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Neferine and isoliensinine from *Nelumbo nucifera* induced reactive oxygen species (ROS)-mediated apoptosis in colorectal cancer HCT-15 cells

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Several scientific reports documented the potent anti-cancer effect of various natural products such as curcumin, epigallocatechin gallate (EGCG), resveretrol via the rise in reactive oxygen species (ROS) and dysfunction of anti-apoptotic gene expressions. The present study evaluated the anticancer potential of lotus derived alkaloids, neferine and isoliensinine on colorectal cancer HCT-15 (CRCs) cells. Neferine/Isoliensinine treatments induced cytotoxicity with sequential enhancement of intracellular ROS, intracellular calcium $[Ca^{2+}]_i$ and mitochondrial membrane potential (ΔψM). Furthermore, cell cycle, Annexin FITC and PI uptake were carried out by flow cytometry. Activation of p38 MAPK and apoptotic genes expression was done by western blotting and reverse transcription polymerase chain reaction (RT-PCR) respectively. Results indicate neferine/isoliensinine induced hyper-generation of ROS was responsible for their cytotoxic effect in CRCs consequently, with a significant increase in $[Ca^{2+}]_i$ followed by a significant decrease in the ΔψM. The above treatments induced cell cycle arrest at G1 phase whereas apoptosis was indicated by upregulation of p38 MAPK, Bax, caspase 9, caspase 3, cleaved poly (ADP-ribose) polymerase (PARP) and down-regulation of Bcl2. In conclusion, this is the first study of anticancer effect of neferine/isoliensinine via apoptotic mechanism in CRCs and the results suggest that neferine/isoliensinine induced apoptosis through the ROS generation, activation of p38 MAPK which in turn induces mitochondrial mediated apoptosis.

Key words: Neferine, isoliensinine, reactive oxygen species (ROS), HCT-15, apoptosis.

INTRODUCTION

Recent research focus has shifted towards natural products as chemotherapeutic agents. Vincristine, irinotecan, etoposide and paclitaxel are some plant-derived natural compounds that are used in cancer

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medicine. The chief source of bisbenzylisoquinoline alkaloids is *Nelumbo nucifera* (Nymphaeceae) often referred to as the medicinal lotus plant. Several vegetative parts plant such as rhizomes, stem, seeds, leaves, and embryos are extensively used in Chinese and Indian traditional medicine to treat skin diseases, cardiovascular diseases and neurodegenerative disorders (Asokan et al., 2018). However, the extracted compounds of this plant have been used in treating hypertension, lung diseases and microbial diseases of humans in several Asian nations. Several chemical constituents such as glycosides, steroids, fatty acids, alkaloids, minerals, and vitamins have been isolated from several parts of the *N. nucifera*. In the present scenario, the isolated alkaloids from seeds, stem, and plumule of *N. nucifera* have shown profound therapeutic efficacy in treating various metabolic diseases including cancer. Neferine is a bis-benzylisoquinoline alkaloid extracted from the seed embryo of *N. nucifera* and several research reports have illustrated that the isolated neferine possess pharmacological efficacy *in vitro* against cancer.

Colon rectal cancer (CRC) is the third most prevalent cancer worldwide. As per the United States Surveillance and Epidemiological studies, the number of CRC patients is predicted to increase to 90% by 2030 (Koosha et al., 2016). The incidence of CRCs has been on the rise in the past few decades due to changes in the lifestyle which include technological development, unhealthy food habits and sedentary life. Current clinical treatment options include surgery followed by chemotherapy. Chemotherapy causes adverse side effects such as alopecia, diarrhoea, myelosuppression and immune suppression which in turn reduce the quality of life in these patients (Bagheri et al., 2018). Hence, there is an urgent requirement for new therapeutic interventions with minimal side effects.

Oxidative stress plays a major role in several pathological conditions such as cancer, cardiovascular diseases and neurodegenerative diseases (Zhang et al., 2015). ROS generation is a cellular event which occurs during normal metabolism as well as in pathological conditions, whether it is beneficial or harmful is based on the cellular antioxidant status. An imbalance of reactive oxygen species (ROS) and cellular antioxidants result in various pathological conditions including cancer. Several studies suggest ROS involvement in various forms of cell death namely apoptosis, autophagy and necrosis (Su et al., 2019). Growing body of evidence also suggests higher ROS levels in cancer cells compared to normal cells (Kumari et al., 2018). Further, these studies indicate that cancer cells are more susceptible to ROS mediated apoptosis induced by exogenous agents compared to normal cells. Further, these studies indicate that cancer cells are more susceptible to ROS mediated apoptosis induced by exogenous agents compared to normal cells (Poornima et al., 2013a). Previous reports have suggested apoptosis induction through ROS mediated activation of p38 (Zhang et al., 2015).

Several studies have reported the anticancer effect of isolated natural product, that is, neferine in various cancer cells (Asokan et al., 2018). Neferine induced apoptosis in osteosarcoma cells through the modulation of stress-activated protein kinase, that is, p38 MAPK signalling (Zhang et al., 2012). This natural compound has been reported to enhance the therapeutic efficacy of anticancer agents namely doxorubicin and cisplatin against A549 cells (Sivalingam et al., 2017) while it enhanced the therapeutic efficacy of mitomycin-C in cervical cancer (Hela) cells (Eid and Abdel-Rehim, 2017). Neferine induced autophagy was reported in lung (Poornima et al., 2013b) and ovarian cancer cells (Xu et al., 2016). Recent reports documented that isoliensinine causes apoptosis mode of cell death in triple negative breast cancer (MDA-MB-231) through ROS mediated MAPK pathway (Zhang et al., 2015) and in hepatocellular carcinoma by modulating NF-kB (Shu et al., 2015) pathway. Further, it induced autophagy-mode cell death in cervical cancer cells (Law et al., 2014). However, there are no reports regarding the anticancer potential of neferine/isoliensinine in CRCs. Hence, the present study evaluates the comparative efficacy of these alkaloids against CRCs.

MATERIALS AND METHODS

Chemicals and cell culture

Neferine (CAS No.2292-16-2) and isoliensinine (CAS.No.6817-41-0) were purchased from Sigma and Baojiher best bio-tech Co. Ltd., China. The cell culture media, RPMI 1640 was purchased from Gibco. PARP, p38 and pP38 primary antibodies were purchased from Cell Signalling Technology. All the primers used for this study as well as propidium iodide were purchased from Sigma chemicals. Annexin V FITC kit was purchased from BD bioscience.

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 and were maintained at 5% CO_2 with 95% humidity. In addition, the cells were supplemented with penicillin (100 units/ml), streptomycin (30 µg/ml) and gentamycin (20 µg/ml) antibiotics. All the experiments were carried out within 20 passages (Passage no. 50 to 70) to conform reproducibility and consistency of cell population.

Cell proliferation and clonogenic assay

1×10^5 cells per well were seeded in 96 well plates and left overnight for cell attachment. Afterwards, cells were treated with neferine and isoliensinine for 24 h time period. At the end of the treatment period, the media was removed and 5 mg/ml of 3-(4, 5-Dimethylthiazol-2-Yl) -2, 5-Diphenyltetrazolium Bromide (MTT) was added and left for 4 h. Subsequently, DMSO was added to dissolve

the formazan crystals and reading was obtained at 570 nm.

For clonogenic assay, 500 viable cells from control and treated groups were cultured for 14 days to form clones. After two weeks, cells were fixed with methanol and stained with 0.3% of crystal violet stain and the colonies were counted.

Lactate dehydrogenase (LDH) and nitric oxide (NO) release

HCT-15 cells were treated with neferine/isoliensinine for 24 h. After treatment, the LDH leakage in culture supernatant was ascertained using lithium lactate (30 mM), glycine buffer ((50 mM), pH-10), and nicotinamide adenine dinucleotide (NAD, 3 mM). The activity of LDH was measured by conversion of lithium lactate to pyruvate, in the presence of NAD, by using dinitrophenylhydrazine (DNPH) as the colorimetric reagent at 420 nm in Synergy H1 Multi-Mode Reader (Biotek). LDH activity was calculated by a standard linear graph on pyruvate using regression analysis and expressed in terms of percent leakage of LDH. Nitric oxide released inside the culture subsequently measured using Greiss reagent (Stuehr and Marletta, 1987) in terms of nitrite content as nitrite is considered as the stable output product of nitric oxide and represented in 'nM'.

Cell cycle analysis

To determine the effect of neferine/isoliensinine on cell cycle phases, 5×10^6 cells were seeded per well in 6 well plates, after overnight attachment, cells were treated with neferine/isoliensinine for 24 h. At the end of the treatment schedule, cells were harvested and fixed with 70% ethanol overnight at 4°C. Later, the cells were subjected to PBS washings subsequently stained with propidium iodide (PI) and incubated for 30 min, at room temperature. Cells were analyzed by BD C6 Accuri.

Reactive oxygen species (ROS) analysis

Intracellular ROS level was determined by the fluorescent probe, DCFDA. Briefly, 2×10^5 cells were seeded in 24 well plates. After attachment, cells were washed with PBS and incubated with DCFH-DA at 37°C for 30 min, in a CO_2 incubator. Subsequently, these cells were treated with neferine (0, 15, 30, 45 min, and 1 h)/isoliensinine (0, 1, 2, 3, 4, and 5 h) at different time points. One treatment group with H_2O_2 (50 µM) was included to serve as a positive control. At the end of the treatment period, the cells were harvested, centrifuged, washed and resuspended in PBS and the fluorescent intensity was measured using spectrofluorimetry as Ex./Em., at 480/520 nm. The estimations were carried out in triplicates, keeping the number of cells per treatment group the same to ensure reproducibility. The fluorescence peak was used to represent the level of ROS production. The values were expressed as percentage of relative fluorescence.

Intracellular Ca^{2+} level

Intracellular calcium level in treated and untreated cells was determined using florescent dye, 'FURA-2/AM' by Spectrofluorimetry. Briefly, 1×10^5 cells were seeded in 24 well plates and treated with neferine/isoliensinine for 24 h. At the end of treatment schedule, the media was removed and 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)); cells were incubated with FURA-2/AM in buffer A for 45 min, and after the incubation, florescent intensity was measured as Ex./Em., at 500/340 nm. The values of calcium release were represented as % of relative fluorescence intensity compared to untreated.

Mitochondrial membrane potential measurement

Mitochondrial membrane integrity in different treatment groups was assessed in terms of retention of the dye Dio6 (3, 3'-dihexloacarbocyanine iodide) in comparison with control. CRCs were treated with neferine/isoliensinine for the 24 h time period. After the treatment period, the media was removed and cells were washed twice using PBS. Then, the cells were loaded with Dio6 dye (50 nm), incubated for atleast 30 min and the fluorescence intensity was measured at 488/500 nm (Ex./Em.,) using spectrofluorometer (Biotek, US). Cells were incubated with CCCP (50 µm) for 15 min (induce mitochondrial depolarization) prior to loading with DiOC6-dye for the positive control. Finally, the mitochondrial membrane potential (ΔψM) was expressed in terms of percentage of relative fluorescence intensity when compared with control.

Scanning electron microscopy (SEM) analysis

Surface morphological changes of neferine/isoliensinine treated and untreated HCT-15 cells were determined by SEM. After treatment, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 1 h. Then, the cells were washed with cacodylate buffer twice. Followed by secondary fixation with 1% Osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 1 h, the cells were dehydrated with increasing concentration of ethanol (30, 60, 90 and 100%) each for 30 min. Finally, the samples were air dried and viewed under scanning electron microscopy.

Western blotting

Total protein was collected from treated and untreated cells using lysis buffer (Sigma Aldrich). Total protein was estimated by Bradford method. Equal amount of proteins were separated in 10% gel using SDS-PAGE unit at 100 V for 2 h. Separated proteins were transferred to nitrocellulose membrane using semidry equipment. After membranes were blocked with 5% non-fat milk protein for 1 h in TBST, they were washed with TBST thrice at room temperature. Then, the blots were incubated with specific primary antibody at 4°C overnight. Next day, the blots were washed with TBST thrice for 5 min, each. Finally, the blots were incubated with HRP-linked secondary antibody for 2 h. Subsequently, DAB and H_2O_2 were added and blots stored in the dark till the bands appeared.

Quantitative real-time PCR analysis

After treatment schedule, RNA was isolated by conventional trizol method and quantified by take3 plate (Biotek). 1 µg or 500 ng of RNA was used for cDNA conversion according to manufacturer's protocol (Takara A1640). Gene expressions of treated and untreated groups were determined by real time PCR analysis and changes in mRNA expression levels normalized by GAPDH. Primers sequences used are listed in Table 1 (Ravi et al., 2015; Shafiee et al., 2016).

Propidium iodide (PI) uptake

Briefly, 2×10^5 HCT-15 cells were seeded in 12 well plates and left for overnight attachment. Subsequently, the cells were treated with neferine/isoliensinine for 24 h. Cells from different treatment groups were harvested and washed with PBS and stained with PI for 10

Table 1. Primer sequences used for real-time RT-PCR analysis.

S/N	Gene	Forward (5'-3')	Reverse (5'-3')
1	*BAX*	TGCTTCAGGGTTTCATCCAG	GGCGGCAATCATCCTCTG
2	*Bcl2*	AGGAAGTGAACATTTCGGTGAC	GCTCAGTTCCAGGACCAGGC
3	*CASPASE-3*	CAGCACCTGGTTATTATTCT	TTGTCGGCATACTGTTTC
4	*CASPASE-9*	CCAGAGATTCGCAAACCAGAGG	GAGCACCGACATCACCAAATCC
5	*GAPDH*	ACCCAGAAGACTGTGGATGG	CAGTGAGCTTCCCGTTCAG

min, subsequently the stained cells were analyzed by flow cytometry.

Annexin V/PI apoptosis assay

Neferine/Isoliensinine-induced apoptosis in HCT-15 cells were determined by flow cytometry. Briefly, HCT-15 cells were treated with neferine/isoliensinine for 24 h. Then, cells were stained with Annexin V and PI according to BD bioscience kit protocol and analyzed using flow cytometry.

Statistical analysis

All the experiments were carried out independently three times. The data were obtained and expressed in terms of mean and standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) to express the difference between the groups using SPSS 20.0 Software.

RESULTS

Cell viability and colony formation assay

Cytotoxic effects of neferine/isoliensinine on colon cancer HCT-15 cells were assessed by MTT assay. The cells were treated with various concentrations of neferine/isoliensinine for 24 h. As shown in Figure 1A, neferine/isoliensinine suppressed the proliferation of colon cancer HCT-15 cells in a dose-dependent manner and the IC_{50} values were found to be 8.95±1.3 and 12.0±1.5 µM, respectively (Figure 1B). As shown in Figure 1D, untreated HCT-15 cells showed normal morphology whereas treatment with IC_{50} dose of neferine/isoliensinine resulted in cellular morphology alterations such as cell shrinking and rounding in addition to floating of dead cells in the medium. Furthermore, the effect of neferine/isoliensinine on colony forming ability of HCT-15 cells was analyzed by clonogenic assay. The results showed that both neferine/isoliensinine significantly decreased the colony formation ability in a dose-dependent manner in HCT-15 cells compared to control (Figure 1C).

NO and LDH release

As shown in Figure 2A and B, when HCT-15 cells were treated with IC_{50} doses of neferine/isoliensinine, a significant increase in the concentration of NO and LDH compared to control were observed in the culture supernatant, representing the cytotoxic effect of neferine/isoliensinine on colon cancer HCT-15 cells.

Generation of ROS level

In order to determine the effect of neferine and isoliensinine treatment on 'ROS generation' in HCT-15 cells, a time course study was carried out with the fluorescence probe (DCFDA) where H_2O_2 was used as positive control. Neferine/Isoliensinine treated cells showed a significant increase in the intracellular ROS levels in a time-dependent manner. As shown in Figure 3A and B, neferine treated HCT-15 cells showed a significant increase in ROS generation within 15 min, which further peaked at 30 min, whereas the isoliensinine treated HCT-15 cells showed a significant ROS generation at 1 h, which peaked at 3 h time point.

$[Ca^{2+}]_i$ release

The intracellular concentration was measured using florescence dye, FURA-2/AM in different treatment groups. As shown Figure 3C, neferine and isoliensinine treatments increased intracellular $[Ca^{2+}]$ level in HCT-15 cells compared to control.

Mitochondrial membrane potential ($\Delta\psi M$)

As mitochondrial integrity plays an important role in apoptotic mode of cell death and the changes in $\Delta\psi M$ in HCT-15 cells were observed by DioC6 dye loading. As shown in Figure 3D, a significant decrease in $\Delta\psi M$ was observed in neferine/isoliensinine treated HCT-15 cells as evidenced by reduction of DioC6 fluorescence compared to control cells. CCCP (50 µM) served as positive control as this could induce mitochondrial membrane damage.

Cell cycle analysis

Cell cycle analysis of different treatment groups was

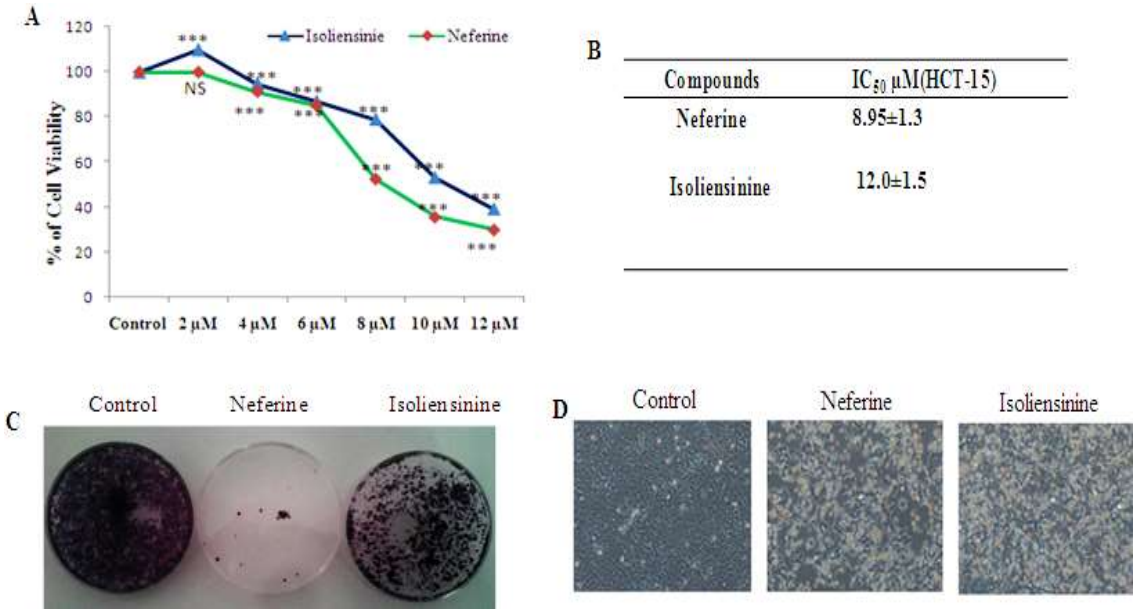

Figure 1. Neferine/Isoliensinine induced cytotoxicity on HCT-15 cells. HCT-15 cells were treated with neferine/isoliensinine for 24 h. (A) Cell viability was assessed by MTT assay and the results were expressed as % cell viability. (B) IC_{50} values (µM) were determined. (C) HCT-15 cells treated with neferine/isoliensinine were subjected to long time colony formation, and the number of colonies formed was observed. (D) Effect of neferine/isoliensinine induced morphological changes were observed by light microscopy. Neferine/Isoliensinine treated cells showed distinct morphological changes, which suggest the cytotoxic nature of the compounds such as cell rounding and detachment compared to control. These figures are representative of one experiment out of three with similar results. The results were shown in three separate experiments carried out in triplicates and expressed as Mean ± S.D (***p<0.001 (0.0001), compared to control, non-significant-p>0.05 (0.12)).

determined by flow cytometry. The results revealed that neferine/isoliensinine treatment for 24 h led to a significant increase in the percentage of cells in G1-phase of cell cycle (62.4% vs. 75.5% and 63.1% vs. 74.4%) whereas a significant decrease was observed in the percentage of cells in G2/M-phase of cell cycle (28.9% vs. 12.2% and 31.6% vs. 12.7%) (Figure 4A). Neferine/Isoliensinine treatments have resulted in a significant increase in a number of cells in the G1 phase of the cell cycle compared to control cells. The results suggest that both neferine/isoliensinine treatments of CRCs resulted in the cell cycle arrest at G1 phase.

Morphological changes

Scanning electron microscopy (SEM) results revealed that cells treated with neferine and isoliensinine induces distinct morphological changes indicated by the appearance of apoptotic bodies and cell shrinkage (Figure 4D).

Apoptosis

Annexin V/PI staining indicates increased percentage of early apoptotic cells from 4.9% (control) to 27.49% with neferine and from 4.9 to 23.44% with isoliensinine (Figure 4C). Propidium iodide uptake increased in neferine/isoliensinine treated cells when compared with control as shown in Figure 4B.

The imbalance in Bax/Bcl2 would result in the release of cytochrome-*c* from mitochondria, resulting in caspase-3 activation and subsequent initiation of apoptosis. Therefore, we observed the level of Bax and Bcl2 by real time PCR in neferine/isoliensinine treated cells. Both neferine/isoliensinine treatments significantly decreased the expression of Bcl2 and increased the expression of Bax compared to untreated cells (Figure 5A and C).

Activation of caspase 3 and PARP cleavage are the hallmarks of apoptosis. We examined the expression of caspase 3 (gene expression) and PARP cleavage through western blot analysis in neferine/isoliensinine treated HCT-15 cells. As shown in Figure 5B and E, neferine/isoliensinine increased the expression of caspase 9, 3 and triggered the cleavage of PARP protein in HCT-15 cells.

Activation of MAPK pathway

Western blot analysis of MAPK pathway proteins such as p38 and pP38 and PARP revealed neferine/isoliensinine

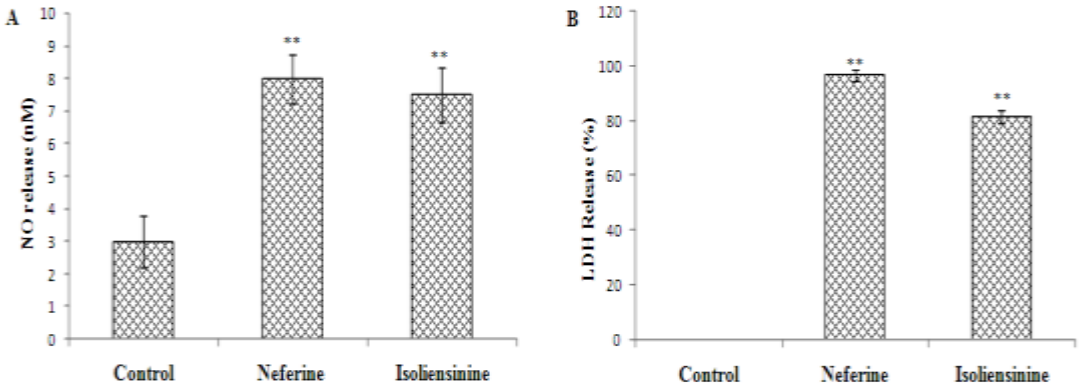

Figure 2. Effect of neferine/isoliensinine treatment on nitric oxide generation (NO) measured in terms of nitrite level (A) and lactate dehydrogenase (LDH) leakage (B) in the extracellular medium (after treatment to HCT-15 cells) were measured to assess membrane integrity (**p<0.01, compared to control).

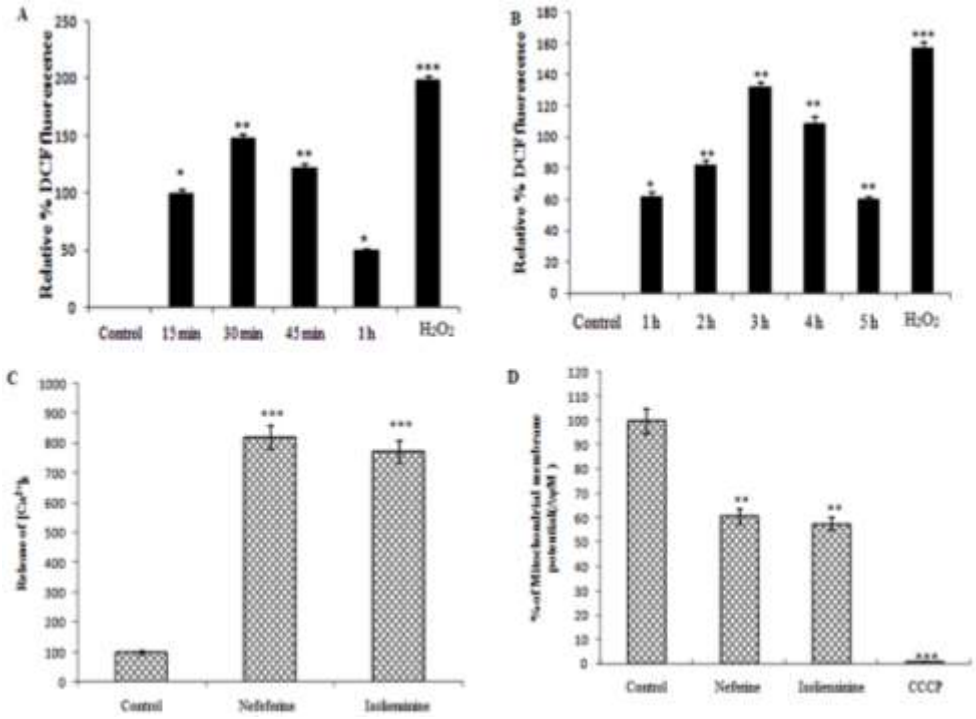

Figure 3. Time course study was carried out to determine the effect of (A) neferine/isoliensinine and (B) treatment on ROS generation in HCT-15 cells. Intracellular ROS concentration was determined by treating DCF-DA loaded HCT-15 cells with neferine/isoliensinine for different treatment periods. Results were expressed as relative DCF florescence in % compared to control and H_2O_2 was used as positive control. (C) Intracellular calcium concentration was measured in neferine/isoliensinine treated HCT-15 cells and results were expressed as relative FURA-2/AM fluorescence. (D) Mitochondrial membrane potential (ΔΨm) was measured in Spectrofluorimetry using DioC6 in neferine/isoliensinine treated HCT-15 cells. To compare the loss of ΔΨm induced by neferine/isoliensinine, CCCP was used as a positive.

treatment of CRCs induced the up-regulation of p38 and pP38 protein expression and cleaved PARP as shown in Figure 5E compared to control CRCs. Together, these data suggest that treatment with neferine/isoliensinine

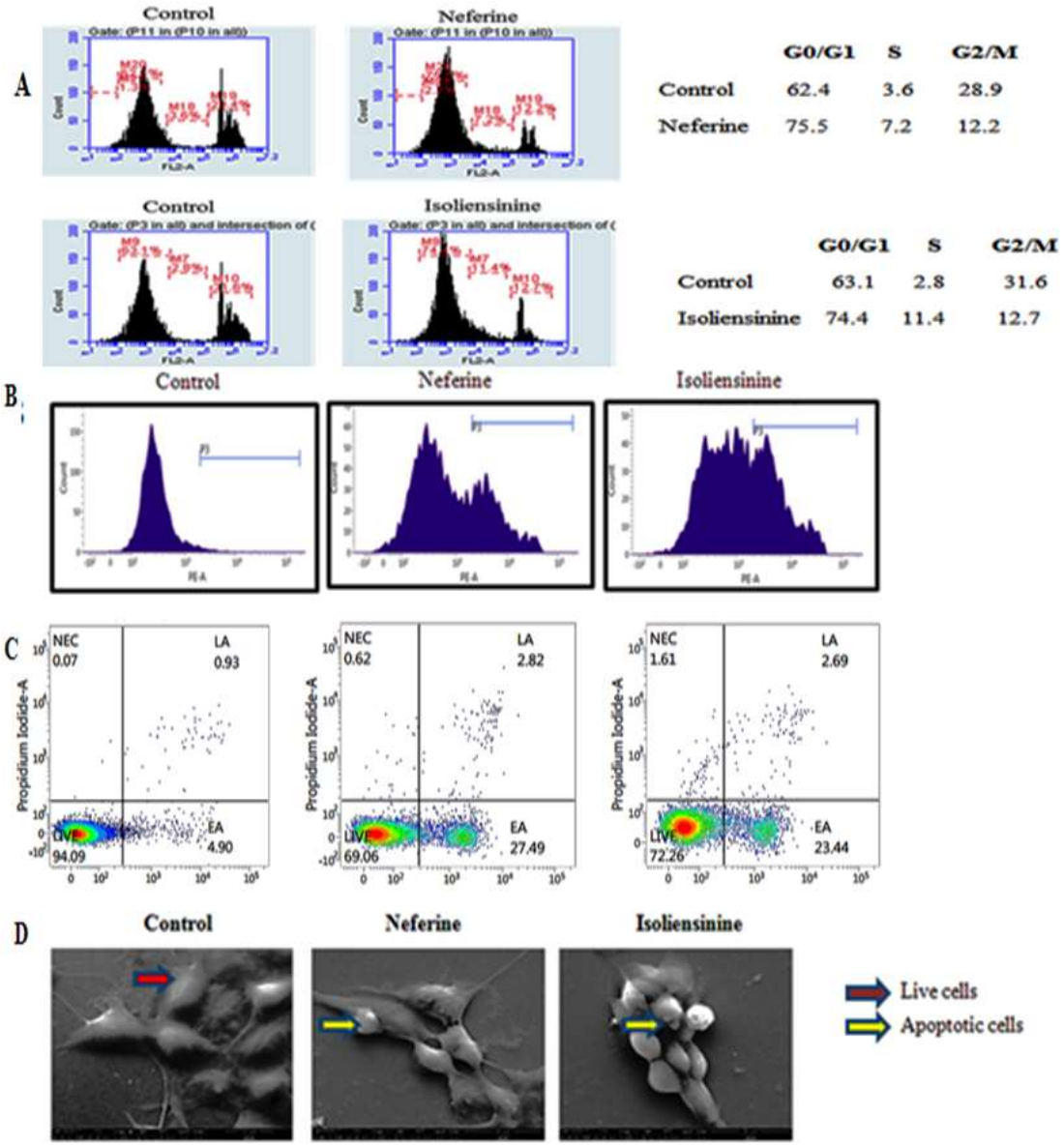

Figure 4. (A) Cell cycle distribution in neferine/isoliensinine treated HCT-15 cells was analyzed by flow cytometry using propidium iodide. (B) Propidium iodide (PI) uptake in neferine/isoliensinine treated HCT-15 cells was determined by flow cytometry. We confirmed the cytotoxic effect and apoptosis inducing nature of neferine/isoliensinine on HCT-15, results revealed that uptake of PI were more in neferine/isoliensinine treated cells. (C) Apoptosis inducing ability of neferine/isoliensinine treatment on HCT-15 cells were analyzed by flow cytometry. The % of Annexin V positive cells indicates the apoptotic induction. The results shown were representative of three separate experiments with similar results. (D) HCT-15 cells were treated with neferine/isoliensinine for 24 h, fixed and analyzed under scanning electron microscopy. Control cells, Neferine and Isoliensinine treated cells.

induce mitochondria-mediated apoptosis in CRCs.

DISCUSSION

Natural compounds serve as an important source of therapeutics especially for the treatment of cancer. Vincristine, irinotecan, etoposide and paclitaxel are some of the natural compounds approved as cancer therapeutic drugs by Food and Drug Administration (Newman and Cragg, 2016). Previous reports of our group and other researchers have documented the

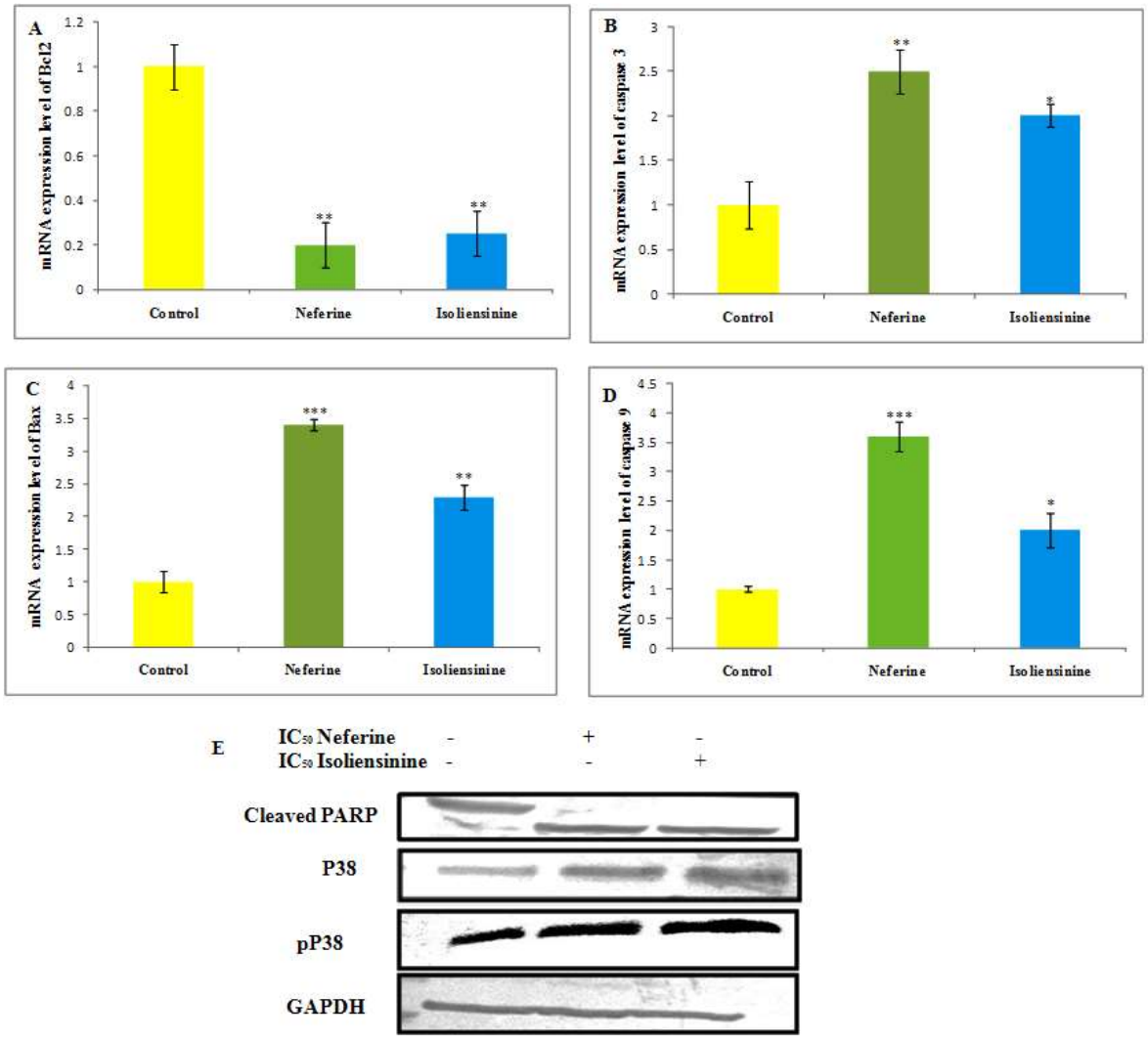

Figure 5. (A-D) Gene expression profile of caspase 9, caspase 3, Bax and Bcl-2 were carried out by Real-time PCR analysis in neferine/isoliensinine treated HCT-15 cells. All the gene expression levels were normalized by GAPDH, used as internal control. All the experiments were repeated three times with same results. (E) Effect of neferine and isoliensinine on MAPK and apoptosis protein expression. Equal amount of protein was loaded in SDS-PAGE, and electrophoresed and transferred on to nitrocellulose membrane subsequently; the expression of protein was detected by specific antibody as mentioned in methodology. GAPDH served as internal control. Each bar represents the mean of triplicate ± S.D (***p<0.001, **p<0.01,*p<0.05 compared to control).

anticancer activity of neferine and isoliensinine through induction of apoptosis and autophagy in various cancer cells (Law et al., 2014; Shu et al., 2015; Xu et al., 2016; Yoon et al., 2013).

Recent reports suggest that isoliensinine selectively induces apoptosis in triple-negative breast cancer cells. Their study assessed cytotoxic effects of isoliensinine, liensinine and neferine on triple-negative human breast cancer cells and has reported that isoliensinine possesses the most potent anti-cancer activity among the three alkaloids. These molecular studies were performed to conclude the protective efficacy of isoliensinine based on cytotoxic studies and authors have failed to demonstrate the molecular mechanism behind the anticancer activity of neferine and liensinine on triple negative breast cells (Zhang et al., 2015). In the present study, we have compared the anticancer efficacy of the two alkaloids of the lotus seed embryo namely neferine and isoliensinine in HCT-15 cells. To our knowledge, this is the first report of anticancer activity of neferine and isoliensinine on colorectal cancer HCT-15cells.

ROS generation plays a vital role in modulation of cell signaling pathways (Subramanian et al., 2016). ROS generation has been implicated in various disease

conditions including cancer and it has also been reported to be the initiator of different modes of cell death in normal as well as cancer cells (He et al., 2016; Mohan et al., 2016; Salimi et al., 2015; Wang et al., 2012). Thus, ROS generation is a key event in cellular homeostasis. Further, it was reported that neferine and isoliensinine increase the ROS generation which in turn induce apoptosis in cancer cells (Zhang et al., 2015). In the present study, neferine and isoliensinine treatment enhanced ROS generation in HCT-15 cells in a time dependent manner.

Several reports suggest that various stress signals including oxidative stress and cytokines induce activation of p38MAPK. Activation of p38 MAPK has been associated with the induction of apoptosis in cancer cells (Hsieh et al., 2014; Kim et al., 2011; Ramiro-cortes et al., 2011). Recent reports have documented that neferine and isoliensinine induce p38 MAPK pathway-mediated cell death in cancer cells including lung, hepatocellular and triple negative breast cancer cells. Neferine and isoliensinine treatment resulted in the increased expression levels of p38 and Pp38 in HCT-15 cells in the current study which is in line with the aforementioned reports.

Neferine and isoliensinine have been shown to arrest the cell cycle at G1 phase in several cancer cells (Yoon et al., 2013; Zhang et al., 2015). In the present study, both neferine and isoliensinine have been shown to arrest a significant proportion of the cells at G1 phase of cell cycle in HCT-15 cells.

Death receptor and mitochondrial cytochrome *c* mediated pathways are major apoptosis signaling pathways (Samejima et al., 2001**;** Pfeffer and Singh, 2018). Contemporary research has shown that the damage of mitochondrial membrane potential leads to mitochondrial mediated apoptosis pathway by release of mitochondrial pro-apoptotic factors (Chen et al., 2018). Several natural products have been reported to induce mitochondrial mediated apoptosis in cancer cells (Liang et al., 2017; Subramanian et al., 2016). Bcl-2 and its homologues inhibit the release of cytochrome c by adhering to the mitochondrial membrane and serve as anti-apoptotic proteins while Bax is known as apoptotic protein which promotes apoptosis. The overall expression rate of these two genes determines the fate of cells. Hence, the proportion of Bcl-2/Bax is typically observed as a standard for the determination of apoptosis. Caspase cascade, a family of cysteine proteases modulates the programmed cell death through sequential activation of caspase 9 and 3 which leads to cleavage of PARP, one of the hallmarks of programmed cell death (McArthur and Kile, 2018).

Past research documented that neferine treatment could induce sequential rise in ROS production in turn trigger increase in intercellular calcium release consequently damages mitochondrial membrane by activating apoptotic cascade through damaged mitochondria (Poornima et al., 2014b). Similar results were reported with isoliensinine treatment in HepG2 cells (Shu et al., 2015). Recent report documented that the isoliensinine treatment significantly decreased the expression of anti-apoptotic genes, Bcl-2, Bcl-x and increased the expression of apoptotic genes caspase 3, 9 in triple negative breast cancer cells (Zhang et al., 2015). Previous reports indicate neferine and isoliensinine activate caspase 3 and induce PARP cleavage in lung A549 and breast cancer MDA-MB-231 cells, respectively (Zhang et al., 2015). Current study analyzed the mitochondrial membrane potential, intracellular calcium levels and the expression profile of the various genes of the intrinsic apoptotic pathway in HCT-15 cells and the results are in line with the aforementioned reports, which suggest that both the alkaloids induce mitochondrial mediated apoptosis in colon cancer cells. In conclusion, isolated natural products such as neferine and isoliensinine induce apoptotic mode of cell death via oxidative stress and activation of p38 MAPK pathway in HCT-15 cells. The present research findings suggest that neferine and isoliensinine may serve as potential anti-cancer agents against colon cancer cells and they can be recommended as natural medicinal agents in targeting CRCs.

Abbreviations: AKT, Protein kinase B; **CCCP,** carbonyl cyanide M-cholorophenyl hydrazine; **DAB,** 3, 3'-diaminobenzidine; **DCFH-DA,** 2',7'-dichlorofluorescin diacetate; **DioC6,** 3, 3′-dihexyloxacarbocyanine Iodide; **Fura-2-AM,** fura-2-acetoxymethyl ester; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **HRP,** horseradish peroxidase; **JNK,** Janus kinase; **mTOR,** mammalian target of rapamycin; **NCCS,** National Centre for Cell Science; **PARP,** poly (ADP-ribose) polymerase; **PI3K,** phosphatidylinositol-4,5-bisphosphate 3-kinase; **TGF,** tumor growth factor; **TNBC,** triple negative breast cancer.

CONFLICT OF INTERESTS

The authors report no conflict of interest in this work

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