Antineoplastic activity of cadmium proline complexes in A549 non-small cell lung cancer cell line

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Abstract— The development of metal complexes with biological activities has had an enormous impact on therapeutics. Amino acid complexes with metals are similar in structure to the natural ones present in the body and release trace elements exactly to cell or tissue that needs them. In this way, we have synthesized cadmium proline complexes using both the isomers of proline (D and L). This present study was aimed to evaluate the antineoplastic activity of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ against human A549 cells. The synthesized compounds were characterized by UV-visible, Fourier transform infrared spectroscopy (FT-IR) and NMR spectroscopy techniques. Cytotoxicity studies were assessed by MTT assay. The initial apoptosis induction was performed by AO/EB and Hoechst staining. Accumulation of reactive oxygen species (ROS) was observed in fluorescence microscope, which was associated with the increased expression of cell cycle regulating factors likes insulin like growth factor binding protein-3 (IGFBP-3), p53 and p21. In addition it down-regulated the expression of MMP_2 and MMP_9. Subsequent apoptosis induction was accompanied with the activation of mitochondrial pathway, in particular a decreased mitochondrial membrane potential ($\Delta\Psi m$) as well as increased Bax and decreased Bcl_2 ratio then followed by caspase activation. In conclusion, our study was the first to report that Cd[L-proline]$_2$ complex has inhibitory effect on the growth of A549 cells. These finding might to be drive potential therapeutic approach of lung cancer in the future.

Key words—Cd[D-proline]$_2$, Cd[L-proline]$_2$, Intrinsic pathway,Lung cancer,p53, Reactive oxygen species.

1 INTRODUCTION

Metal-based compounds have received great attention in medicinal chemistry and have been widely studied in modern medicine for the diagnosis and treatment of different human malignancies. For instance, metallo-pharmaceutical agents have been described for the treatment of cancer, arthritis and manic depression, for use as anti-microbial and anti-parasitic agents. Many transition metal complexes have been used throughout history for the diagnosis and treatment of a wide variety of disorders. The unique properties of transition metal complexes, such as their structural diversity, interesting photochemical and photophysical properties and their ability to form specific interactions with biomolecules, make them versatile alternatives to organic small molecules for drug discovery[1]. Structurally, metal complexes consist of a metal centre surrounded by a number of organic or inorganic ligands via coordination bonds. Metal complexes can adopt a large range of structural types, including octahedral or square-planar geometries, which are unavailable to purely organic small molecules. This feature can allow metal complexes to sample regions of chemical space within biomolecules that cannot be accessed by organic compounds. Additionally, metal complexes can perform ligand exchange a reaction with biological molecules, forming covalent adducts that can perturb cellular function. Importantly, the metal-ligand bonds can be tuned by the choice of metal ion, its oxidation state, and auxiliary ligands. Finally, metal complexes can coordinate ligands that are themselves biologically active, leading to dual function or even synergistic effects between the metal ion and the ligand. Cadmium (Cd), more harmful than necessary to people, is a widespread environmental pollutant with increasing worldwide concern. Cd has been shown to play an important role in enhancing DNA mutation rates and stimulating mitogenic signaling pathways and expression of proteins, which control cellular proliferation.

Amino acids exist as zwitterionic structures in solution phase. In the gas phase, these charges separated species lack the stabilization of the solvation shell, but can be stabilized by metal cations[2]. Proline is a non-essential amino acid because it can be synthesized by the body through the breakdown of L-glutamate, another amino acid. To body will have the right amount of amino acids it needs to produce proline, an important compound responsible for tissue repair, collagen formation, arteriosclerosis prevention and blood pressure maintenance. Tumor metabolic reprogramming driven by non-genetic or genetic factors including oncogenes and tumor suppressors has been recently linked to cancer progression. The development of metal complexes with biological activities has had an enormous impact on therapeutics. In this way, we have synthesized cadmium proline complexes using both the isomers of proline (D and L). This present study was aimed to evaluate the antineoplastic activity of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ against human A549 cells.

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2 MATERIALS AND METHODS

2.1 Synthesis of Cd[proline]$_2$ complex

The metal-proline complex was synthesized using L-proline and D-proline as the starting material. To the stirred solution of proline (2 mM) in methanol (5 ml) was added triethylamine (2 mM), then after 10 mins cadmium nitrate tetra hydrate (1 mM) was added. After stirring for 1 h, the precipitate was washed with methanol and collected by filtration to get Cd proline complexes as white amorphous solid with 100% yield.

2.2 Characterization of Cd[proline]$_2$ complex

Electronic spectra were recorded in H_2O with a double beam UV-visible spectrophotometer (UV-1800, Shimadzu, Japan) in the range of 200-800 nm. Fourier transform infrared spectroscopy (FT-IR) was recorded as KBr pellets (JASCO FT-IR 4600, Japan) in the range of 400-4000 cm^{-1}. ^1H NMR spectra was recorded on a Bruker AV III 500 MHz instrument using D_2O as solvent at SAIF, Indian Institute of Technology Madras, Chennai, Tamil Nadu.

2.3 Cell culture

A549 cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM: Hi Media Laboratories Mumbai, India), supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hi Media Laboratories Mumbai, India) in a 5% CO_2 humidified atmosphere at 37˚C.

2.4 Cytotoxicity assay

MTT (dimethyl thiazolyltetrazolium bromide) assay was performed by using the method of Yuvan *et al.,* 2004[3]. For cytotoxicity assays, cells in monolayer containing approximately 1×10^4 were added to each well of a 96-well culture plate and incubated for overnight at 37°C with 5% CO_2. A549 cells were treated with a 0.5 to 5.0 µM concentration of chemically synthesized compounds Cd[D-proline]$_2$ and Cd[L-proline]$_2$. After treatment at 12 h of incubation 20 µL of MTT (dimethyl thiazolyltetrazolium bromide) was added then the cultures were further incubated for 4 h. After incubation, MTT was aspirated and then 200 µL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm (measurement) and 630 nm (reference) using a micro plate reader (Bio-Rad, USA). DMSO by itself was found to be non-toxic to the cells. Results were collected for triplicate each and used to calculate the mean and standard deviation.

2.5 Acridine Orange/Ethidium Bromide (AO/EB) staining assay

Influence of apoptosis by Cd[D-proline]$_2$ and Cd[L-proline]$_2$compound against A549 cells were confirmed using Acridine Orange (AO) and Ethidium Bromide (EB) (1 mg/mL for both AO and EB in 1x PBS) staining method[4]. In brief, $5x10^5$ cells/well were cultured on cover slip in 6-cell plate and incubated overnight for attachment. After attachment, the cells were treated with fresh medium containing 2 µM of Cd[D-proline]$_2$ and Cd[L-proline]$_2$compound. After 12 h incubation, cover slip was removed and stained with AO/EB (10 µL) for 30 min and washed with 1x PBS for removing of excess staining. Cover slip was mounted on 20x objective glass and cells images were captured using an inverted fluorescence microscope (Floid cell imaging station).

2.6 Apoptosis assay using Hoechst 33258 stain

The A549 cells in logarithmic growth phase were seeded at a final concentration of 5×10^5/mL in a 6-well culture plate. The cells were exposed to 2 µM of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ for 12 h. Then they were stained by Hoechst 33258, and the changes in the nuclei of cells were examined and photographed by using a fluorescence microscope (Floid cell imaging station)[5].

2.7 Assessment of reactive oxygen species (ROS)

For assessment of intracellular ROS, 5×10^5 cells were seeded on a coverslip in 6-well plate and incubated overnight for attachment. Next day the cells were treated with fresh medium containing 2 µM of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ and incubated for 12 h. At the end of incubation cover-slip was removed from the culture plate and stained with 40 µM of 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) dye for 30 min. The stained cover slip was washed with 1x PBS. Using 20x objective fluorescence microscope the cell's image was captured[6].

2.8 Assessment of mitochondrial membrane potential level ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using Rhodamine-123 dye. In brief, 5×10^5 cells/well were seeded in 6-well plates and incubated overnight for attachment. After overnight attachment the cells were treated with fresh medium containing 2 µM of Cd[D-proline]$_2$ and Cd[L-proline]$_2$. After 12 h, the cover-slip was stained with 50 µL of Rhodamine-123 dye (10 µg/mL) for 30 min, excess dye was removed by washing with 1x PBS and cell images were captured using 20x objectives under fluorescence microscope[7].

2.9 mRNA isolation & Reverse Transcription–PCR

Total mRNA was isolated from Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treated and untreated A549 NSCLC cells using TRIzol reagent (Invitrogen, Grand Island, NY, USA). Integrity of RNA was checked by agarose gel electrophoresis and Ethidium bromide staining. 2 µg of RNA was used as a template for each reverse transcriptase mediated PCR (RT-PCR) by using aiScript cDNA Synthesis Kit (Bio-Rad). The single-strand complementary DNA (cDNA) was synthesized. 1 µL of this cDNA pool was used for PCR. PCR was performed in a DNA thermal cycler for 30–35 cycles and the cycling condition was described as follows: initial denaturing at 94˚C for 2 min, annealing at optimal Tm for 1 min & final extension at 72˚C for 7 min. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control. All PCR products (10 µL) were analyzed by electrophoresis on 1.0% agarose gel.

2.10 Western blot analysis

The 50 µg of protein samples were mixed with 4X SDS-PAGE sample buffer (100 mmol/L Tris-HCl, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 5% β-mercaptoethanol). Samples were then boiled, for 5 min at 95˚C and resolved by 12% SDS-PAGE at 100 V for 2 h. Polypeptides were transferred to

nitrocellulose membranes for 60 min at 60 V in a transfer buffer containing 39 mmol/L glycine, 48 mmol/L Tris and 20% methanol. Membranes were blocked for 1 h in 5% milk diluted in TBS containing 0.1% Tween 20 (TBST). Membranes were incubated overnight at 4˚C with specific primary antibodies (1:1000 dilutions). Blots were washed 3 times for 5 min in TBS and washed 3 times for 5 min in TBST before incubation with secondary antibodies goat anti-rabbit IgG-ALP antibody, Goat anti-mouse IgG-HRP antibody (GeNei-Merck, India, 1:1000 dilution) for 1 h at 4˚C. Blots were washed 3 times for 5 min in TBS and washed 3 times for 5 min in TBST and developed by BCIP/NBT, TAB-HRP (GeNei-Merck, India).

2.11 Statistical analysis

One-way ANOVA with Dunnett'spost test was performed using GraphPad Prism version 6.04 (La Jolla California USA). A value of *P < 0.05, **P < 0.01, ***P < 0.001 was considered to indicate statistical significance. All the results were expressed as mean S.D.

3 Results and Disscussion

3.1 Electronic spectra

The electronic spectra of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ complexes in H_2O are shown in *Fig. 1*. The maximum wavelength of Cd^{2+} complex appeared at 240 nm for both the isomers. This complex was further confirmed by FTIR analysis.

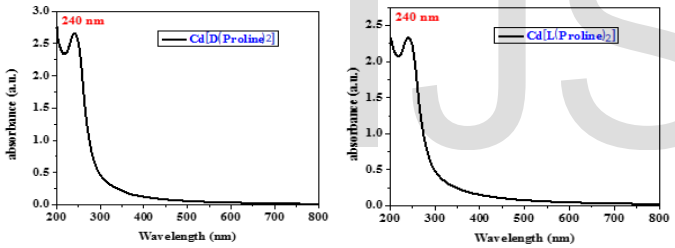

Figure 1. Electronic spectra of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ complexes in H_2O.

3.2 FT-IR spectra

FT-IR has become an important tool in understanding the involvement of functional groups. *Fig. 2* shows spectral peaks suggesting, the occurrence of bands relevant to data present in the table 1. These functional groups are further investigated by NMR spectra.

3.3 NMR spectra

The NMR spectra of the compound were recorded in D_2O solvent for confirming the binding mode of the complex shown in *Fig. 3*. ^{1}H NMR (500 MHz, D_2O): δ (ppm) 1.74 (m, 4H) indicates acyclic ring –CH_2 proton, 2.20 (m, 4H) indicates acyclic ring –CH_2 proton, 2.86 (s, 2H) indicates acyclic ring N-H proton, 3.12 (m, 4H) indicates acyclic ring N–CH_2 proton, 3.79 (t, 2H, J= 7.5Hz) indicates acyclic ring carbonyl carbon attached –CH_2 proton.

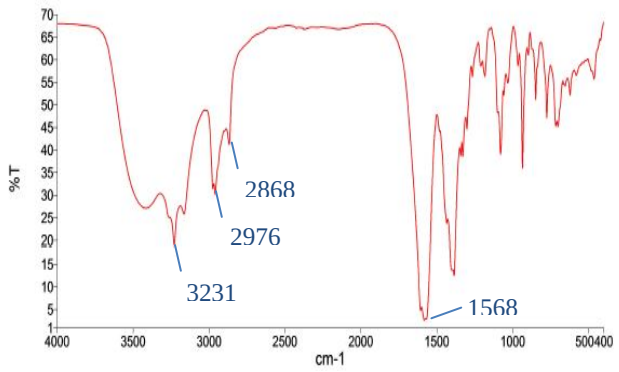

Figure 2. FT-IR spectrum of Cd[proline]$_2$ complex

Wave number (cm-1)	Assignment
3231	N-H stretching for secondary amine
2976, 2868	Aliphatic C-H stretching frequency
1568	N-H bending

Table 1: FT-IR spectrum assigmentforCd[proline]$_2$ complex

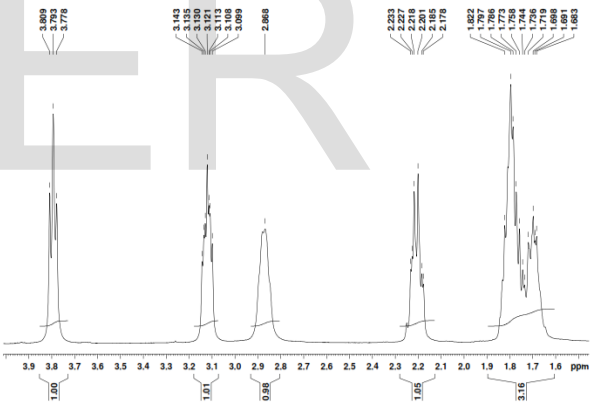

Figure 3. ^{1}H NMR spectra of Cd proline complex in D_2O

3.4 Cytotoxicity assessment using MTT cell proliferation assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a tetrazolium salt that is reduced to yield a purple-coloured water-insoluble formazan product. This assay measure cellular metabolic activity via NADPH dependent cellular oxidoreductase enzymes regulated by active mitochondria hence it reflects the number of viable cells present. Formazan salt is produced in proportion to the active cell number and accumulates within the cells, since it is not membrane permeable[8].

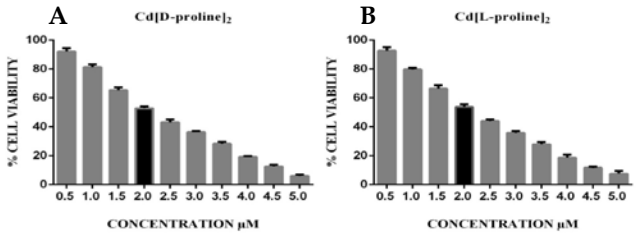

Figure 4. Decrease in viability of A549 NSCLC cells caused by the Cd[D-proline]$_2$ and Cd[L-proline]$_2$. Survival rate of A549 cells after treated with different concentrations of Cd[D-proline]$_2$ **(A)**Cd[L-proline]$_2$ **(B)** for 12 h

In the present study, the cytotoxicity of Cd[D-proline]2 and Cd[L-proline]2 against A549 cells was evaluated by determining the IC50 values of Cadmium D&L proline. The percentage of viable cells decreased significantly in a dose dependent manner. The IC50 value for Cd[D-proline]2 and Cd[L-proline]2 was obtained at a concentration of 2 µM. (Fig. 4). This shows that both Cd[D-proline]2 and Cd[L-proline]2 can inhibit A549 cell growth . It was observed that Cd[D-proline]2 and Cd[L-proline]2 are more active than the free amino acid. This indicated enhancing antitumor activity upon amino acid complexes with metal coordination. The enhancement of cytotoxic activity may be assigned to the positive charge of the metal which increases the acidity of coordinated ligand that bears protons, leading to stronger hydrogen bonds which enhanced the biological activity[9]. In the present study, we found that Cadmium proline complex inhibits the growth of A549 cells at a dose of 2 µM for 12 hours. We selected this dose for further studies. This suggested that treatment with Cadmium proline complexes inhibited the growth and reduced the viability of A549. The induction of apoptosis has been described as a standard and best strategy in anticancer therapy[10]. Previous studies showed that cadmium inhibited the proliferation of MDA-MB-231, pancreatic cancer cell lines Mia Paca-2, BxPC-3 and Panc-1[11]. In the present study, we showed that Cadmium proline complexes inhibited proliferation in human lung cancer A549 cells with IC50 at the range of 2 µM, which suggested tht Cadmium proline complexes possesses a potential cytotoxicity to lung cancer cells.

3.5 Cadmium proline complexes induced morphological features of apoptosis in A549 NSCLC cells by AO/EB staining

Apoptosis is a type of genetically regulated programmed cell death that controls the development of multicellular organisms and tissues by eliminating physiologically redundant, physical damaged and abnormal cells. Acridine orange/Ethidium bromide (AO/EB) fluorescent staining, visualized under a fluorescent microscope, can be used to identify apoptosis-associated changes of cell membranes during the process of apoptosis. This method can also accuratelydistinguishcells in different stages of apoptosis [12]. Morphological changes of A549 cells occurred by the treatment with cadmium proline complexes. After treatment with Cd[D-proline]$_2$ and Cd[L-proline]$_2$ for 12 h the A549 cells showed the characteristic morphological changes of apoptosis, including shrinking of the cytoplasm and nuclear fragmentation with an intact cell membrane or a contracted nucleus and condensed chromatin fragments. Staining was examined under a fluorescent microscope. No significant apoptosis was detected in the control (Fig.5). Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treatment shows early-stage apoptotic cells, condensed nuclei and appeared crescent-shaped or granular yellow-green colour (Fig.5). Late apoptotic cells appeared in red colour with condensed and fragmented nuclei. In necrotic cells, Ethidium bromide penetrated into the membranes of dead cells and stains their nuclei which appear red (Fig.5). AO/EB staining is a qualitative and quantitative method to detect apoptosis [13]. We speculate that AO penetrated normal and early apoptotic cells with intact membranes, fluorescing green when bound to DNA. EB can enter cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies. Furthermore, AO/EB staining is able to detect mild DNA injuries.

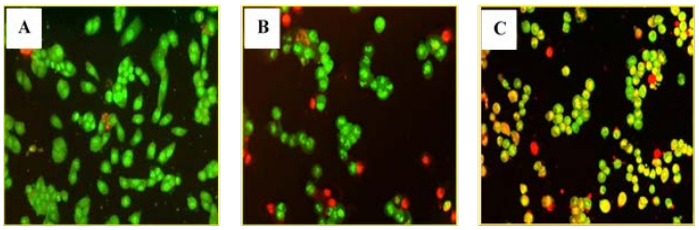

Figure 5. Induction of apoptosis in A549 NSCLC cells by AO/EB staining after the Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treatment. **(A)** Control**(B)**Cd[D-proline]$_2$**(C)** Cd[L-proline]$_2$

Therefore, to distinguish normal, early apoptotic, late apoptotic cells and dead cells, nuclear morphology, A459 cells treated with Cd[D-proline]$_2$ and Cd[L-proline]$_2$ are assessed. Fluorescent staining using AO alone has been used in the past; however, detection of cell apoptosis using AO/EB is a relatively new approach and few papers have reported its use.

3.6 Apoptosis induction in A549 cells by Hoechst staining

Hoechst 33258 staining was also carried out to investigate the apoptosis induction in A549 cells treated with 2 µM of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ for 12 hours. After treatment with Hoechst 33258, control cells were seen with uniformly light blue nuclei under fluorescence microscope; while Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treated A549 cells exhibited bright blue colour because of chromatin condensation (Fig.6). Hoechst staining exhibited the morphological features of apoptosis such as chromatin condensation and membrane blebbing in Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treated A549 cells. The imbalance between proliferation and apoptosis can lead to unlimited cell proliferation, which can ultimately develop into a tumor.

Our study further verified that Cd[D-proline]$_2$ and Cd[L-proline]$_2$ suppresses A549 cell proliferation through the induction of cell apoptosis. These results demonstrate that Cd[D-proline]$_2$ and Cd[L-proline]$_2$ having anti-cancer effects through the induction of apoptosis. These findings prompted us to evaluate further the changes in mitochondrial membrane potential and in the production of ROS in the cytosol, since

these two events are often associated with apoptosis.

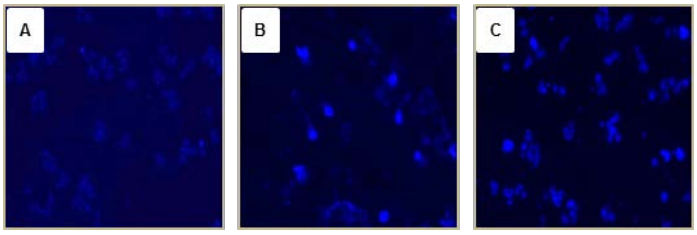

Figure 6. Induction of apoptosis in A549 NSCLC cells by Hoechst 33258 staining after the Cd[D-proline]$_2$ and Cd[L-proline]$_2$treatment**(A)** Control**(B)** Cd[D-proline]$_2$**(C)**Cd[L-proline]$_2$

3.7 Cadmium proline complex repress cancer cell invasion via ERK/MAPK mediated down-regulation of MMP-2 and MMP-9 in Non-Small Cell Lung Cancer

The role of ERK1/2 in cell invasiveness has been elucidated in many recent evidences. Cell invasiveness primarily involves the breakdown of extra cellular matrix. The family of zinc dependent endopeptidases, which are involved in the breakdown of extra cellular matrix, normal tissue remodelling and repair process are known as matrix metalloproteinase (MMP). In cancer they play a critical role to facilitate cell migration by removing physical barriers promoting angiogenesis. Among MMPs, MMP2 and MMP9 (gelatinase A and gelatinase B) have found to be related in major cases of tumor invasion and metastasis with their ability to degrade collagen type IV, a major component basement membrane [14]. Many recent evidences have been linked with the activation of ERK1/2 pathway with MMPs. Specific ERK inhibition could inhibit MMP2 and MMP9 expression in human gallbladder cell line [15]. In this present study we aimed to look at the role of ERK1/2 in modulating MMP2 and MMP9 in a Cd[D-proline]$_2$ and Cd[L-proline]$_2$ induced system. We studied the effect of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ on expression of MMP2 and MMP9 and the expression of pERK1/2 and ERK1/2 in A549 NSCLC cells. To confirm the role of MMPs, gene expression studies on MMP-2 and MMP-9 was performed following treatment with Cd[D-proline]$_2$ and Cd[L-proline]$_2$ in A549 cells by RT-PCR.

The expression of MMP-2 and MMP-9 mRNA was significantly decreased in Cd[D-proline]$_2$ and Cd[L- proline]$_2$ in A549 cells (Fig.7). To further confirm the role of pERK1/2 and ERK1/2, protein expression of MMP-2 and MMP-9 carried in Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treated A549 cells. The expression of active MMP-2 &9 proteins were significantly decreased in Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treated A549 cells (Fig.8). Cd[D-proline]$_2$ and Cd[L-proline]$_2$ shown appreciable decreased expression in phosphorylation of ERK1/2 and its leads to down regulation of MMP-2 and MMP-9 (Fig.8). Interestingly, the total ERK1/2 protein levels seem unaffected indicating the importance of activated protein in transducing signals in the cell. ERK1/2 holds a tight selectivity on their activation mechanism and gets phosphorylated by MEK1/2 making them excellent targets for the development of therapeutic agents against cancer. This suggested a possible role of ERK1/2 in the modulation of MMP.

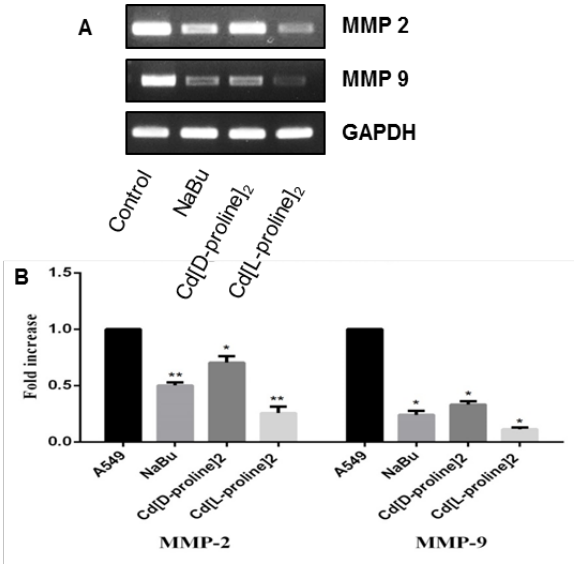

Figure 7. (A) Effect of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ on MMP-2 and MMP-9 mRNA expression in A549 NSCLC cells by RT-PCR. Cellular RNA of A549 cells were harvested 12 h after indicated treatment and analysed for MMP-2 and MMP-9. GAPDH served as an internal control. **(B)** Bar chart displaying the gene expression fold change by Cd[D-proline]$_2$ and Cd[L-proline]$_2$

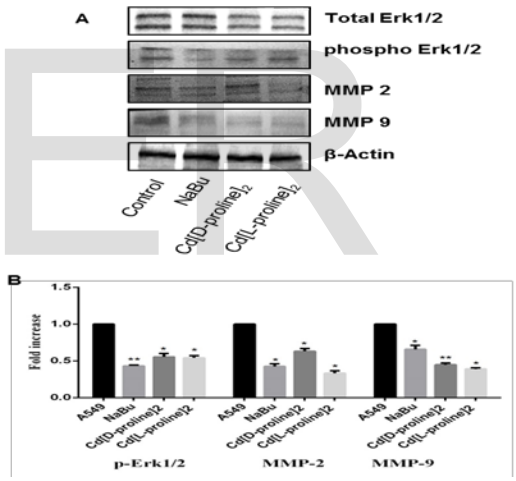

Figure 8. A549 cells were treated with indicated concentrations of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ for 12 h. **(A)** Cell lysates were analysed by western blotting. Blots were incubated with Total ERK1/2, phosphoERK1/2, MMP-2 and MMP-9 and β-actin. **(B)** Bar chart displaying the protein expression fold change by Cd[D-proline]$_2$ and Cd[L-proline]$_2$ (*P<0.05, **P<0.01)

Our study, which investigates the possible role of ERK1/2 in modulating MMP-2 and MMP-9, suggests that there might be a direct correlation between ERK1/2 activity and MMP-2 and MMP-9. MMP-2 and MMP-9, which were taken as an indicator of tumor invasiveness along with other phenotypical parameters like cell invasion and cell migration, were shown to be regulated by ERK1/2 modulation.

8 Effect of Cadmium proline complexes on generation of Reactive Oxygen Species (ROS) in A549 cells

ROS is known to play important role in regulating cellular functions, including regulation of cellular growth arrest, increased cellular proliferation, senescence, apoptosis and necrosis. Cellular damage caused by ROS includes lipid peroxi-

dation, DNA adduct formation, protein oxidation and enzyme inactivation, which can lead to apoptosis. Excess ROS can cause irreversible peroxidation of lipids, amino acids, nucleic acids and carbohydrates. Although excess ROS is associated with having negative consequences in the cell, a certain level of ROS is needed to maintain the normal physiological processes of the cell, such as defence against infectious agents and the induction of signalling pathways. ROS serve as a promising treatment target for cancer cells.To determine whether an ROS is involved in Cd[D-proline]$_2$ and Cd[L-proline]$_2$ induced apoptosis in A549 cells, ROS production was examined at indicated time points after Cadmium proline complexes treatment. As shown in Fig.9 observed ROS levels reached a maximum at about 12 h in the cells after exposure to Cd[D-proline]$_2$ and Cd[L-proline]$_2$.

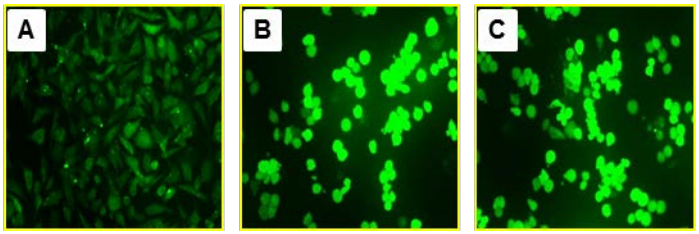

Figure 9. Cd[D-proline]$_2$ and Cd[L-proline]$_2$ on generation of reactive oxygen species (ROS) in A549 cells for 12 h **(A)** Control **(B)** Cd[D-proline]$_2$, C. Cd[L-proline]$_2$. Presence of ROS sensitive fluorescent dye DCFH-DA and capture using fluorescence microscope

Control cells showed negligible production of ROS (Fig.9). The cells incubated with Cd[D-proline]$_2$ and Cd[L-proline]$_2$ resulted in the production of ROS. ROS generation increased in the presence of a 2 µM concentration of Cd[D-proline]$_2$ and Cd[L-proline]$_2$ in A549 cells resulted in nuclear condensation. Apoptotic induction can also be mediated through ROS intermediates. It was reported that ROS could play a role in down regulating Bcl$_2$[16] as well as in triggering the release of cytochrome C from the mitochondria into the cytoplasm along with Fas associated proteins recruitment and finally leading to the activation of caspase 3 and apoptosis [17]. In our present study, increased levels of ROS in the treatment group indicated that the apoptotic mode of cell death induced by the Cadmium proline complexes is through the generation of ROS. Our result shows that Cadmium proline complexes are a potential cytotoxic metal complex. Instead of apoptosis, it induces ROS-triggered autophagy which inhibits the growth of human lung cancer A549 cells.

3.9 Cadmium proline complex upregulate the expression of IGFBP-3, p21 and p53 levels

Tumour suppressor gene p53 is a key element in the induction of cell cycle arrest and apoptosis following DNA damage or cellular stress in human cells. The tumor suppressor protein p53 is targeted by a wide variety of intracellular and extracellular stimuli, such as withdrawal of growth factors, hypoxia, irradiation, chemicals and defects in nucleotide synthesisOf the many functions of p53, the first ones identified were inhibition of abnormal growth of cells and triggering of programmed cell death. Because these processes ensure genomic integrity or destroy the damaged cell, p53 has been called the "guardian of the genome" [18].

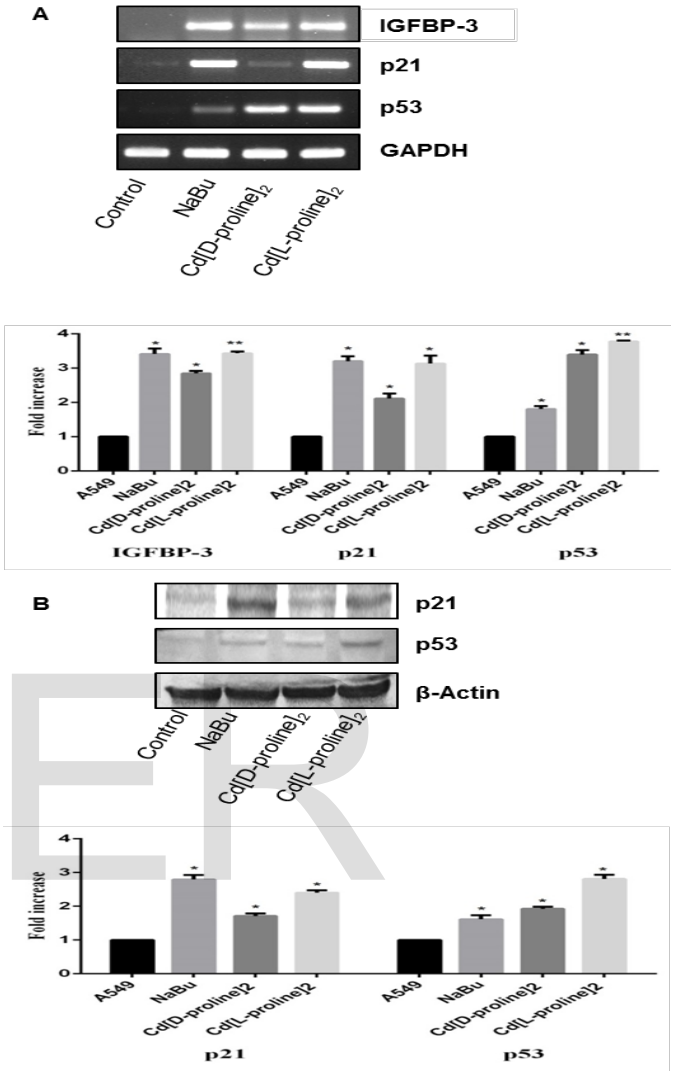

Figure 10. Increase in the expression ratio of IGFBP-3, p21 and p53 induced by the Cd[D-proline]$_2$ and Cd[L-proline]$_2$ **(A)** Reverse-transcriptase PCR of the gene expression of IGFBP-3, p21 and p53 ratio in A549 cells after the indicated treatment (*P<0.05). **(B)** Western blot analysis showing protein levels of p21, p53 and ß-actin after the indicated treatment (*P<0.05)

p21 codes a protein that mediates p53 induced growth arrest of the cell cycle and is also a regulator of cyclin dependent kinase (CDK) activity. p21 can inhibit CDK activity and arrest the cell cycleat the G1 or G2 cell cycle checkpoint. This gives time for DNA repair before replication or mitosis and thus links p21 directly to the tumour suppressor function of p53 [19]. The role of IGFBP-3 as a growth-inhibitory protein has been previously demonstrated by us and others in various cell types.Initially, IGFBP-3 was thought to inhibit growth by binding to IGFs and sequestering them from their receptor. Later, the cell growth-inhibitory effect of IGFBP-3 was suggested to also be IGF-independent and to involve cell growth arrest[20]. Recently, this inhibitory effect of IGFBP-3 was suggested to be mediated by interaction with a putative IGFBP-3 receptor. Although the IGF-independent growth-inhibitory

role of IGFBP-3 has been recently investigated, an apoptosis-inducing role for IGFBP-3 has not been previously determined.

The role of IGFBP-3 in mediating p53 effects was proposed when p53 was demonstrated to activate the IGFBP-3 promotor [21]. Induction of IGFBP-3 expression by wild-type p53 leads to an active form of IGFBP-3 capable of inhibiting cell growth. This suggests that there may be a link between p53 and IGFBP-3 in regulating cellular growth, transformation, and survival. We first assessed the status of IGFBP-3, p21 and p53 in cadmium proline complexes treated A549 cells by a reverse transcription-PCR (RT-PCR). Exposure of cells to 2 µM results indicates that igfbp-3, p21 and p53 mRNA is significantly increased by Cd[D-proline]$_2$ and Cd[L-proline]$_2$. In compare, Cd[L-proline]$_2$ upregulate the p53 expression more than Cd[D-proline]$_2$ (Fig.10). We first assessed the status of IGFBP-3, p21 and p53 in cadmium proline complexes treated A549 cells by a reverse transcription-PCR (RT-PCR). Exposure of cells to 2 µM results indicates that IGFBP-3, p21 and p53 mRNA is significantly increased by Cd[D-proline]$_2$ and Cd[L-proline]$_2$. In compare, Cd[L-proline]$_2$ upregulate the p53 expression more than Cd[D-proline]$_2$ (Fig.10).

3.10 Induction of apoptosis by Cadmium proline complexes involved collapse of Mitochondrial Membrane Potential (ΔΨm)

The alteration of mitochondrial membrane potential (ΔΨm) is a crucial step occurring in cells undergoing apoptosis. Apoptosis the most desired mechanism of cell death in case of cancer drug screening associates changes in mitochondria. A membrane-permeable cationic Rhodamine 123, was used to evaluate the ΔΨm in A549 cells. Green fluorescence is observed in cells with high membrane potential. We found that, the highest level of green fluorescence was seen in untreated cells upon treatment with the Cadmium proline complexes, the ΔΨm was decreased inA549 cells as evidenced by the decrease in the fluorescence compared to the untreated cells (Fig.11). Evaluation of apoptosis is crucial to differentiate it from necrosis. Nuclear fragmentation is one of the characteristic features of apoptotic mode of cell death. The treatment group showed fragmented apoptotic bodies, shrunken and marginated nuclei in contrast to the normal and large nucleus in the untreated cells, proving the apoptotic potential of the Cadmium proline complex. Decrease in ΔΨm usually indicates apoptosis and helps to distinguish the mode of cell death from necrosis [22].

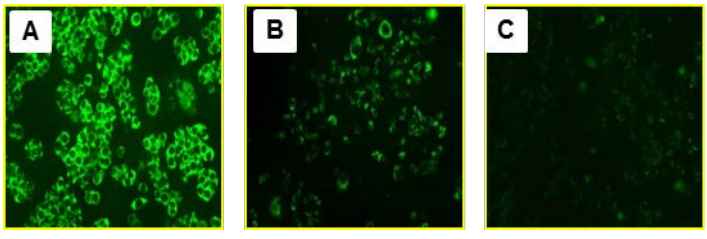

Figure 11. (A)control**(B)**Cd[D-proline]$_2$**(C)**Cd[L-proline]$_2$ Mitochondrial membrane potential was determined by Rhodamine-123

Impairment of mitochondria membrane is involved in the induction of apoptosis. Many researches have demonstrated the changes in mitochondrial structure and the collapse of mitochondrial membrane potential, ΔΨm prior to apoptosis. When the ΔΨm collapse and the dye will move out of the mitochondria resulting in the drop of green fluorescence [23]. Treatment of A549 cells with Cd[D-proline]$_2$ and Cd[L-proline]$_2$ resulted in a drop of green fluorescence in a dose-dependent manner. The cells were treated with Cd[D-proline]$_2$ and Cd[L-proline]$_2$ for 12 h for mitochondrial studies. Mitochondria are sensitive to changes in the redox state of cells. Maintenance of mitochondrial membrane integrity is a dynamic process.

Therefore, mitochondrial dysfunctions including; mitochondrial permeability transition and changes in ΔΨm leads to release of cytochrome c, followed by Caspases activation in cytosol contributing to apoptosis [24]. Rhodamine 123 staining experiments showed disruption of ΔΨm in the Cadmium proline complex treated cells (Fig.11), indicating that the mitochondrial apoptotic pathway plays a pivotal role in Cd[D-proline]$_2$ and Cd[L-proline]$_2$ induced apoptosis in A549 cells. Our findings confirmed that IC_{50} dose of Cd[D-proline]$_2$ and Cd[L-proline]$_2$decreases mitochondrial membrane potential in A549 cells which is evident from the decreased Rhodamine 123 fluorescence intensity.

3.11 Mechanism of Cadmium proline complexes induced apoptotic cell death by intrinsic pathway

Hoechst unlike the extrinsic and intrinsic pathway of apoptosis is initiated within the cell, most often through the disruption of cellular homeostasis. The mitochondria play a very significant role in the execution of this pathway leading to it being commonly referred to as simply the mitochondrial apoptotic pathway.

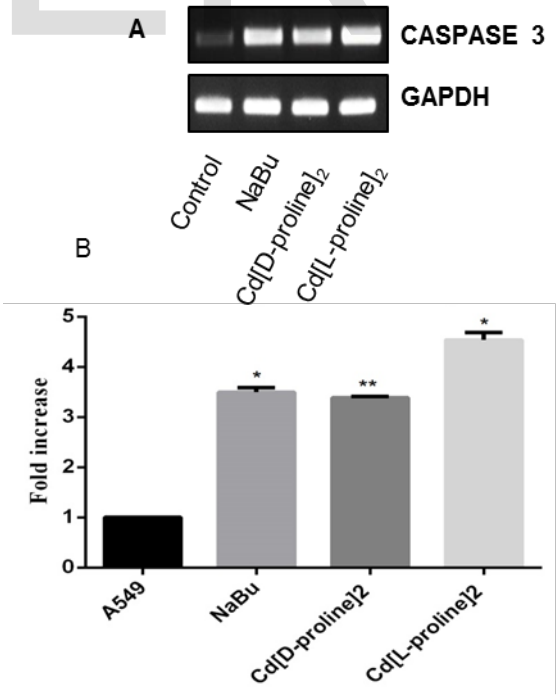

Figure 12. Cadmium proline complexes induced apoptotic cell death by intrinsic pathway**(A)** A549 cells were treated with nothing, Cd[D-proline]$_2$ and Cd[L-proline]$_2$ for 12 h. Caspase-3 and GAPDH transcriptions was determined by RT-PCR**(B)** Bar chart displaying the gene expression fold change by Cd[D-proline]$_2$ and Cd[L-proline]$_2$ (*P<0.05)

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tissue type, as well as the environmental stresses leading to the need of cellular death[25].The instability of the mitochondrial membrane results in the release of cytochrome c which binds to apoptotic activating factor-1 (APAF-1) and activates caspase-9 leading to the activation of caspase-3. Caspase-3 is the most potent effector caspase with many substrates. Mitochondrial membrane permeability is regulated through a family of proto-oncogenes. The Bcl_2 family of proto-oncogenes is anti-apoptotic (Bcl_2) or pro-apoptotic (Bad, Bax). Once activated, Bax is inserted into the mitochondrial membrane and increases membrane permeability leading to cytochrome-c release, thereby promoting apoptosis. The anti-apoptotic protein, Bcl_2, inhibits the ability of BAX to increase membrane potential. As such, the balance of these proteins may determine cell fate[26].We examined the gene expression of caspase 3 in Cadmium proline complex treated A549 cells. They are significantly induced by Cd[D-proline]$_2$ and Cd[L-proline]$_2$ as compared with NaBu and control in A549 cells (Fig.12).

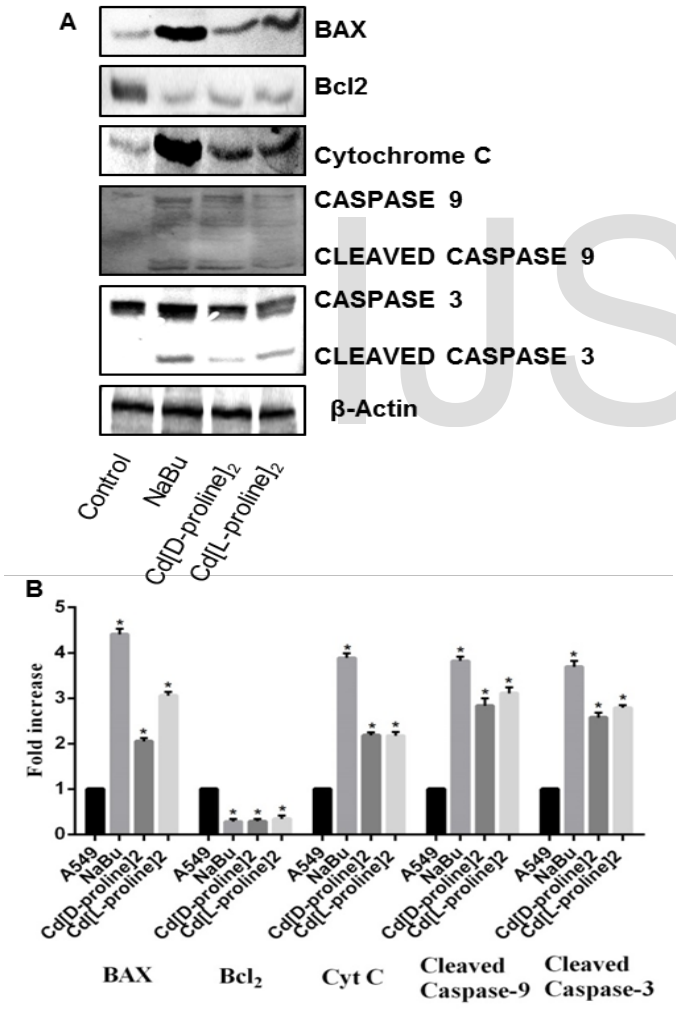

Figure 13. (A) A549 cells were treated with nothing, Cd[D-proline]$_2$ and Cd[L-proline]$_2$. Western blots were performed with antibodies for BAX, Bcl_2, Cytochrome C, Caspase-9 &3 **(B)** Bar chart displaying the protein expression fold change by Cd[D-proline]$_2$ and Cd[L-proline]$_2$ (*P<0.05)

We also examined the protein expression of Bcl_2, BAX, Cytochrome C, Caspase 9 and Caspase-3 in Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treated A549 cells. Cd[D-proline]$_2$ and Cd[L-proline]$_2$ treatment resulted in clear decrease of Bcl2 and increase of BAX, Cytochrome C, Cleavage of caspase 9 and caspase 3. As shown in figure.13.Cd[D-proline]$_2$ and Cd[L-proline]$_2$ lead to slightly increasedBAX, Cytochrome C, Cleavage of caspase 9, cleavage of caspase 3, and decreased Bcl2 protein levels. Cleavage of procaspase-9, an intrinsic associated caspase, associated with caspase-9 activation and initiation of intrinsic apoptotic pathway.Our caspase investigates propose that while caspase-3 is downstream from the initiator effects of caspase-8 and 9.These interpretations identify a mechanism of positive apoptosis control that operates directly at the level of an essential initiator caspase in the intrinsic pathway. Our results confirmed that the Cd[D-proline]$_2$ and Cd[L-proline]$_2$ which up regulate Caspases-8 can also up regulate Caspase-9 there by activate caspase-3 by proteolytic cleavage, and caspase-3 then cleaves vital cellular proteins, leading to intrinsic apoptotic cell death.

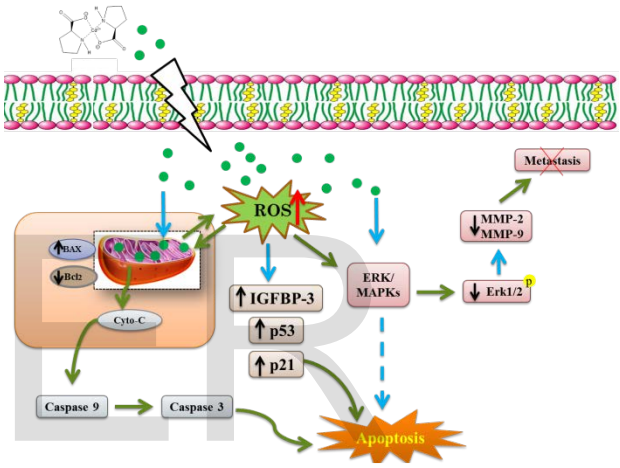

Figure 14. Overall schematic representation of cadmium proline complexes involed in apoptosis

4 Conclusion

Recent advances in medicinal inorganic chemistry gives significant prospects for the utilization of metal complexes in the development of anticancer drugs. Novel Cd[D-proline]$_2$ and Cd[L-proline]$_2$ complexes were successfully synthesized and characterized using UV, FTIR, NMR. The analyses confirmed the metal based amino acid complex. Complexes showed a high potential cytotoxic activity against growth of human lung cancer A549 cell. To confirmed the apoptosis induction using AO/EB and Hoechst staining. The Cd[D-proline]$_2$, Cd[L-proline]$_2$ complexes treated human lung cancer cell line showed up regulated cell cycle regular, apoptotic (p21, p53 & IGFBP-3) mRNA and protein expression. Overall the results are compared to sodium butyrate (NaBu) is a standard drug revealed significant up regulated tumour suppressor genes and down regulate oncogenes in human lung cancer cell A549. The activation of mitochondrial intrinsic pathway through production of ROS, loss of mitochondrial membrane potential, release of cytochrome C, inhibition of antiapoptotic proteins and overexpression Bax with the activation of caspase 9 & 3.

Finally to form a caspase cascade to induce cell death. Our study provides strong *in vitro* evidence that Cd[D-proline]$_2$ and Cd[L-proline]$_2$ complexes, will inhibits cancer growth by triggering an intrinsic apoptotic pathway via a caspase-dependent manner. The present study concludes that Cd[L-proline]2 comples shows better anticancer activity than with-compare to Cd[D-proline]$_2$.Thus, Cd[L-proline]$_2$ complexes will be a novel drug for chemotherapy.

ACKNOWLEDGMENT

First author is grateful thank to Bharathidasan University, Tiruchirappalli, India for providing financial support by their University Research fellowship (URF) scheme (Ref.No:055441/URF/K7/2013). Thrid author thanks Department of Science and Technology, Science and Engineering Board (DST-SERB, SB/FT/CS-086/2013), India, for providing research fund. We acknowledge Dr. C. Prahalathan and Dr. A. Antony Joseph Velanganni, Department of Biochemistry, Bharathidasan University, Tiruchirappalli, India for their help with gel documentation and fluorescence microscopic studies. We are grateful to the Department of Science and Technology-Fund for Improvement of S&T Infrastructure in Universities and Higher Educational Institutions (DST-FIST) for their infrastructure support to our department.

REFERENCES

[1] D. E. Reichert, J. S. Lewis, and C. J. Anderson, "Metal complexes as diagnostic tools," *Coord. Chem. Rev.*, vol. 184, no. 1, pp. 3–66, 1999.

[2] M. Gutowski, P. Skurski, and J. Simons, "Dipole-bound anions of glycine based on the zwitterion and neutral structures," *J. Am. Chem. Soc.*, vol. 122, no. 41, pp. 10159–10162, 2000.

[3] J.-P. Yuan, G.-H. Wang, H. Ling, Q. Su, Y.-H. Yang, Y. Song, R.-J. Tang, Y. Liu, and C. Huang, "Diallyl disulfide-induced G2/M arrest of human gastric cancer MGC803 cells involves activation of p38 MAP kinase pathways," *World J. Gastroenterol.*, vol. 10, no. 18, pp. 2731–2734, 2004.

[4] S. Kasibhatla, G. P. Amarante-Mendes, D. Finucane, T. Brunner, E. Bossy-Wetzel, and D. R. Green, "Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis," *CSH Protoc*, vol. 3, 2006.

[5] J. Wu, L. Jing, H. Yuan, and S. Peng, "T-2 toxin induces apoptosis in ovarian granulosa cells of rats through reactive oxygen species-mediated mitochondrial pathway," *Toxicol. Lett.*, vol. 202, no. 3, pp. 168–177, 2011.

[6] C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2', 7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress," *Chem. Res. Toxicol.*, vol. 5, no. 2, pp. 227–231, 1992.

[7] L. V Johnson, M. L. Walsh, and L. B. Chen, "Localization of mitochondria in living cells with rhodamine 123," *Proc. Natl. Acad. Sci.*, vol. 77, no. 2, pp. 990–994, 1980.

[8] Y. Kondo, T. Aoki, A. Abe, and A. Tanabe, "MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) colorimetric assay for mitogen-induced activation of chicken peripheral blood lymphocytes," *Anim. Sci. Technol.*, 1995.

[9] G. Feng, J. C. Mareque-Rivas, and N. H. Williams, "Comparing a mononuclear Zn (II) complex with hydrogen bond donors with a dinuclear Zn (II) complex for catalysing phosphate ester cleavage," *Chem. Commun.*, no. 17, pp. 1845–1847, 2006.

[10] A. Russo, M. Terrasi, V. Agnese, D. Santini, and V. Bazan, "Apoptosis: a relevant tool for anticancer therapy," *Ann. Oncol.*, vol. 17, no. suppl 7, pp. vii115–vii123, 2006.

[11] T. Karmakar, Y. Kuang, N. Neamati, and J. B. Baruah, "Cadmium complexes and cocrystals of indium complexes of benzothiazole derivatives and anticancer activities of the cadmium complexes," *Polyhedron*, vol. 54, pp. 285–293, 2013.

[12] M. Leite, M. Quinta-Costa, P. S. Leite, and J. E. Guimarães, "Critical evaluation of techniques to detect and measure cell death--study in a model of UV radiation of the leukaemic cell line HL60," *Anal. Cell. Pathol.*, vol. 19, no. 3–4, pp. 139–151, 1999.

[13] W. L. Biffl, E. E. Moore, F. A. Moore, and C. C. Barnett, "Interleukin-6 delays neutrophil apoptosis via a mechanism involving platelet-activating factor," *J. Trauma Acute Care Surg.*, vol. 40, no. 4, pp. 575–579, 1996.

[14] S. Moulik, S. Pal, J. Biswas, and A. Chatterjee, "Role of ERK in modulating MMP 2 and MMP 9 with respect to tumour invasiveness in human cancer cell line MCF-7 and MDA-MB-231," *J. Tumor*, vol. 2, no. 2, 2014.

[15] H. Horiuchi, H. Kawamata, T. Furihata, F. Omotehara, H. Hori, Y. Shinagawa, Y. Ohkura, M. Tachibana, T. Yamazaki, T. Ajiki, and others, "A MEK inhibitor (U0126) markedly inhibits direct liver invasion of orthotopically inoculated human gallbladder cancer cells in nude mice.," *J. Exp. Clin. cancer Res. CR*, vol. 23, no. 4, pp. 599–606, 2004.

[16] D. Li, E. Ueta, T. Kimura, T. Yamamoto, and T. Osaki, "Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination," *Cancer Sci.*, vol. 95, no. 8, pp. 644–650, 2004.

[17] K.-C. Chen, P.-H. Kao, S.-R. Lin, and L.-S. Chang, "Upregulation of Fas and FasL in Taiwan cobra phospholipase A2-treated human neuroblastoma SK-N-SH cells through ROS-and Ca2+-mediated p38 MAPK activation," *J. Cell. Biochem.*, vol. 106, no. 1, pp. 93–102, 2009.

[18] F. Krepulat, J. Löhler, C. Heinlein, A. Hermannstädter, G. V Tolstonog, and W. Deppert, "Epigenetic mechanisms affect mutant p53 transgene expression in WAP-mutp53 transgenic mice," *Oncogene*, vol. 24, no. 29, pp. 4645–4659, 2005.

[19] C. J. Sherr and J. M. Roberts, "CDK inhibitors: positive and negative regulators of G1-phase progression," *Genes Dev.*, vol. 13, no. 12, pp. 1501–1512, 1999.

[20] R. Rajah, B. Valentinis, and P. Cohen, "Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-$β$1 on programmed cell death through a p53-and IGF-independent mechanism," *J. Biol. Chem.*, vol. 272, no. 18, pp. 12181–12188, 1997.

[21] L. Buckbinder, R. Talbott, S. Velasco-Miguel, I. Takenaka, B. Faha, B. R. Seizinger, and N. Kley, "Induction of the growth inhibitor IGF-binding protein 3 by p53," *Nature*, vol. 377, no. 6550, pp. 646–649, Oct. 1995.

[22] S. Pajaniradje, K. Mohankumar, R. Pamidimukkala, S. Subramanian, and R. Rajagopalan, "Antiproliferative and

apoptotic effects of sesbania grandiflora leaves in human cancer cells," *Biomed Res. Int.*, vol. 2014, 2014.

[23] H. Tuschl and C. Schwab, "Cytotoxic effects of the herbicide 2, 4-dichlorophenoxyacetic acid in HepG2 cells," *Food Chem. Toxicol.*, vol. 41, no. 3, pp. 385–393, 2003.

[24] K. Kannan and S. K. Jain, "Oxidative stress and apoptosis," *Pathophysiology*, vol. 7, no. 3, pp. 153–163, 2000.

[25] M. A. Hayat, *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging: Volume 3-Role in Specific Diseases*, vol. 3. Academic Press, 2013.

[26] S. Desagher and J.-C. Martinou, "Mitochondria as the central control point of apoptosis," *Trends Cell Biol.*, vol. 10, no. 9, pp. 369–377, 2000.