Pharmacol 2016; 11: 844-850**
**ISSN:** 1991-0088

# Antitumor effects of iridomyrmecin in HeLa cervical cancer cells are mediated via apoptosis induction, loss of mitochondrial membrane potential, cell cycle arrest and down-regulation of PI3K/Akt and up-regulation of lncRNA CCAT2 expression

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## Article Info

Received: 28 April 2016
Accepted: 31 May 2016
Available Online: 14 October 2016

DOI: 10.3329/bjp.v11i4.27539

Cite this article:
Lin L, Cheng XL, Li MZ, Wang T, Dong MH, Wang ZY, Liao M. Antitumor effects of iridomyrmecin in HeLa cervical cancer cells are mediated via apoptosis induction, loss of mitochondrial membrane potential, cell cycle arrest and down-regulation of PI3K/Akt and up-regulation of lncRNA CCAT2 expression. Bangladesh J Pharmacol. 2016; 11: 844 -850.

## Abstract

The main purpose of the current study was to study the antitumor effects of iridomyrmecin against human cervical cancer cells (HeLa). Its effects on apoptosis induction, cell cycle phase distribution, PI3K/Akt signalling pathway and long non-coding RNA (lncRNA) were also investigated. Cytotoxic effects of iridomyrmecin were evaluated by MTT assay while the apoptotic effect was assessed by flow cytometry using annexin V-FITC assay. Western blot assay was used to study effects on PI3K/Akt signalling pathway. Results exhibited that iridomyrmecin led to concentration-dependent as well as time-dependent growth inhibitory effects. Iridomyrmecin-treated cells showed signs of early and late apoptosis. Iridomyrmecin treatment also led to sub-G1 cell cycle arrest as well as induced loss of mitochondrial membrane potential ($\Delta\Psi m$). Further, Western blot assay revealed that iridomyrmecin treatment resulted in down-regulation of PI3K/Akt protein expressions in a dose-dependent manner while as it up-regulated lncRNA CCAT2 expression.

## Introduction

China represents one of the few countries with a very high occurrence of cervical cancer and is accountable for more than 45% of all the new cases diagnosed globally. In China, cervical cancer is the third prominent cause of cancer related mortality (Chen et al., 2004; Shi et al., 2008; Wen, 2005). Among the risk factors, *Helicobacter pylori* infection remains one of the most prominent risk factors of cervical cancer (Fiedler et al., 2004).

Cervical cancer treatment strategy involves a combination of surgical resection, radiotherapy and chemotherapy depending on the tumor location and size. Among the chemotherapeutic agents used against cervical cancer include 5-fluorouracil, leucovorin, cisplatin, epirubicin, capecitabine, oxaliplatin or their different combinations (Peter et al., 1999; Yoshihiro et al., 2000).

In the present study, antitumor effects of iridomyrmecin were tested against HeLa, a human-cervical cancer cell line, along with evaluating its efficacy in inducing effects on apoptosis, cell cycle and PI3K/Akt signalling pathway.

## Materials and Methods

### *Chemicals and other reagents*

Iridomyrmecin (purity ≥98%), annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit and MTT were obtained from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin and phosphate buffered saline (PBS), supplemented with calcium chloride and magnesium chloride, were obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (China). All other chemicals and solvents used were of the highest purity grade.

### *Cell line*

HeLa, a human-cervical cell line was purchased from the Shanghai Institute of Cell Resource Center of Life Science (China). The cells were maintained in DMEM supplemented with 10% (v/v) FBS under a humidified atmosphere of 5% $CO_2$ at 37°C. The medium was replaced every 2 days and the cells were subcultured every 4 days.

### *MTT assay for cell proliferation*

The cytotoxic effects of iridomyrmecin on HeLa cells was evaluated by MTT assay. Briefly, HeLa cells at a density of 1 x $10^6$ cells per well were seeded into a 96-well plates and then incubated for 4 hours for cell adherence. The cells were then treated with iridomyrmecin at varying doses (0, 10, 20, 80 and 160 µM) and then incubated for 24, 48 and 72 hours time periods. After treatment, MTT solution (10 µL) was added to the cells for 4 hours at 37°C. The formazan crystals were solubilized with dimethyl sulfoxide and the absorbance was measured on a microplate reader at a wavelength of 490 nm. The effects of iridomyrmecin on cell viability were calculated as an inhibition ratio (I%) using the following formula:

$$I\% = [OD_{490} \text{ (control)} - OD_{490} \text{ (treated)}]/OD_{490} \text{ (control)} \times 100$$

Where, $OD_{490}$ is the optical density at 490 nm

### *Annexin V-FITC assay of cell apoptosis*

HeLa cells at a density of 2 x $10^6$ cells per mL were seeded into 12-well plates and incubated overnight. After treating cells with different doses (0, 10, 20 and 80 µM) of iridomyrmecin, the cells were washed with PBS and then resuspended in binding buffer containing annexin V-fluorescein isothiocyanate and propidium iodide for 30 min. Cells grown in media containing an equivalent amount of 0.11% DMSO without any drug served as control. Fluorescence intensity was measured using flow cytometry (Becton, Dickinson and Company, USA).

### *Cell cycle analysis*

Briefly, HeLa cells were seeded at a density of 2 x $10^5$ cells/mL into a 6-well plates and incubated for 48 hours. The cells were treated with different doses (0, 10, 20 and 80 µM) of iridomyrmecin for 48 hours. The cells were trypsinized, centrifuged at 12,000 rpm for 15 min and then washed with PBS twice and fixed with ice-cold 70% ethanol at -20°C for 3 hours. After the cells were resuspended in 300 µL of PBS, 50 µL PI and 50 µL RNase A, the cells were finally analyzed on a FACSCalibur flow cytometer (Becton, Dickinson and Company, USA). Data on 15,000 cells were acquired and processed with CellQuest software.

### *Mitochondrial membrane potential (ΔΨm) loss assay*

The effect of iridomyrmecin on mitochondrial membrane potential in HeLa was evaluated by using Rhodamine-123 (5 mM) fluorescent probe using flow cytometry. The cells (2 x $10^5$ cells/dish) were treated with increasing doses of iridomyrmecin (0, 10, 20 and 80 µM) for 48 hours. Rhodamine-123 (5 mM) was added 2 hours before the termination of experiment. Mitochondrial membrane potential was measured by flow cytometry, BD FACSCalibur flow cytometer and Cell Quest software 3.0 (Becton-Dickinson).

### *Western blot analysis*

Next we involved Western blot assay to demonstrate the main proteins involved in the biofunctions of these cancer cells. HeLa cells were harvested and lysed with RIPA buffer, and collected protein samples were quantified by using bichinconinic acid protein assay kit (Pierce Biotechnology, USA). About 100 µg of cellular protein from each sample was applied to 8–10% SDS-polyacrylamide gels and probed with specific antibodies followed by exposure to horseradish per-oxidase -conjugated goat anti-mouse antibodies. Blots were then developed using the West Pico Chemiluminescent substrate (Pierce, USA).

### *Quantitative realtime PCR (qRT-PCR)*

Total RNA from tissues was extracted using trizol reagent (Invitrogen). qRT-PCR assays were performed to detect CCAT2 expression using the Prime Script RT reagent Kit and SYBR Premix ExTaq (Takara) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and lncRNA CCAT2 values were normalized to GAPDH. qRT-PCR reactions were performed by the ABI7900 system (Applied Biosystems). The PCR primers used were as follows: 5′-CCACATCGC-TCAGACACCAT-3′ (sense) and 5′-ACCAGGCGC-CCAATACG-3′ (antisense) for GAPDH; and 5′-CCCTGGTCAAATTGCTTAACCT-3′ (sense) and 5′-TTATTCGTCCCTCTGTTTTATGGAT-3′ (antisense) for CCAT2.

### *Statistical analysis*

Each experiment was performed in triplicate. The data were expressed as the mean value ± standard deviation. Significance of difference was indicated as [a]p<0.05, [b]p<0.01.

## Results

### *Cytotoxic activity of iridomyrmecin against human cervical cancer cells (HeLa)*

The chemical iridomyrmecin belongs to the polyphenolic group of naturally occurring compounds with significant anti-oxidant activity. The cytotoxic activity of iridomyrmecin in HeLa cells is shown in Figure 1 which shows that iridomyrmecin exhibits significant cytotoxic effect in these cells. The cytotoxic

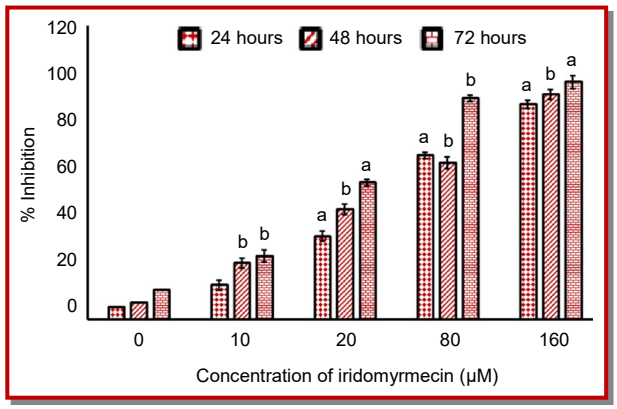


Figure 1: Inhibition of cell proliferation by iridomyrmecin in human cervical cancer cells (HeLa). The cells were treated with increasing doses of iridomyrmecin and then anti-proliferative effect was assessed by MTT cell viability assay. Data are shown as the mean ± SD of three independent experiments. [a]p<0.05, [b]p<0.01 vs 0 μM (control)

effect was related to not only dose of the compound but it also depended on the incubation times to which these cells were subjected. The $IC_{50}$ values which were calculated to indicate the efficacy of the drug against these cancer cells were found to 17.2, 24.1 and 46.2 μM respectively at 24, 48 and 72 hours incubation time intervals respectively.

### *Iridomyrmecin induced early and late apoptosis in HeLa human cervical cancer cells*

Figure 2A-D show the effect of iridomyrmecin on apoptosis induction as well as its quantification using annexin V-FITC assay along with flow cytometry. The results indicate that as compared to the untreated control cells Figure 2A, which show very less cells undergoing apoptosis. However, on treating cells with 10, 20 and 80 μM dose of iridomyrmecin, the percentage of early and late apoptotic cells increased dramatically showing a symbolic dose-dependence. The percentage of apoptotic cells increased from 4.9% in control cells to 13.1, 34.2 and 66.4% in cells treated with 10, 20 and 80 μM of iridomyrmecin respectively (Figure 2B-D). The upper right and lower right quadrants in Figure 3 represent early and late apoptotic cells respectively.

### *Iridomyrmecin induces sub-G1 and G2/M cell cycle arrest*

The effects of iridomyrmecin on cell cycle phase distribution were evaluated by flow cytometry using propidium iodide as a staining agent. The results of this assay are shown in Figure 3 indicating that iridomyrmecin induces potent and concentration-dependent effects on cell cycle progression. Specifically, it affected mostly sub-G1 phase (apoptotic phase) as well as G2/M phase of the cell cycle. The population of cells in G1 and S phase almost remained the same on different doses of the drug, however, sub-G1 cells showed a significant increase as the dose of iridomyrmecin was increased from 0 to 10, 20 and then 80 μM. G2/M phase cells only showed a slight increase in their percentage with increase in iridomyrmecin dose.

### *Iridomyrmecin induces loss of mitochondrial membrane potential loss (ΔΨm) in HeLa human cervical cancer cells*

The effect of iridomyrmecin on mitochondrial transmembrane potential was evaluated by flow cytometry using Rh-123 as fluorescent probe. The results of the assay are shown in Figure 4 which exhibit that iridomyrmecin led to significant loss of mitochondrial transmembrane potential (MMP) in a dose-dependent fashion. As compared to the untreated control with no loss of MMP, but iridomyrmecin-treated cells showed significant loss of MMP. Loss of mitochondrial membrane potential has been reported in apoptosis process. Opening of the mitochondrial permeability transition pore has been demonstrated to induce depolarization of the transmembrane potential (Δψm), release of apoptogenic factors and loss of oxidative phosphorylation.

### *Iridomyrmecin down-regulates PI3K/Akt-related protein expressions*

In this assay, the effect of iridomyrmecin on PI3K/Akt-related protein expressions in human cervical cancer cells was evaluated by Western blot assay. The results indicated that iridomyrmecin targets PI3K/Akt pathway by down-regulating expressions of these proteins. The results are shown in Figure 5 revealing that iridomyrmecin led to reduction of phosphorylation of PI3K and Akt proteins. The down-regulation of PI3K/Akt pathway has been implicated in many oncogenesis processes.

### *Iridomyrmecin treatment led to up-regulation of long non-coding RNA colon cancer associated transcript 2 (CCAT2) up-regulation in HeLa cervical cancer cells*

LncRNA CCAT2 expression was detected in 42 pairs of

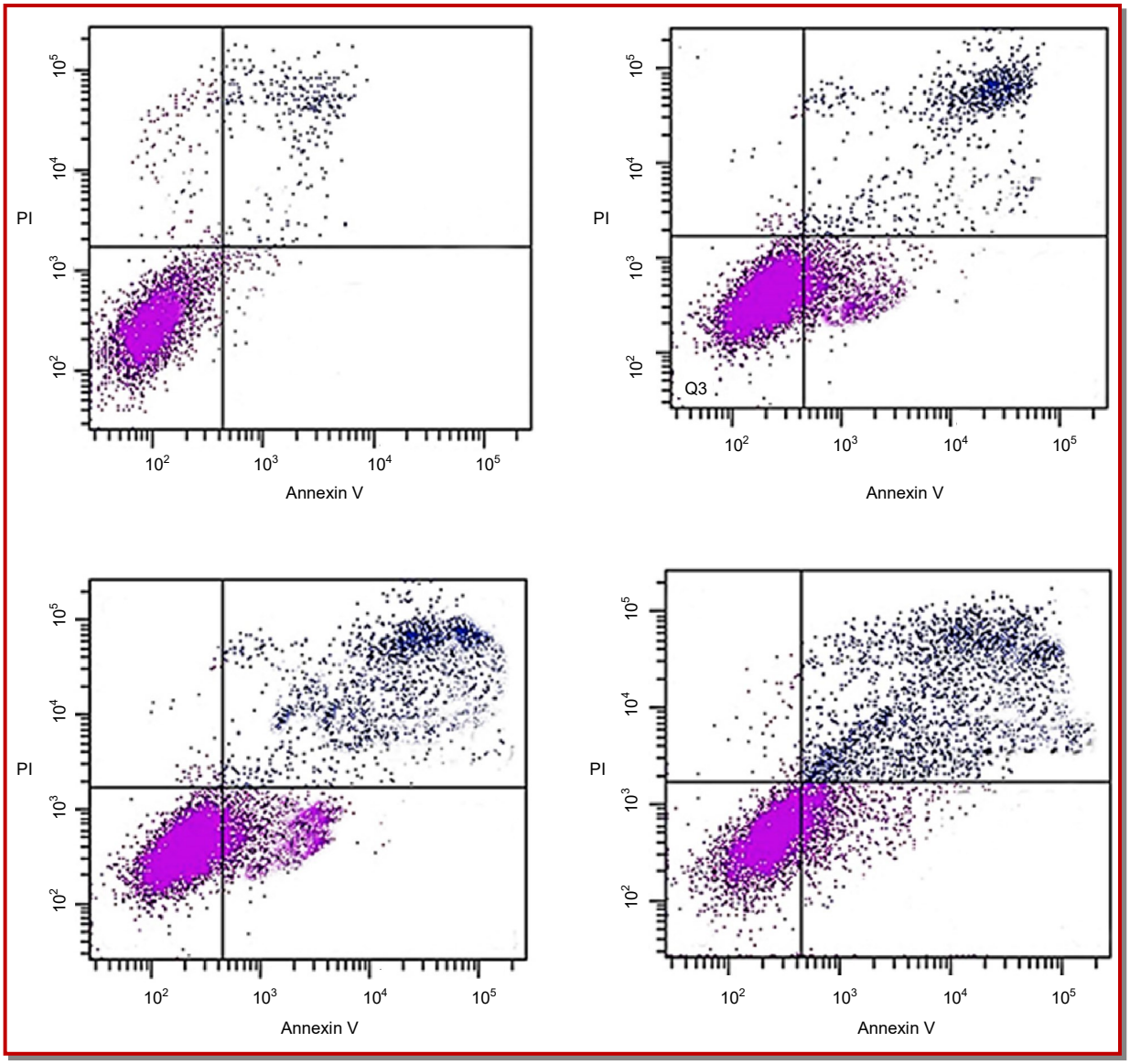


Figure 2: Evaluation of apoptosis by annexin V-FITC assay in human cervical cancer cells (HeLa) induced by iridomyrmecin. After the cells were treated with 0, 10, 20 and 80 µM dose of iridomyrmecin for 48 hours and then analyzed by flow cytometry. With increase in the dose of iridomyrmecin, there was a significant increase in the percentage of early and late apoptotic cells

cervical cancer and adjacent non-tumor tissues by qRT-PCR. As shown in Figure 6, after normalization to GAPDH expression levels, the expression level of lncRNA CCAT2 in cervical cancer cells treated with iridomyrmecin was significantly higher than that in adjacent non-tumor tissues as well as cancer tissues without any drug treatment.

## Discussion

The results revealed that iridomyrmecin could induce potent cytotoxic effects in HeLa human cervical cancer cells in a dose-dependent as well as in a time-dependent manner with $IC_{50}$ values of 17.2, 24.1 and 46.2 µM respectively at 24, 48 and 72 hours incubation time intervals respectively. Flow cytometry using annexin v assay revealed that iridomyrmecin led to early and late apoptosis in these cells. The percentage of apoptotic cells increased from 4.9% in control cells to 13.1, 34.2 and 66.4% in cells treated with 10, 20 and 80 µM of iridomyrmecin respectively. Further, it was seen that iridomyrmecin induced sub-G1 and G2/M cell cycle arrest. Sub-G1 cells showed a significant increase as the dose of iridomyrmecin was increased from 0 to 10, 20 and then 80 µM. The population of cells in G1 and S phase almost remained the same on different doses of the drug. Iridomyrmecin also led to significant loss of mitochondrial transmembrane potential (MMP) in a dose-dependent fashion. Iridomyrmecin also led to

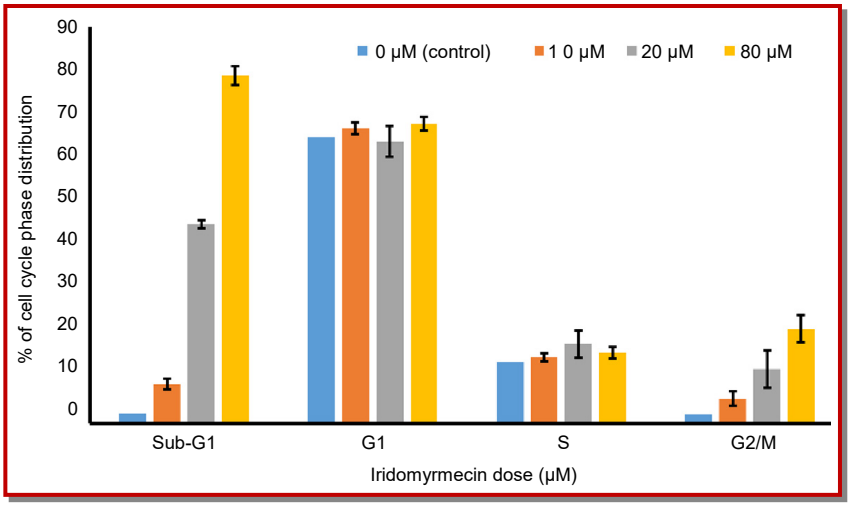


Figure 3: Iridomyrmecin induced significant sub-G1 and mild G2/M cell cycle arrest in human cervical cancer cells (HeLa). The cells were treated with 0, 10, 20 and 80 µM dose of iridomyrmecin for 48 hours and then analyzed by flow cytometry. There was a significant increase in the sub-G1 and G2/M phase cells as the dose of iridomyrmecin was increased

down-regulation of PI3K/Akt-related protein expressions.

To the best of our knowledge, there are no such reports on the anti-cancer activity of iridomyrmecin against HeLa human cervical cancer cells, as such the present study is the first such report. Like iridomyrmecin, oleanolic acid methyl ester derivative shows anti-cancer effect in HeLa cervical cancer cells which is mediated through apoptosis induction and reactive oxygen species production (Song et al., 2015). The ethanol extract of banana (*Musa paradisiaca*) flower or *Cordia dichotoma* leaf also shows anti-cancer activity on the cervical cancer cell line HeLa (Nadumane and Timsina, 2014; Rahman and Hussain, 2015).

Long non-coding RNA (LncRNA) is an RNA molecule that is longer than 200 nucleotides and is not translated into a protein. There is compelling evidence that LncRNA has a role to play in a diverse biochemical functions including cell proliferation, cell apoptosis, cell invasion and cell migration, cell differentiation etc. There are various published reports which indicate that there is a potential involvement of LncRNA in different human malignancies (Mercer et al., 2009; Gibb et al., 2011; Ng et al., 2012; Geng et al., 2011). Apoptosis induction in tumor cells is one of the principal characteristics of various antitumor drugs which ultimately leads to cancer prevention by controlling cell death. Apoptosis is a highly specialized biochemical process

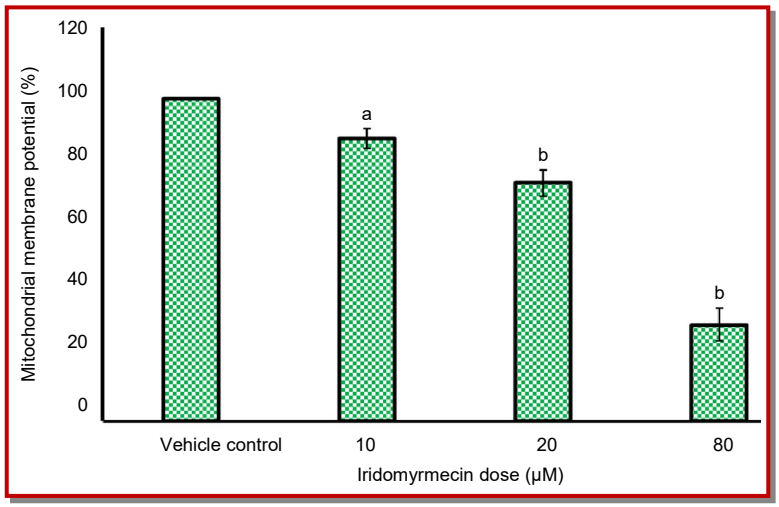


Figure 4: Iridomyrmecin led to loss of mitochondrial membrane potential loss in HeLa human cervical cancer cells. The cells were treated with 0, 10, 20 and 80 µM dose of iridomyrmecin for 48 hours and then stained with Rh-123 fluorescent dye and finally analyzed by flow cytometry. Data are shown as the mean ± SD of three independent experiments. $^{a}p<0.05$, $^{b}p<0.01$ vs 0 µM (control)

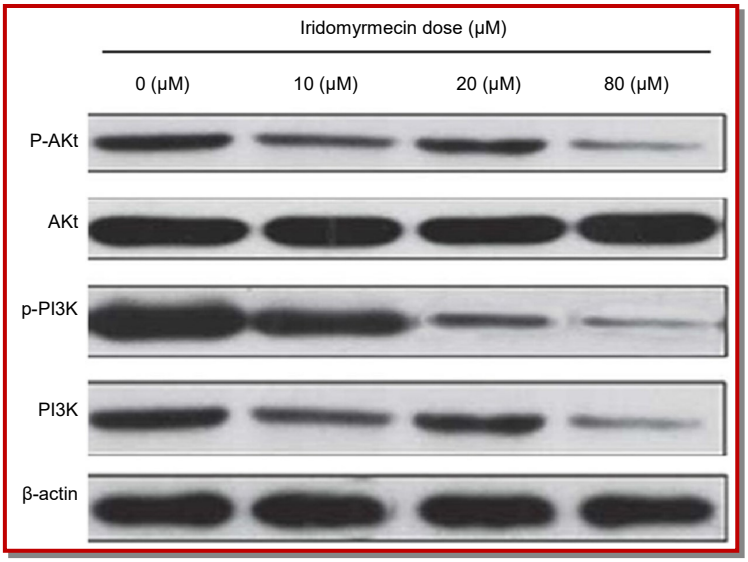


Figure 5: Iridomyrmecin leads to down-regulation of PI3K/Akt signalling pathway in human cervical cancer cells (HeLa). The changes in protein expressions were detected using Western blot. The cells were treated with 0, 10, 20 and 80 μM dose of iridomyrmecin for 48 hours

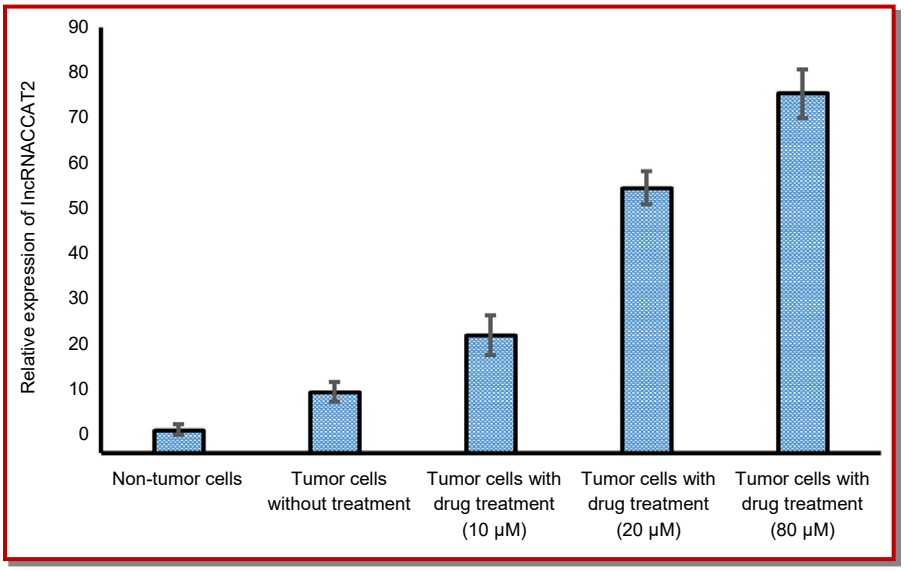


Figure 6: Graphical representation of LncRNA CCAT2 expression in iridomyrmecin treated and untreated cervical cancer and nearby non-cancer tissues detected by qRT-PCR

which eradicates redundant cells from the body and as such is the key for maintaining tissue homeostasis. Any disturbance in this process eventually results in numerous diseases including cancer. Both intrinsic as well as extrinsic stimuli can trigger the process of apoptosis which finally lead to the activation of proteases (caspases) and nucleases, resulting in destruction of the cell (Chen et al., 2016; Wang et al., 2016). The results of the current study indicated that iridomyrmecin, which is a plant iridoid compound, exhibits potent cytotoxic effects in HeLa cells via inducing early and late apoptosis, sub-G1 cell cycle arrest and down-regulation of PI3K/Akt protein expressions.

## Conclusion

Iridomyrmecin exhibits antitumor activity by inducing early and late apoptosis, loss of mitochondrial membrane potential loss and sub-G1 cell cycle arrest, leading to down-regulation of PI3K/Akt protein expressions and up-regulation of lncRNA CCAT2 expression.

## Financial Support

Self-funded

## Conflict of Interest

Authors declare no conflicts of interest

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