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Neferine and isoliensinine enhance 'intracellular uptake of cisplatin' and induce 'ROS-mediated apoptosis' in colorectal cancer cells – A comparative study

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ABSTRACT

Cisplatin (CDDP) is a potent platinum-based chemotherapeutic agent used to treat solid tumors including colorectal cancer via inducing cytotoxicity. CDDP usage is limited due to the chemoresistance and associated adverse effects. A combinatorial regimen of phytochemicals with anticancer activity along with approved anticancer drugs seems to be a hopeful strategy against cancer treatment. Lotus-derived compounds such as neferine and isoliensinine have proven significant chemosensitizing activity in different cancer cells. Present study aims to compare chemosensitizing activity/anticancer potential of neferine/isoliensinine in combinatorial regimen with CDDP. Results documented that neferine/isoliensinine with CDDP augmented 'intracellular uptake of cisplatin' consequently apoptosis in HCT-15 cells exemplified by 'apoptotic morphological changes', 'S phase cell cycle arrest', 'ROS mediated oxidative stress' with the concomitant escalation in intracellular calcium & dissipation of MMP and activation of MAPK/PI3K/AKT pathway'. Furthermore, isoliensinine combination with CDDP exclusively enhanced CDDP uptake and induced more ROS-mediated apoptosis compared to other treatment regimens. Combination regimens induced downregulation of Bcl2 and upregulation of cytochrome *c*, caspase 3, 9, PARP cleavage indicating apoptosis induction through the intrinsic pathway. Thus, the results of the present study suggest that CDDP combination with neferine/isoliensinine augments the anticancer potential of CDDP in an additive manner and decrease CDDP dose requirement.

1. Introduction

Colon Rectal Cancer (CRC) is the most prevalent cancer among aged individuals. Epigenetic alterations and improper dietary patterns have been reported to be the main contributors to its development. As per the United States Surveillance and Epidemiological studies prediction, the incidence rate of CRC will rise to 90% of all the cancers by 2030 worldwide (Koosha et al., 2016). Lack of selective nontoxic chemotherapy has been suggested as the main reason behind the high mortality of CRC patients.

Majority of chemotherapeutic agents against colorectal cancer (CRC) tumors induce toxicity to non-target tissues. Hence, their therapeutic efficacy and applications are constrained by the dose-limiting toxicity, development of drug-resistance and adverse effects (Hsu et al., 2018). Cisplatin (cis-diammedichloro platinum, DDP) is a potent first-line anticancer agent and it is used against several cancers pertaining to lung, ovary, breast, neck including human colorectal cancer (He et al., 2016; Selvi et al., 2017). Cytotoxic effect of cisplatin involves its interaction with DNA to form adduct which further activates several signaling pathways, ultimately leads to apoptosis (Sivalingam et al., 2017). However, the therapeutic efficacy of cisplatin is hampered due to the drug resistance and several side effects such as nephrotoxicity, peripheral neuropathy, cardiotoxicity and ototoxicity (Florea and Büsselberg, 2011; Kesavan et al., 2015). In order to overcome these problems, there is an urgent need to identify small molecules which can be used along with cisplatin in combinatorial strategy to reduce the dose-dependent side effects induced by cisplatin.

Recently, a combination of dietary natural products with chemotherapeutic drugs has gained increasing attention as the preferred therapeutic strategy against several cancers (Selvi et al., 2017). Neferine and isoliensinine are the major bisbenzylisoquinoline alkaloids found in the seed-embryos of *Nelumbo nucifera*. Several studies have

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reported the medicinal properties of neferine which include: anti-tumor, anti-oxidant, anti-inflammatory, anti-fibrosis, anti-arrhythmic and anti-platelet effects (Mukherjee et al., 2009; Poornima et al., 2013b; Xiong and Zeng, 2003). Anticancer potential of neferine against hepatocellular and osteosarcoma cells has been reported earlier (Poornima et al., 2013a; Zhang et al., 2012). Neferine could induce autophagy mode of cell death in lung and ovarian cancer cells (Poornima et al., 2013b; Xu et al., 2016). Several *in vitro* studies suggest that neferine could potentiate antitumor effects of cisplatin, doxorubicin and Mitomycin C in lung cancer (A549) and cervical cancer (HeLa) cells respectively (Eid and Abdel-Rehim, 2017; Poornima et al., 2014; Selvi et al., 2017; Sivalingam et al., 2017). Chemosensitizing ability of neferine against multidrug-resistant cancer cells has been reported in MCF-7 cells (Cao et al., 2004). Neferine has been reported to protect against the cisplatin-induced nephrotoxicity in kidney cells (NRK-52E cells) through the AMPK/mTOR signaling pathway (Li et al., 2017).

Recent investigations have illustrated various pharmacological activities of isoliensinine which include: anticancer, anti-diabetic and anti-pulmonary fibrosis (Shu et al., 2015; Xiao et al., 2005; Yang et al., 2017; Zhang et al., 2015). Anticancer activity of isoliensinine against the triple negative breast cancer cells (MDA-MB-231) has been shown to involve the ROS-mediated activation of MAPK pathway (Zhang et al., 2015). Shu et al. (2015) have illustrated that isoliensinine induced apoptosis in hepatocellular carcinoma by modulating NF-kB signaling pathway (Shu et al., 2015). Furthermore, the treatment with a combinatorial regimen of isoliensinine and dauricine resulted in chemosensitization of the dauricine-resistant hepatocellular carcinoma (HepG2 and Huh-7 HCC cells) (Li et al., 2018).

ROS-mediated oxidative stress plays a significant role in carcinogenesis and modulates various cell signaling pathways leading to cancer phenotype as well as induces cellular damage and apoptosis by sequential damage to protein, lipids and nucleic acids. ROS generation seem to be involved in the initiation of the mitochondrial apoptosis pathway, involving the alteration of Bax/Bcl-2 ratio, dysfunction of mitochondrial membrane potential, release of cytochrome *c* and activation of a caspase cascade (Sivalingam et al., 2017). However, the cellular uptake of CDDP and its consequent accumulation in cancer cells may also induce higher apoptotic cell death in a dose-dependent manner. Hence, the present study evaluates the comparative efficacy of the combinatorial regimen of cisplatin and neferine/isoliensinine against colorectal cancer cells.

2. Methods and materials

2.1. Chemicals and antibodies

Neferine (CAS No.2292-16-2) and isoliensinine (CAS.No.6817-41-0) were purchased from Sigma and Baojiherbest bio-tech Co. Ltd, China. RPMI media used for cell culture studies was purchased from Gibco. Cisplatin (CDDP) and propidium iodide were purchased from Sigma, Bangalore, and India. Primary monoclonal antibodies for Caspase 3, caspase 9, Akt, pAkt, PI3K, Cyto C, cleaved PARP, Bax, Bcl2, MAPK (p38 and p-P38), and GAPDH were obtained from 'Cell Signaling Technology' (Beverly, MA). Secondary conjugated antibodies such as anti-mouse and anti-rabbit IgG horseradish peroxidase were procured from GE Healthcare (UK). Western blotting membranes and other Western blotting reagents were obtained from PerkinElmer Inc. (USA). Annexin V kit was purchased from BD bioscience for flow cytometry. DCF–DA, DiOC6 & CCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone] were purchased from Calbiochem (USA).

2.2. Cell culture and treatment

Colorectal cancer HCT-15 cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in RPMI1640 media containing 10% fetal bovine serum. Subsequently, these cells were supplemented with antibiotics such as 'penicillin (100 units/ml), streptomycin (30 μg/ml) gentamycin (20 μg/ml) and were maintained at 5% CO_2 in 95% of humidity. Cultured cells at 80% confluence were used for all the subsequent molecular assays. All the experiments were carried out within 20 passages (Passage no. 50 to 70) to confirm the reproducibility and consistency of the cell population.

2.3. Determination of cytotoxicity through MTT assay

Approximately, [1×10^4] HCT-15 cells per well were seeded in a '96-well plate' and incubated overnight for the cell attachment. Later, the cells were treated as per the treatment schedule (neferine/isoliensinine/CDDP alone or combination of neferine/isoliensinine + CDDP or CDDP alone at individual IC_{50} values) for 24 h time period. At the end of the treatment period, the media was aspirated and 20 μl of MTT (3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyl tetrazolium Bromide - 5 mg/ml) was added and incubated for 4 h. Afterward, 200 μl of DMSO was added to dissolve the purple formazan crystals and the absorbance was read at 570 nm (Bio-Tek Instruments, VT) and the values were expressed in terms of % of cell viability. The combination index (CI) was assessed as per the Chou-Talalay protocol with Calcusyn (Biosoft) software. CI values were ascertained during cell viability assay with neferine/isoliensinine treatment and their individual combination with CDDP. As per this analysis, the combination effect determined whether combination efficacy is antagonistic ($CI > 1$) or additive ($CI = 1$) or synergistic ($CI < 1$) against HCT-15 cells.

2.4. Cell cycle analysis

Approximately [5×10^6] HCT-15 cells per well were seeded per well in a "6-well plate". Cells were treated with neferine/isoliensinine in combination with 'CDDP' and incubated for 24 h to assess their effect on cell cycle phases based on DNA contents. At the end of the treatment protocol, the cells were harvested & fixed with 70% ethanol overnight at 4 °C. Finally, these cells were stained using propidium iodide (PI)/RNase and incubated for 30 min at room temperature in dark. These cells were analyzed using flow cytometry (BD C6 Accuri).

2.5. Estimation of intracellular ROS level

Intracellular ROS level was determined using a fluorescent probe, DCFH-DA. In order to assess ROS level, approximately, [2×10^5] HCT-15 cells/well were seeded in 24-well plates. After attachment, the cells were washed using PBS and incubated with DCFH-DA probe at 37 °C for 30 min in a CO_2 incubator. These cells were treated with/without IC_{50} dose of neferine/cisplatin, isoliensinine/cisplatin alone or combination or for different time periods. H_2O_2 treated cells were served as a positive control. At the end of the treatment period, the cells were washed with PBS, harvested and were resuspended in PBS solution and the fluorescent intensity was measured using Hitachi spectrofluorometer at Ex.,/Em., 480/520 nm. Above experiment was carried out in triplicates to ensure reproducibility. The values were expressed in terms of percentage (%) of relative fluorescence to indicate the intracellular 'ROS' level.

2.6. Intracellular [Ca^{2+}] concentration

The intracellular concentration of calcium in treated and untreated HCT-15 cells was determined using FURA-2AM (a fluorescent divalent calcium dye) based on the fluorescence intensity measured using a spectrofluorometer. Briefly, [1×10^5] HCT-15 cells were seeded in 24-well plates and subsequently treated with/without neferine/CDDP, isoliensinine/CDDP alone or combination or IC_{50} dose of CDDP for different time periods/24 h. At the end of the treatment schedule, the

media was removed and the cells were washed twice with buffer-A [(Na_2HPO_4 (0.2 mM), $CaCl_2$ (2 mM), NaCl (137 mM), KH_2PO_4 (0.5 mM), $NaHCO_3$ (4 mM), KCl (5 mM)]. Afterward, the cells were loaded using FURA-2AM in buffer-A, and incubated for 45 min. Finally, the fluorescent intensity was measured at the Em.,/Ex., wavelengths of 500/340 nm and the % of relative fluorescence intensity was indicated for intracellular calcium release compared to untreated cells.

2.7. Mitochondrial membrane potential (ΔψM) assessment

Mitochondrial membrane integrity was assessed in terms of retention of the dye, DioC6 (3, 3′-dihexloacarbocyanine iodide) in drug-treated HCT-15 cells compared to untreated cells (control). Normally, this dye retention is lesser at the time of mitochondrial-membrane collapse due to the subsequent exclusion of dye from mitochondria. Initially, HCT-15 cells were treated with/without neferine/CDDP, isoliensinine/CDDP alone or combination or IC_{50} dose of CDDP for different time periods for 24 h. After the treatment period, the media was removed and cells were washed twice using PBS. Then, the cells loaded with 'Dio6 dye' (50 nM) and incubated for 30 min at 37 °C, finally fluorescence intensity was measured at 488/500 nm using spectrofluorometer (Biotek, US). Here, the cells incubated with a mitochondrial uncoupler, CCCP (50 μm) for 15 min prior to loading with [DiOC6-dye] served as a positive control. The mitochondrial membrane potential was expressed in terms of % relative fluorescence compared to control values.

2.8. SEM analysis

Alterations in the surface morphology of HCT-15 cells can be studied through SEM analysis in various treatment groups. Cells from the various treatment groups as mentioned in the previous section were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 1 h. Afterward, the cells were washed using cacodylate buffer twice, subsequently, post-fixation was performed with OsO4 (1%) in 0.1 M cacodylate buffer at room temperature for 1 h. These cells were dehydrated with increasing concentration (30%, 60%, 90% & 100%) of ethanol (30 min each). Finally, samples were air-dried and examined under SEM.

2.9. Western blotting

Total protein content was isolated from the cells in various treatment groups with lysis buffer (Sigma Aldrich) and total protein was estimated using the Bradford method. Equal amount of proteins was separated in 10% gel using SDS-PAGE by applying 100 V into the sample for 2 h. These isolated proteins were transferred onto the nitrocellulose membrane using semidry equipment. The membranes were blocked with skimmed milk (5%) protein for 1 h in TBST buffer. Later, washings with TBST buffer were performed thrice at room temperature subsequently the blots were incubated with 'specific primary antibody' at 4 °C overnight. Next day, the blots were washed with TBST buffer again 3 times for 5 min each. These blots were incubated with 'HRP-linked secondary antibody' for 2 h. Afterward, the DAB substrate and H_2O_2 were added to the above sample finally they were stored in the dark till the bands appeared.

2.10. Propidium iodide (PI) uptake

Approximately [2×10^5] HCT-15 cells per well were seeded in 12-well plates and incubated overnight. After the cell attachment, the cells were subjected to various treatments as mentioned previously. At the end of the treatment schedule, cells were isolated and washed using PBS finally, stained with PI for 10 min. Later, these stained cells were analyzed using flow cytometry (FACS Verse).

2.11. Annexin V assay

In order to study the proportion of cells undergoing early apoptosis, cells were subjected to various treatments as mentioned previously. At the end of the treatment schedule, cells were harvested through trypsinization, washed with PBS and were stained using Annexin V/PI as per the manufacturer instructions. The proportion of cells undergone early vs. late 'apoptosis' was analyzed using flow cytometry.

2.12. Intracellular platinum measurement (ICP-MS)

Approximately, HCT-15 cells of [5×10^5] per well were seeded into a 6 well plate and left for one day in an incubator at 37 °C. The cells were subjected to various treatments as mentioned previously. At the end of the treatment schedule, the cells were collected and washed with PBS. Later, the cells were subjected to overnight digestion with conc. nitric acid. The intracellular platinum concentration was determined using ICP-MS and expressed in terms of 'ppm' (Kesavan et al., 2015).

2.13. Statistical analysis

All the experiments were carried out in triplicates and performed three times independently. Data obtained were expressed in terms of "Mean ± Standard Deviation (SD)". Statistical analysis was performed with a One-way ANOVA followed by Tukey's multiple comparison test to express the difference between the groups using SPSS-17.0.

3. Results

3.1. Determination of cytotoxicity through MTT assay

HCT-15 cells were treated with different concentrations of neferine/isoliensinine/CDDP for 24 h and IC_{50} values were found to be 8.95 μM, 12.5 μM and 32 μM respectively. The effective doses for the combinatorial regimen were chosen based on the MTT assay and they were 15 μM for CDDP whereas 6 μM for neferine and 8 μM for isoliensinine. Nearly, 50% cell viability was observed with the combinatorial regimens of 'neferine (6 μM) + CDDP (15 μM)' and 'isoliensinine (8 μM) + CDDP (15 μM)'. Both the combination regimens substantially induced 50% cell death equivalent to IC_{50} dose of CDDP (Figure-1A, 1B). The results suggested that the combinatorial regimens potentially reduced the dose requirement of CDDP compared to individual treatments and the combinatorial regimens work in an additive manner to cause cell death (Figure-1C, 1D).

Combinatorial regimens (CDDP with neferine/isoliensinine) have induced cytotoxicity exemplified by significant morphological changes such as cell rounding and cell detachment from culture surface compared to individual treatment groups (as shown in the Figure-1E). A significant increase in distinct morphological features such as membrane blebbing and cell shrinkage indicative of apoptosis was also observed in combinatorial treatments compared to individual treatments (as shown in Figure-1F).

3.2. Neferine, isoliensinine combinatorial regimen with 'CDDP' augments the 'uptake of CDDP' in HCT-15 cells compared to individual treatments

Intracellular concentration of CDDP determines cytotoxicity in cancer cells. Hence, we have studied the 'intercellular accumulation of CDDP' in various treatment groups. As shown in Figure-2, the intercellular concentration of CDDP was increased significantly in cells treated with combinatorial regimens compared to CDDP alone treatment. Furthermore, CDDP uptake was clearly higher in its combination with isoliensinine compared to its combination with neferine. Results have illustrated that the combination of 'CDDP with neferine/isoliensinine' is an effective and novel method to increase the intracellular accumulation of CDDP leading to enhanced cell death of HCT-15 cells.

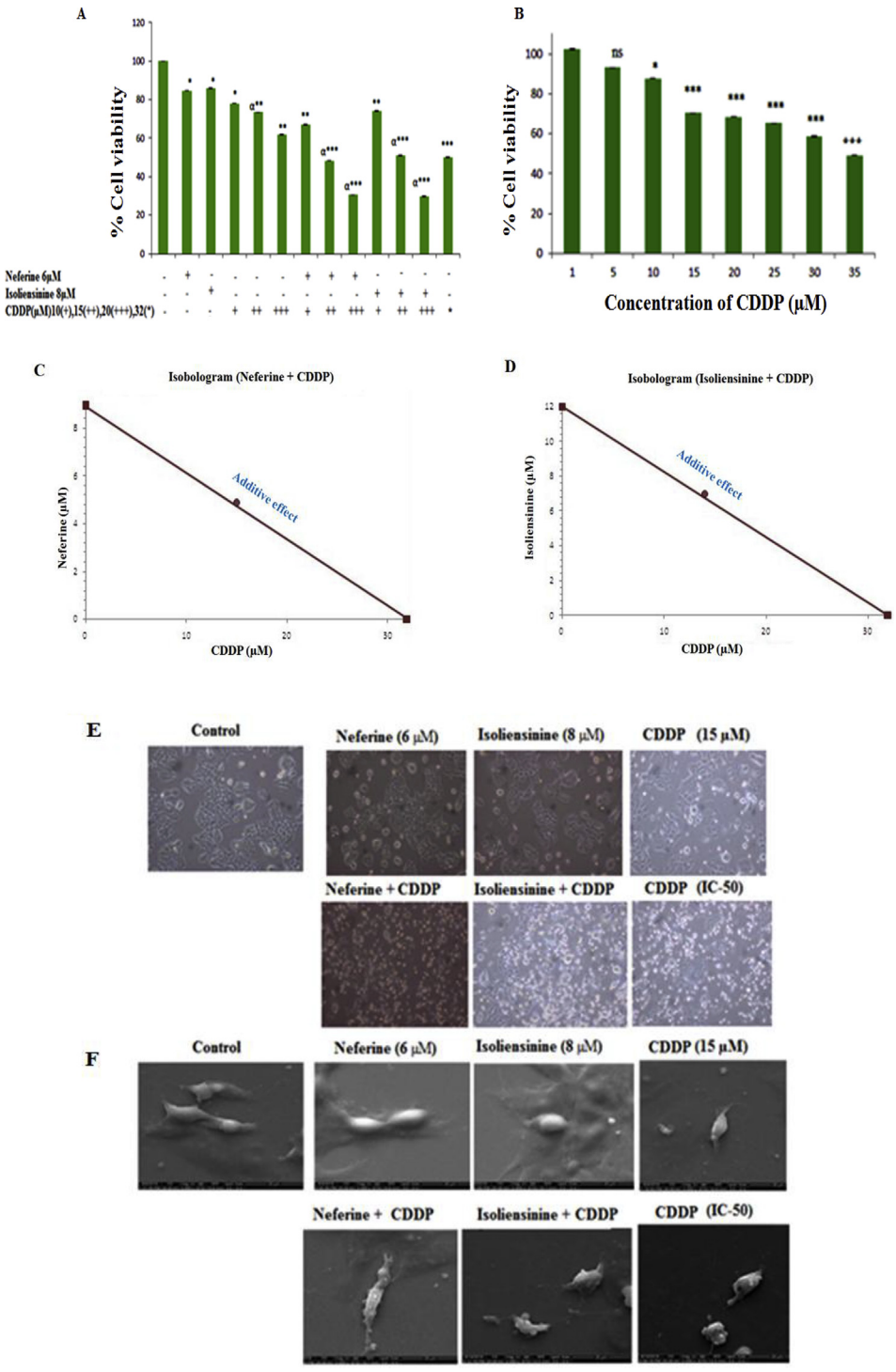

Fig. 1. (A). Combinatorial regimen of neferine/isoliensinine with CDDP (at a dose much lower than IC_{50} dose of individual drugs) exhibited cytotoxicity equivalent to IC_{50} dose of CDDP in HCT-15 cells compared to individual treatment groups. Data were obtained & analyzed from experiments carried out in triplicates and expressed as Mean ± S.D. ***p < 0.001, **p < 0.01,*p < 0.05, NS-No Significance when compared to control (One-way ANOVA followed by Tukey's multiple comparison test). **(B).** Cell viability of HCT-15 cells was performed for different concentrations of CDDP to determine IC_{50} value. The IC_{50} doses for neferine (8.95 μM) and isoliensinine (12.5 μM) were also determined in a similar manner (data not shown) (Allen and Spiteri, 2001). ***C, D.*** *Isobologram analysis:* MTT data were analyzed to ascertain combination index (CI) using CalcuSyn software. CI values at IC_{50} illustrate the additive effect (neferine & CDDP), (isoliensinine & CDDP) on HCT-15 cells. The following concentrations of CDDP (alone) IC_{50} – 32 μM, neferine (alone) IC_{50}- 8.95 μM, isoliensinine (alone) IC_{50} - 12.5 μM, neferine 6 μM + CDDP 15 μM and isoliensinine 8 μM + CDDP 15 μM were used for CI index analysis (CI > 1 antagonism, CI = 1 additive effect, CI < 1 synergism). ***E.*** *Light microscopy:* Morphological changes in various treatment groups as observed through light microscopy indicate that cells treated with combination of neferine/isoliensinine with CDDP, exhibited significant cytotoxic changes (similar to IC_{50} dose CDDP) such as cell rounding and floating cells compared to individual treatments groups (magnification, 200x). ***F.*** *SEM analysis:* The surface morphological changes observed through SEM in different treatment groups revealed significant apoptotic features such as membrane blebbing in cells treated with combinatorial regimen (similar to IC_{50} dose of CDDP) compared to individual treatments.

3.3. Cell cycle analysis

Cell cycle distribution was ascertained in various treatment groups using propidium iodide uptake and flow cytometry. The combinatorial regimens resulted in an increase in the proportion of cells in the S phase indicating higher fraction of cell cycle arrest at S phase (Figure-3A). Furthermore, the 'isoliensinine + CDDP' induced high % of cell cycle arrest in S phase compared to individual treatments, where IC_{50} dose of CDDP was used as a positive control (Figure-3B).

3.4. Intracellular ROS generation

ROS production is the principal indicator of oxidative stress in apoptotic cancer cells. The results of the time course study (data not shown) revealed that the ROS production was initiated within 15 min and consequently peaked at 1 h as indicated by a significant rise in DCF relative fluorescence. Here, 32 μm of CDDP treated cells served as a positive control and NAC (ROS inhibitor) served as a negative control. So, 1 h time period was chosen for further study. As shown in Figure-4A, 4B, the intracellular ROS generation was significantly higher in combinatorial regimens (equivalent to IC_{50} dose of CDDP) compared to individual treatment groups.

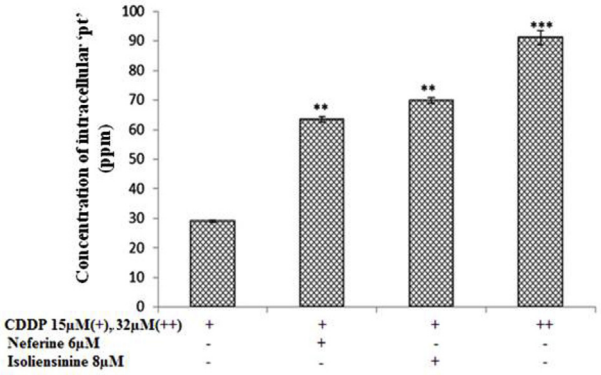

Fig. 2. Intracellular platinum measurements (ICP-MS analysis) – Isoliensinine combination with CDDP treatment induced more CDDP uptake into the HCT-15 cells compared to neferine with CDDP and other individual treatment groups (15 μM CDDP alone), where IC_{50} (32 μM) of CDDP used as a positive control and the platinum concentration in HCT-15 cells was expressed in 'ppm'. Data were obtained from triplicate experiments and expressed as Mean ± S.D (**p < 0.01, ***p < 0.001) compared to CDDP alone.

3.5. Intracellular calcium concentration

Intracellular Ca^{2+} accumulation in programmed cell death is well documented for several cancer cell lines. As shown in Figure-4C, the $[Ca^{2+}]_i$ was significantly augmented during combinatorial regimen treatments (equivalent to IC_{50} of CDDP) compared to the individual treatment groups.

3.6. Mitochondrial membrane potential (MMP)

Combinatorial regimens (CDDP with neferine/Isoliensinine) induced a significant decrease in the mitochondrial membrane potential (ΔΨm) as indicated by a decrease in fluorescence intensity compared to individual treatments. CCCP was used as a positive control (Figure-4D).

3.7. Annexin FITC/PI assay & PI uptake assay for apoptosis determination

Flow cytometry analysis of Annexin FITC/PI stained cells have depicted that the early apoptotic population was significantly higher in combinatorial regimens compared to the individual treatments (Figure-5A). Cells pretreated with 'NAC' significantly decreased the early apoptotic events indicating ROS-mediated oxidative stress of HCT-15 cells. Furthermore, the apoptosis mediated by the above combinatorial groups was also confirmed by the enhanced number of PI-positive cells compared to control and individual treatment groups (Figure-5B).

3.8. Apoptotic protein expression analysis through Western blotting

Additive apoptotic effects of 'neferine + CDDP', 'isoliensinine + CDDP' combinatorial regimens were compared as per the cell survival/apoptotic/proapoptotic protein expression profiles with other treatment groups using Western blotting. Analysis of Bcl2 protein, PARP-mediated simultaneous activation of 'caspase-cascade protein expression' can critically ascertain apoptotic fate of cells. The above combination regimens have significantly decreased the Bcl2 (anti-apoptotic protein) expression with a concomitant increase in the apoptotic protein expression of caspase −3, −9, cytochrome *c*, and PARP cleavage compared to individual treatment groups indicating mitochondria-mediated programmed cell death via intrinsic pathway. PARP was completely cleaved and expressed in the form of cleaved PARP (Lu et al., 2006) (Figure-6).

3.8.1. MAPK/PI3K/pAKT pathway

Combinatorial regimens effect on PI3K/pAKT pathway in HCT-15 cells was analyzed since this pathway mainly involved in ROS-mediated intrinsic apoptosis. As shown in Figure-6, the combinatorial regimens have significantly declined PI3K expression with a consequent decline in pAKT expression level compared to individual treatments.

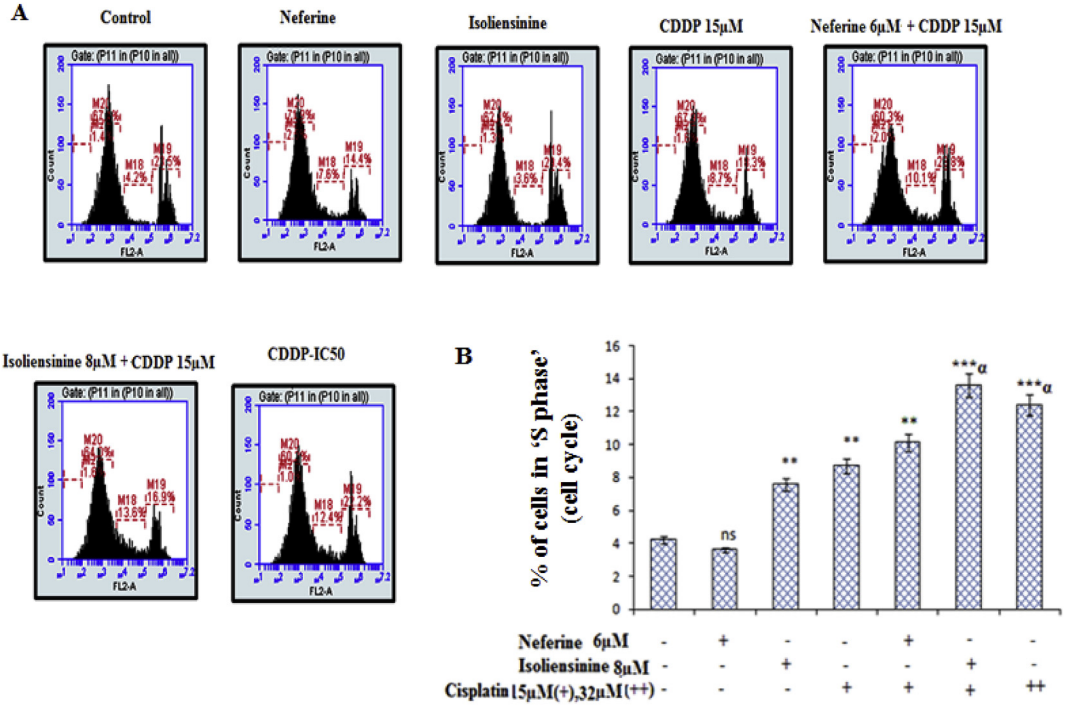

Fig. 3. The results of cell cycle analysis through flow cytometry in different treatment groups. (A). The % of cells arrested in 'S phase' of cell cycle was significantly increased in cells treated with combinatorial regimen (neferine/isoliensinine and CDDP) compared to individual treatments.**(B).** Graphical representation of % of HCT-15 cells arrested in 'S phase' of cell cycle in different treatment groups. Data were obtained from experiments carried out in triplicates and expressed as Mean ± S.D (**p < 0.01, NS-No Significance when compared to control, ***α p < 0.001 when compared to 15 μM cisplatin).

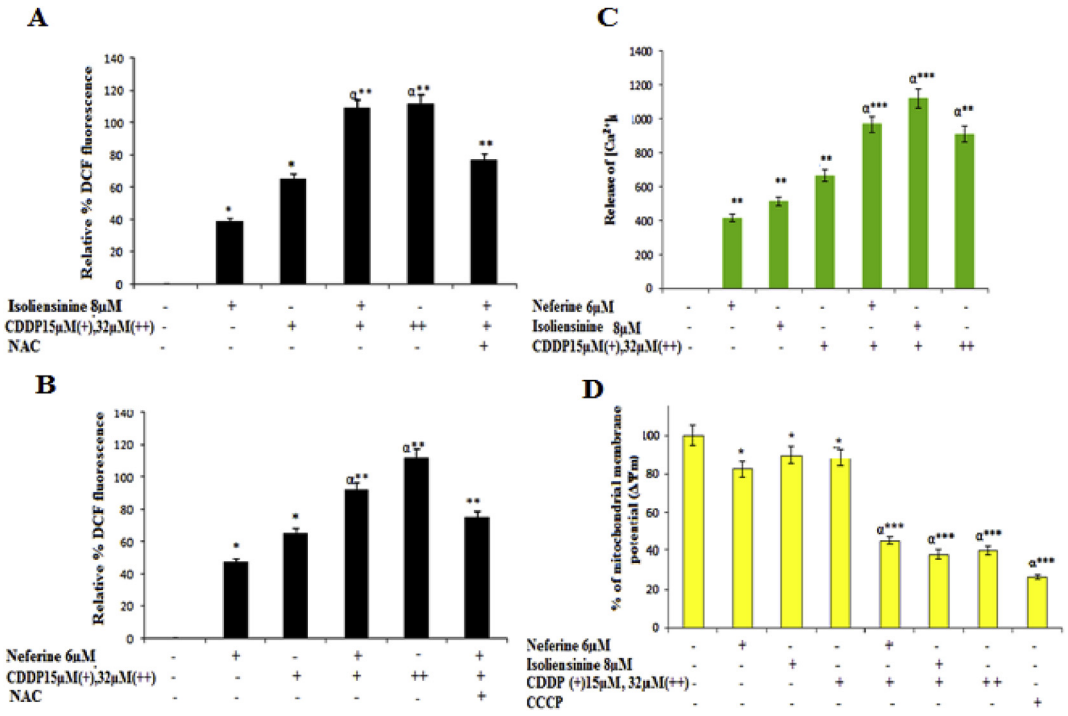

Fig. 4. ROS production was analyzed in the presence/absence of 'NAC inhibitor' in various treatment groups.(A) (B). Time course study was carried out to assess intracellular ROS concentration in different treatment groups (data not shown). ROS generation was initiated within 15 min which consequently peaked at 1 h. ROS production was significantly increased in the cells which received combination regimens of CDDP with neferine/isoliensinine than the individual treatment groups. Further, it was observed that isoliensinine and CDDP combination induced the highest ROS generation compared to other treatment groups. Whereas, NAC pretreatment has significantly decreased the level of ROS generation in various treatment groups of HCT-15 cells. Treatment with cisplatin (50 μM) served as a positive control. Results were expressed in relative DCF-fluorescence intensity compared to control. Data were expressed in terms of Mean ± SD. (*$p < 0.05$, **$p < 0.01$ when compared to control and α**$P < 0.01$, when compared 15 μM cisplatin alone treated groups. **(C).** Intracellular [Ca^{2+}] levels at different time points were observed in various treatment groups after loading with FURA-2AM through spectrofluorimetry and the rise in $[Ca^{2+}]_i$ was depicted as relative % FURA-2-AM fluorescence. Intracellular calcium concentration was significantly increased in combinatorial regimens equivalent to IC_{50} dose of CDDP. Data were obtained from separate experiments executed in triplicates and expressed as Mean ± S.D (**$p < 0.01$, when compared to control, α***$p < 0.001$, **$p < 0.01$ when compared to 15 μM cisplatin alone treated groups).**(D).** HCT-15 cells treated with the neferine/isoliensinine and CDDP showed a significant decrease in mitochondrial membrane potential as indicated by the decline in fluorescence intensity compared to other treatment groups. Data were obtained from separate experiments executed in triplicates and expressed as Mean ± S.D (*$p < 0.05$ when compared to control, α***$p < 0.001$ compared to 15 μM cisplatin alone treated groups).

Furthermore, we investigated the effect of above combinatorial regimens on the expression of P38 and pP38 in HCT-15 cells and the results educe the enhanced expression of pP38 protein when compared to their expression level in control and individual treatment groups. These results suggest that combination regimens induced the apoptosis through activation of p38 MAPK pathway in HCT-15 cells.

4. Discussion

Cancer chemotherapy is manifested by the nonselective cytotoxicity to other non-target tissues. Combinatorial strategies include phytoconstituents as adjuncts with chemotherapy and these approaches have been gaining pace to act against the cancer cells selectively by modulating multiple molecular signaling mechanisms responsible for cell growth, survival and differentiation (Ashkenazi and Dixit, 1998; Omabe and Okorocha, 2011; Selvi et al., 2017). A recent study by Afrin et al. (2019a) showed that strawberry tree honey (STH) exerted anticancer efficacy in both metastatic LoVo and HCT-116 colorectal cancer cells by inhibition of NF-κB & Nrf2 signaling pathways. STH has been reported to induce cell cycle arrest and augment apoptosis in colon cancer cells by modulating EGFR and MAPKs signaling pathways (Afrin et al., 2019b). On a similar note, Manuka honey (MH) has been shown to induce ROS-mediated inhibition of Nrf2-signaling and augmentation of mitochondrial-mediated intrinsic pathway of apoptosis. It also inhibited colorectal cancer cell survival in metabolic stress situations through AMPK mediated downregulation of PGC1α and SIRT1(Afrin et al., 2018b). MH has also been shown to induce ER stress-mediated apoptosis in colorectal cancer cells (Afrin et al., 2018a).

Recently, Brosimone I has been reported to induce ER stress-mediated cell death through ROS-mediated modulation of CaMKKβ-AMPK signaling (Zhao et al., 2019). 8-C-(E-phenylethenyl) quercetin (8-CEPQ), a quercetin derivative induced autophagy mode of death in colorectal cancer cells via activation of ERK (Zhao et al., 2017). Lebein, a snake venom, exerted its inhibitory effect on human colon adenocarcinoma cells (LS174, HCT116, HT29) proliferation through modulation of cell cycle regulating proteins (Zakraoui et al., 2017). Kaempferol mediated chemosensitization was reported in 5-FU-chemoresistant LS174-R colorectal cancer cells through modulation of JAK/STAT3, MAPK signaling as well as PI3K/AKT and NF-κB signaling pathways (Riahi-Chebbi et al., 2019). The present study investigated the effect of neferine/isoliensinine combinatorial regimen with CDDP on the role of ROS-mediated activation of p38 MAPK pathway and apoptosis of HCT-15 cancer cells.

Intracellular concentration of cisplatin (CDDP) plays a major role in CDDP-mediated cytotoxicity in cancer cells (Kilari et al., 2016). Present study has demonstrated the high 'CDDP accumulation' in HCT-15 cells during combinatorial regimens of 'neferine/isoliensinine with CDDP' compared to CDDP alone treatment, consequently greater cytotoxicity was observed in the cells treated with combinatorial regimens compared to other treatment groups. A possible reason for the 'increased sensitivity of HCT-15 cells to CDDP-induced cell death' in the combinatorial regimens is higher CDDP uptake by the cells exposed to

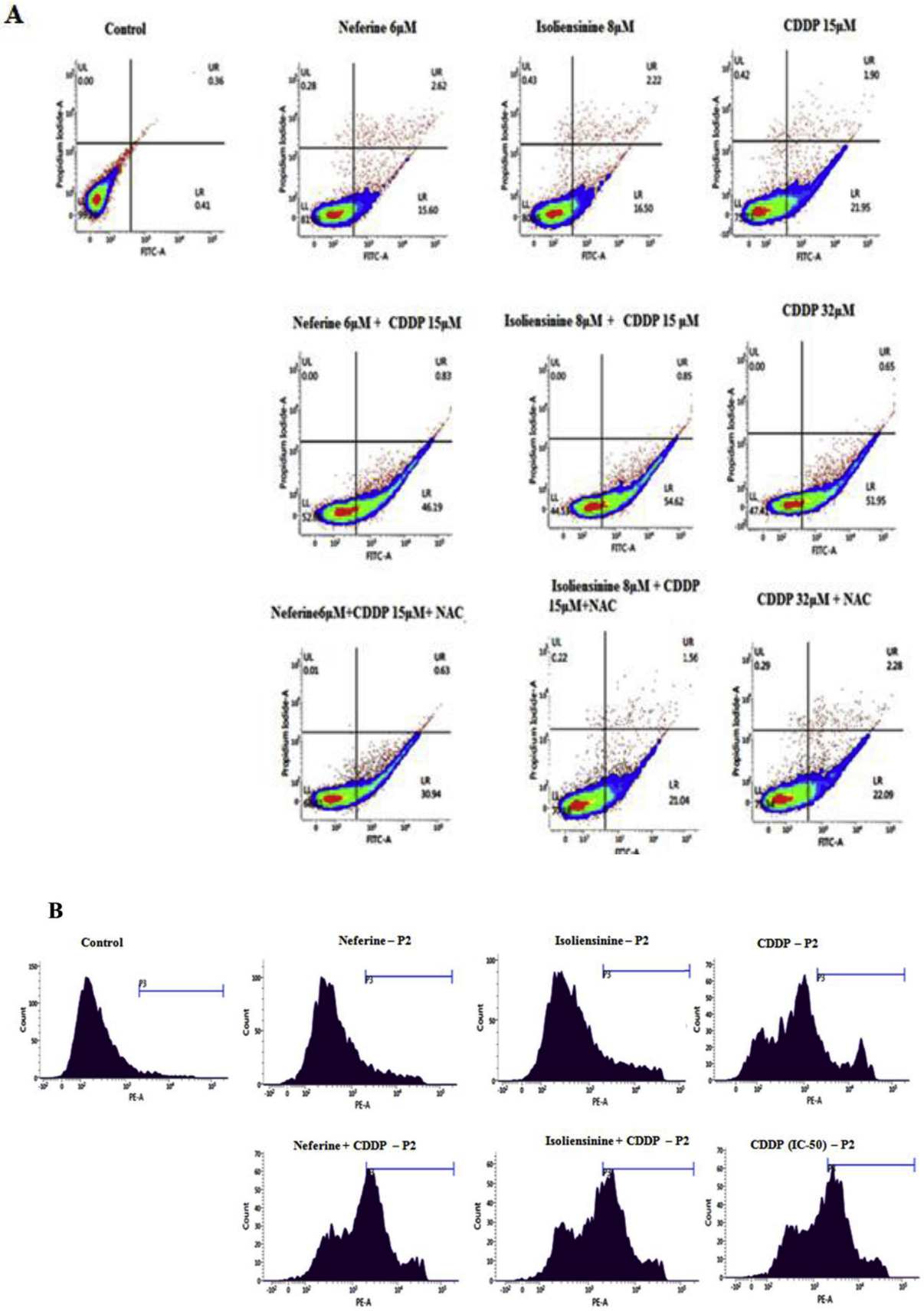

Fig. 5. Flow cytometry/Annexin V-PI staining analysis - **(A).** Combinatorial regimen of neferine/isoliensinine with CDDP augmented early apoptosis in HCT-15 cells than the other individual treatment groups, where isoliensinine with CDDP showed a significant increase in the number of early apoptotic cells compared to the combinatorial regimen of neferine and cisplatin.**(B).** A significant increase in the PI uptake was observed in cells treated with combination regimen of neferine/isoliensinine with CDDP (equivalent to IC_{50} dose of CDDP) compared to individual treatment groups.

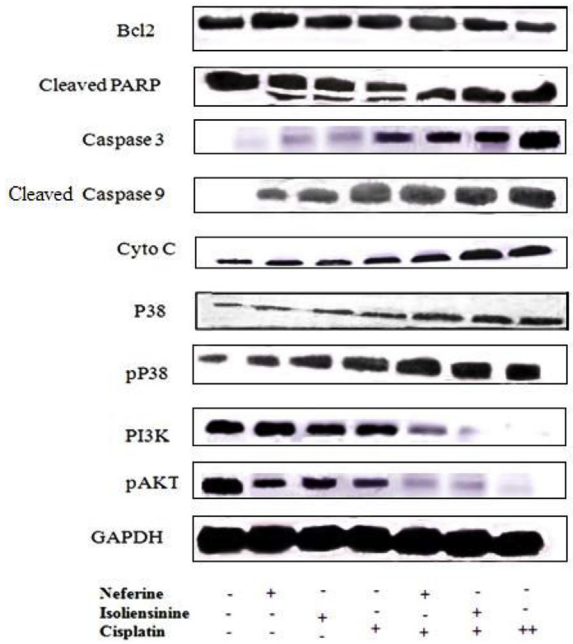

Fig. 6. Neferine/isoliensinine with CDDP combination induced a significant increase in apoptosis through activation of the MAP kinase pathway and inhibited cell survival compared to individual treatments in HCT-15 cells. Western blot analysis elucidated the apoptotic protein expression profiles in various treatment groups. All expression values were normalized to the value of GAPDH, which was used as an internal control.

combinatorial regimen with CDDP & neferine/isoliensinine. Thus, it is clearly indicating that combinatorial regimen with CDDP & isoliensinine induced higher CDDP uptake into the cells compared to other treatments.

Reactive oxygen species (ROS) play a pivotal role in cellular homeostasis and physiological processes ranging from cell signaling, cell aging and inflammation etc. Abnormal generation of ROS leads to a sequential rise in the stress on cell organelles and other cells structures which in turn alter molecular signaling pathways leading to cell death (Selvi et al., 2017; Sivalingam et al., 2017). Several cytotoxic agents such as phytochemicals, intrinsic and extrinsic factors, chemotherapeutic agents enhance intracellular ROS generation leading to increased oxidative stress inside the cancer cells (Vallejo et al., 2017). However, excess ROS production is normally neutralized by the intrinsic cellular antioxidant system but a notable uncontrolled escalation of ROS will cause the failure of intrinsic antioxidant system to neutralize the ROS resulting in enhanced oxidative damage to all essential macromolecules such as protein, lipids and nucleic acid, which lead to irreversible cell damage and cell death. Several phytochemicals could induce a notable escalation of ROS, enhance oxidative damage consequently induce cell death or apoptosis (Vallejo et al., 2017).

CDDP is a proven chemotherapeutic drug that can induce oxidative stress in cancer cells which ultimately leads to cellular apoptosis (Marullo et al., 2013; Scherz-Shouval et al., 2007). The present study demonstrated that the combinatorial regimens of CDDP withneferine/isoliensinine induces ROS-induced mitochondrial mediated intrinsic pathway of apoptosis in HCT-15 cells.

Moreover, the ROS production is the critical signal that could induce MAPK and JNK pathways (Chen et al., 2008). It has also been reported that activation of MAPK plays a critical role in combinatorial regimen of 'neferine and doxorubicin'-induced apoptosis in lung cancer A549 cells (Poornima et al., 2014). Consistent with these reports, the high amount of apoptotic cell death through intrinsic pathway has exclusively occurred in combination of CDDP with neferine/isoliensinine via activation of ROS-mediated P38 MAPK pathway in colon cancer HCT-15 cells.

PI3K/AKT signaling pathway has been reported to be activated in several types of cancers including colorectal cancer and this signaling affects a variety of biological process, including cell proliferation, cell migration, apoptosis and differentiation (Yu et al., 2016). Akt is a nodal point that crosslink PI3K signaling pathway and it has recently been proven that this protein could promote cell survival by inhibiting apoptosis through blocking of pro-apoptotic protein production and consequent inhibition of cytochrome *c* release from mitochondria (Kalimuthu and Se-Kwon, 2013). However, some researchers have found that Akt could influence cell senescence and induce apoptosis by modulating other cell survival signaling pathways. Nogueira et al. (2008) have illustrated the Akt-mediated oxidative stress and consequent ROS-mediated cell death by abolishing the expression of ROS scavengers and downstream cascades of FoxO such as sestrin3 (Nogueira et al., 2008). In our study, we observed that combination of CDDP with neferine/isoliensinine decreased expression of 'phosphorylated-AKT' and further the combination also decreased the expression of PI3K level in HCT-15 cells resulted in apoptosis.

Mitochondrial dysfunction is initiated by a various cascade events like ROS production, high intracellular Ca^{2+} release and other intrinsic/extrinsic factors (Poornima et al., 2014). Intrinsic ROS production could induce the high influx of intracellular calcium that damages the mitochondrial membrane. Disruption of mitochondrial trans-membrane potential ($\Delta\Psi M$) results in increased mitochondrial permeability that could enhance the release of cytochrome *c*/apoptogenic proteins from mitochondria (Sivalingam et al., 2017). Thus, the mitochondrial-mediated apoptosis involves permeability changes in mitochondrial membrane, accompanied by the release of cytochrome *c* into cytosol (Li et al., 2013). Results of the present study revealed a significant increase in the intracellular Ca^{2+} with a concomitant decline in '$\Delta\Psi M$' of HCT-15 cells subjected to combinatorial regimens compared to individual treatments, indicating ROS-mediated oxidative stress.

Bcl2, an anti-apoptotic protein inhibits apoptosis by regulating mitochondrial membrane potential whereas Bax, Bad and cytochrome *c* are apoptotic proteins, required for activation of caspase 9 & caspase 3 to trigger apoptosis (Li et al., 2013). Caspase 3 is required for the activation of PARP cleavage, a process to endorse apoptosis. In our study, cells treated with the combinatorial regimens showed significant increase in expression of the apoptotic proteins such as cytochrome *c*, caspase-9, caspase-3, complete PARP cleavage and decreased expression of the anti-apoptotic protein, Bcl2 compared to individual treatments. In contrast, pretreatment with NAC decreased the early apoptotic positive cells in cells subjected to combinatorial regimens.

In conclusion, the combinatorial regimen of CDDP with neferine/isoliensinine induced high CDDP uptake with a simultaneous increase in ROS-induced mitochondiral mediated apoptosis via intrinsic pathway. These results indicate that both neferine and isoliensinine could sensitize the HCT-15 cells to a low dose of cisplatin by increasing the intracellular uptake of cisplatin, finally, induce hypergeneration of ROS, resulting in cell cycle arrest and apoptosis via P38 MAPK/PI3K/pAKT pathway, where isoliensinine + CDDP regimen proved to be more effective in inducing the above changes in HCT-15 cells.

Conflicts of interest-

The authors declared no conflict of interest to this work.

Ethical standards –

No animal models were chosen for this research.

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