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INVESTIGATION OF HEAVY METAL INDUCED CELL DEATH THROUGH OXIDATIVE STRESS MEDIATED DNA DAMAGE

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Abstract

Most of the researcher says about soil that contaminated with heavy metals through the water due to the unregulated discharge of industrial wastewater, the release of metallic mines, application of pesticides that contain heavy metals, and many other anthropogenic activities. Agriculture fields and rivers near the industrial areas are usually contaminated with heavy metals such as Magnesium (Mg), lead (Pb), zinc (Zn), and copper (Cu). Those metals cause toxic effects on human health upon entering into the food chain. Acute intake of heavy metals that can produce harmful effects on human health and that can easily disrupt the normal cellular processes of the human body. However, we investigate the heavy metal that having the property of oxidative stress-induced DNA damage via the ability to scavenge free radicals, cell-killing property, induction of oxidative stress, Mutagenicity nature, and ability to damage the DNA. Finally, these findings provide scope for future studies on heavy metal-based drug development for the treatment of various diseases.

Keywords: heavy metal, oxidative stress, soil contamination, human health, DNA damage, Mutagenicity nature.

1. Introduction

Environmental pollution and food safety are the most important issues in the current scenario. Particularly the Soil and water pollution those having a historical impact on food safety and causes the human health-related problems (Lu et al. 2015). However, the source of heavy metals is producing from the soil pollution that can absorb by the roots and it transfers to the plant's seeds (Zhang et al. 2015). Those metals cause toxic effects on human health upon entering into the food chain. Acute intake of heavy metals can produce harmful effects on health and can easily disrupt the normal cellular processes of the human body (Mahaffey et al. 1975). One of the most broadly investigated mechanisms the generation of radicals that can cause pathophysiological diseases. The reactive oxygen species (ROS) can produce oxidative stress. The production of reactive species like hydroxyl radical (HO•), hydrogen peroxide (H_2O_2) and singlet oxygen that can able to annoys oxidant-antioxidant balance it is called oxidative stress. These reactive oxygen species react with the bio-molecules causes lipids peroxidation, proteins and DNA damage and their disturbing the functional properties of the cells, tissues, and organs, etc. which turns up to deadly diseases. Involvement of ROS in metal-induced cell death is widely reported (Monoj et al. 2013).

2. Materials and method

EQUIPMENT USED:

- Autoclave - Equitro
- CO2 incubator - NAPCO series 5400
- Balance - Denver instruments Apx 203.
- Deep freezer (-85°C) - Krisp cold.
- Elisa-micro plate reader - Bio rad 550.
- Filtration Unit - Millipore.
- Glassware's - Borosil.
- Hot air oven - American universal.

- Inverted microscope - Olympus IX 70.
- Laminar air flow - Klenzaids.
- Liquid nitrogen container - Cryocan BA 20.
- Magnetic stirrer - Remi instruments.
- Microtitre plates- Tarsons.
- Milli Q - Millipore.
- pH meter - U Tech
- Gel electrophoresis - Genie electrophortiic
- Water bath maintained at 37°C - NSW India.

CHEMICALS USED

- DCFH-DA(dichloro-dihydro-fluorescein diacetate) - Sigma Aldrich, Mumbai
- Phosphate buffer – S.D Fine Chemicals Ltd., Mumbai.
- Ascorbic acid - S.D Fine Chemicals Ltd., Mumbai.
- Ferric chloride - S.D Fine Chemicals Ltd., Mumbai.
- TCA(Trichloroacetic acid) - S.D Fine Chemicals Ltd., Mumbai.
- TBA(Tert-butyl alcohol) - Hi-Media Lab Pvt. Ltd, Mumbai.
- Hydrogen peroxide - Hi-Media Lab Pvt. Ltd, Mumbai.
- Agerose(Low EEO) - Hi-Media Lab Pvt. Ltd, Mumbai.
- ethdium bromide - Hi-Media Lab Pvt. Ltd, Mumbai.
- DNA loading buffer (sodium dodecyl sulphate,bromophenol blue,glycerol,tris-cl) - S.D Fine Chemicals Ltd., Mumbai.
- TE buffer (EDTA,Tris-cl) - S.D Fine Chemicals Ltd., Mumbai.
- PBR322 DNA - Sisco Research Laboratories Pvt. Lt
- Microbial Mutagenicity by Ames kit - MEDOX-BIO® AMES Test Teaching Kithttp://www.medoxbio.com/

MICROORGANISM USED

- Escherichia coli - NCTC 12923
- Klebsiella pneumoniae -NCTC 14097
- Pseudomonas aeruginosa - NCTC 14207
- Staphylococcus aureus - NCTC 14139
- Mycobacterium phlei - NCTC 8151

VERO CELL LINE

- Source : Cercopithecus aethiops (African Green Monkey)
- Tissue / Organ: Normal, Kidney
- Morphology: Fibroblast
- Usage : Cytotoxicity, Virology, Virus titration, Virus replication, Plaque assays, Viral vaccines

HEAVY METALS USED

- Magnesium Sulphate ($MgSO_4$) – Sigma Aldrich, Mumbai
- Copper(II) sulfate ($CuSO_4$) - Sigma Aldrich, Mumbai
- Zinc acetate ($ZnC_4H_6O_4$) - Sigma Aldrich, Mumbai
- Lead(II) acetate ($Pb(C_2H_3O_2)_2$) - Sigma Aldrich, Mumbai

2.1. Hydrogen peroxide radical scavenging assay

Principle: hydrogen peroxide is generated by enzymatic reactions. Even in normal conditions, the hydrogen peroxide production in commonly importance and its leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. In plant and animal cells, hydrogen peroxide produced by superoxide dismutase by disputation of O_2 thus donating to the lowering of oxidative reactions. To remove hydrogen peroxide contributes by a natural combination of dismutase and catalase and thus

will be a true cellular antioxidant activity. Hydrogen peroxide will be diffuse with trouble through cellular membranes. The generation of hydrogen peroxide by activated phagocytes is known to play a significant role in the killing of several bacterial and fungal strains.

Preparation of Test and Standard solutions: 30 mg of each sample were accurately weighed and separately dissolved in 10 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

Method: various concentrations of 1 ml of the heavy metal and ascorbic acid were added to 2 ml solutions in PBS. Then hydrogen peroxide was added. Then absorbance was measured at 230 nm against a blank solution that contained heavy metal and ascorbic acid in PBS without H_2O_2 (Srinivasan et al. 2007).

2.2. Lipid peroxidation assay

Principle: The principles behind the assay polyunsaturated fatty acids (PUFAs) almost present in the cell membranes are oxidized by free radical chain reactions or both enzymatic and auto-oxidative peroxidation. LPO takes place in three stages: initiation, propagation, and termination. In the initial stage of LPO, free radicals block hydrogen and it triggers to form PUFA into lipid radical. In this propagation stage, the lipid radical was formed by imitation stage which breaks down into produce more free radicals. In the termination stage, the free radical species react together or with antioxidants to form inactive products.

Preparation of Egg lecithin: Eggs yolks have to separate from the egg and collect the egg yolk into a separate beaker and treated with 50ml acetones, stirred it with the help of magnetic stirrer for 2-3 minute, and remove the supernatant layer. Repeat the process again and again finally; acetone helps to reduce the yellow color into white color. Once yolk gets decolorized, then incubate in 37°C. Once it evaporates the vapors of egg lecithin. Finally, purified egg lecithin powder was formed. Egg lecithin 3mg dissolved in 1ml of DMSO. Egg lecithin (3 mg/ml) concentration was prepared.

Method: The heavy metal & Ascorbic acid (100 µl) of different concentrations separately added into 1 ml of egg lecithin mixture, control without a sample. Lipid peroxidation has to induce by adding 5 µl $FeCl_3$ (200 mM) and 5µl L-ascorbic acids (200 mM). After incubation for 1 hour at 37°C, the reaction is stopped by adding 2 ml of 0.25 N H Cl containing 15% TCA and TBA 0.375%. Have to add and start heating by using a water bath for 30 minutes. Finally, the color change will appear (purple color). Then absorbance has to measure at 530 nm (Srinivasan et al. 2007).

2.3. Microbial mutagenicity by Ames test

Principle: This assay measures genetic damage at the single base level in DNA by using an E. coli test strain. The E. coli strain used in the assay has a unique mutation that has turned off histidine biosynthesis (HIS). Because of this original mutation, the bacteria require exogenous histidine to survive and will starve to death if grown without this essential nutrient (auxotrophy). The key to the assay is the bacteria can undergo a reverse mutation turning the essential gene back on permitting the cell to grow in the absence of histidine (prototropy). The bacterial strain will be created by a specific type of mutation either a base-pair substitution or frame shift mutation. Because a reverse, compensating mutation usually must occur by the same mutagenic mechanism, mechanistic toxicology information also available from Ames assay result based on the pattern of which strain reverted (His- or His+) (Stasio, 1979).

Preparation of broth: Take 20ml of distilled water and add 500mg of LB broth powder mix completely then autoclave.

Preparation of M910X Salt

- K_2HPO_4 : 2.25g
- KH_2PO_4 : 2.25g
- Ammonium sulphate : 0.5g
- Tri-sodium citrate : 0.25g
- Distilled water : 50ml

Preparation of minimal agar plate (HIS) (50ml)

- Agar : 1.5g
- Distilled water : 75ml

The agar will be melted and autoclave, after autoclaving, then add the following components at around 55°C

- $M_{910}X$ Salt : 7.5 ml
- $MgSO_4$: 0.75ml
- Glucose : 1.5ml
- Proline : 0.75ml
- Arginine : 0.75ml
- Threonine : 0.75ml
- Vitamin B_2 : 0.75ml

Mix it thoroughly and then pour in sterile petri dishes.

Stock solution preparation

- $MgSO_4$: 2.46g in 10ml
- All amino acids : 0.1g/10ml
- Vitamin B_2 : Take 10 ml sterile water and 0.1g of vitamin B_2 under aseptic conditions.

  (Do not autoclave after adding vitamin B_2).

Method: Day 1 culture Revive (host culture of Escherichia coli AB1523)

- Prepare 20ml of broth • Inoculate 0.5ml of host culture: incubate at 37 °C

Day 2 AMES test (Before starting the experiment, prepare the minimal agar plate and minimal soft agar tube)

- Take 1.5ml of culture and centrifuge it at 10,000 rpm for 5 min at 4°C and collect the pellets. Wash the pellets 2-3 times with 0.85% sterile normal saline and re-suspended the pellets in equal volume of sterile normal saline.
- Obtain three tubes containing 10ml of minimal soft agar from 55°C.
- Mark the three plates as 1, 2, 3.
- Aseptically add 0.5ml of re-suspended culture and 0.8 ml of freshly prepared hydroxylamine (NH_2OH) to tube 1. Add 0.5ml of re-suspended culture and 0.8ml of sterile saline in to plate 2 that will serve as a control (without NH_2OH). • Add 0.5ml of re-suspended culture and 0.8ml of test solution in tube 3.
- Quickly mix well bubbles and allow solidifying. Try to eliminate all bubbles and allow solidifying.
- Incubate the plate at 37°C for 48-72 hrs. After 72hrs, inoculated plate will be observed for colony formation

2.4. Evaluation of DNA damage by UV radiation

Principle: The principle behind the assay was DNA undergoes mutation when it examined with UV radiation. In this study examined by whether the addition of heavy metal along with the DNA that can able to protects DNA from damage induced by UV radiation that can be examined using by gel electrophoresis method. Finally compared with the control and test concentration we can clearly say the drug or compounds protect the mutation.

METHOD: In this DNA nicking assay was carried out using 10µL plasmid DNA, 10µL of different concentration of heavy metals, 4 µL of loading dye (bromophenol blue dye (0.25% in 50% glycerol) was added to form a mixture. This mixture examined in UV radiation and standard used as a without examined with UV radiation. Then the mixtures (24 µL) was load in a 0.7% agarose gel {prepared by dissolving 0.35 g of agarose in 50 mL TBE Buffer(made up of 2mL of TBE in 50 ml of distilled

water)}and electrophoresis was carried out at 50 V for 2 hours followed by ethidium bromide staining (Rastogi et al. 2010), (Ahsan et al. 1999).

2.5. Evaluation of DNA damage in microorganism induced by H_2O_2

Principle: The principle behind the assay was hydroxyl radicals produced by the hydrogen peroxide that cause DNA damage. The hydrogen peroxide travels into the cell membrane of microorganism cause DNA damage. That the study examined by whether the addition of drug along with the hydrogen peroxide that can able to protects microorganism cells from DNA damage induced by scavenging hydroxyl radical that can be examined using by gel electrophoresis method.

METHOD: In this DNA nicking assay was carried out in 96 well plates. First 200 µL microorganisms have to add in each well of a 96-well plate, (10µL of different concentration of heavy metals and ascorbic acid) was treated and incubate it for 24 hours. Then from supernatant layer 20 µL was taken out and mixed with 4 µL of loading dye (bromophenol blue dye (0.25% in 50% glycerol) was added to form a mixture. This mixture examined in UV radiation and standard used as a without examined with UV radiation. Then the mixtures (24 µL) was load in a 0.7% agarose gel {prepared by dissolving 0.35 g of agarose in 50 mL TBE Buffer(made up of 2mL of TBE in 50 ml of distilled water)}and electrophoresis was carried out at 50 V for 2 hours followed by ethidium bromide staining (Longum et al. 2015).

2.6. Involvement of ROS induced oxidative stress

Method: The animal cells was seeded at a 96 well plate.in the density of 5,000 cells per well and it incubate at co2 incubator for overnight for cell attachment. Cells are pre-treated with 0.1 mm ascorbic acid for 6 h before treatment with drug. Untreated are marked as a control. After 24 and 48 h, 20 µl of 5 mg/ml of MTT was added into each well, and the plate has to incubate for 3 hours. Supernatant layer will be removed and 100µl of DMSO are added to solubilize the purple blue formazan. The absorbance has to measure with the help of Micro plate Reader at wavelength of 570 nm, and 630 nm as reference wavelength. A graph of percentage of cell viability versus various concentrations of extracts was plotted, and the IC50 was determined.

2.7. Determination of the involvement of ROS in heavy metal induced oxidative stress

Method: The dichlorodihydro fluorescein diacetate (DCFH-DA) assay was used to determined level of intracellular reactive oxygen species (ROS). The animal cells was seeded at a 96 well plate.in the density of 5,000 cells per well and it incubate at co2 incubator for overnight for cell attachment. Then, the cells have to wash with 1X PBS and DCFH-DA are added incubate it for one hour in co2 incubator. Subsequently, the cell was wash with PBS and treated with various concentration of drug, 50 µM of H2O2 solution (positive control) for 3 h. Fluorescence intensity was measured at wavelength of 485 nm and emission wavelength of 535 nm (Tor et al. 2015).

2.8. Mechanism of induction of cell death in Vero cell line

Principle: Generally, cell death occurs in three different types .There are apoptosis, autophagic cell death, and necrosis. This method used to distinguishing between apoptotic and necrotic cell death that can be visualised by DNA fragmentation study. Generally the Apoptosis caused by fragmentation of DNA, necrosis caused without fragmentation of DNA.

Method: In this DNA nicking assay was carried out in the animal cell. First cells have to add in the each well of a 96-well plate (5000 µl/well), 10µL of different concentration of heavy metals was treated and incubate it for 24 hours. Then from supernatant layer 20 µL was taken out and mixed with 4 µL of loading dye (bromophenol blue dye (0.25% in 50% glycerol) was added to form a mixture. This mixture examined in UV radiation and standard used as a without examined with UV radiation. Then the mixtures (24 µL) was load in a 0.7% agarose gel {prepared by dissolving 0.35 g of agarose in 50 mL TBE Buffer(made up of 2mL of TBE in 50 ml of distilled water)}and electrophoresis was carried out at 50 V for 2 hour followed by ethidium bromide staining (Abid-Essefi et al. 2003).

3. Results and discussion

3.1. Hydrogen peroxide radical scavenging assay

In hydrogen peroxide radical scavenging assay, ascorbic acid showed a strong free radical scavenging effect. Other compounds are not able to scavenge free radicals produced during with the reaction. $Zn(CH_3CO_2)_2$ and $(Pb(CH_3COO)_2)$ showed highest production radicals during the reaction and showed less scavenging ability against radical with -43.76% and -43.87% respectively.(Figure 1)

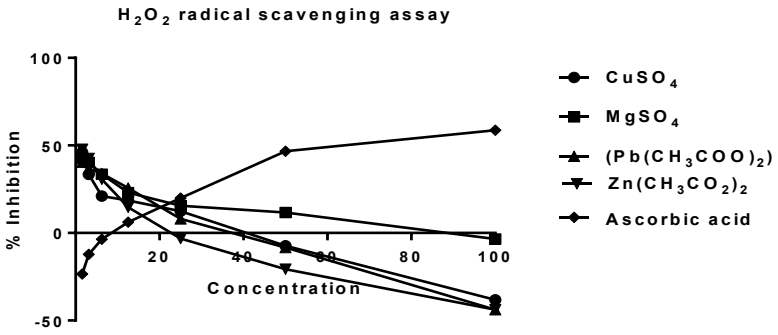


Figure 1

3.2. Lipid Peroxidation assay

Lipid peroxidation assay was carried out for all the compounds. Among the compound tested for lipid peroxidation assay, ascorbic acid showed a strong scavenging effect than heavy metals. All the heavy metals showed a reduction in inhibition according to their concentration. The higher concentration of compounds showed no inhibition. In graph (Figure.2) it is clear that all the heavy metals showed lesser than 50% inhibition. This inhibition may be because of lower concentration. At 100 µg/ml concentration, ascorbic acid showed 58.62% inhibition of lipid peroxidation.

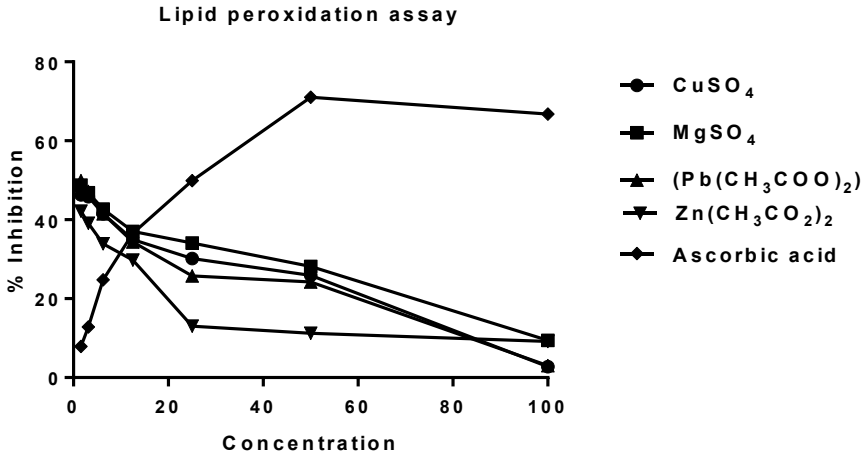
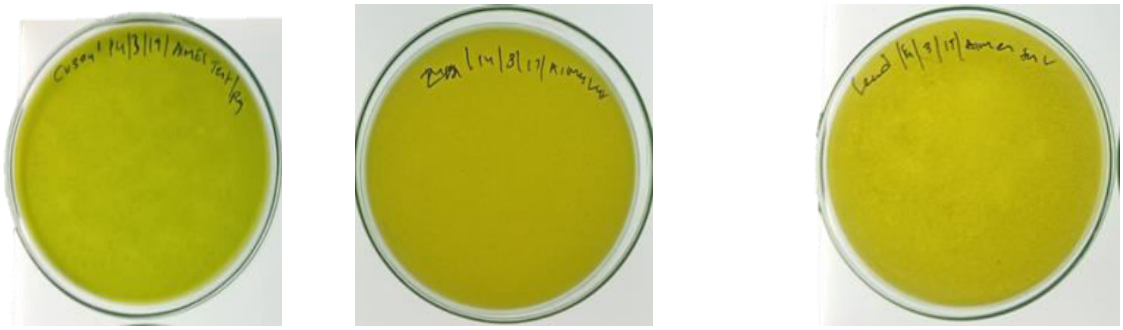


Figure 2

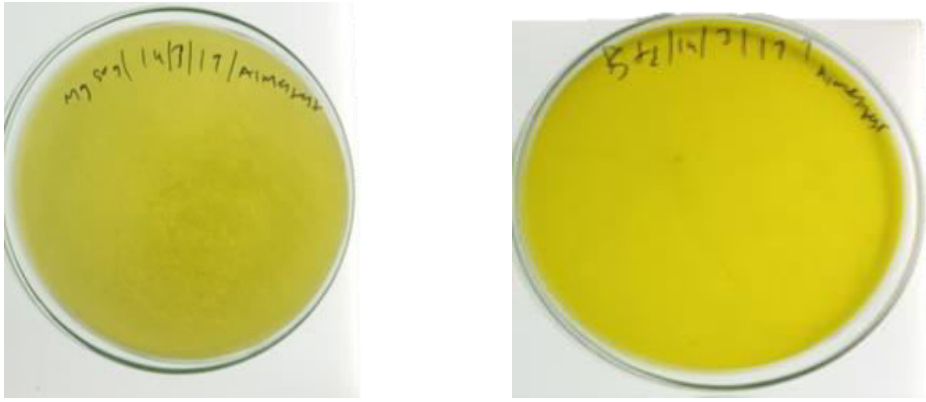
3.3. Microbial Mutagenicity by Ames assay

Ames test was carried out to know the mutagenicity nature of the heavy metals. In this assay E.coli which is used in this assay was mutated with their histidine genes. So, the growth of the organism increases gradually since histidine independently. In this assay, all the heavy metals turned to mutate the histidine gene off to on. In the following figure, it is clear that heavy metals showed mutation on histidine that is the reason the organism has grown with heavy metals shown growth. It indicates that

heavy metals are mutating the histidine gene and has carcinogenicity. This may be due to the production of oxidative stress by them might induce the mutation. (Figure 3)



CuSO4 *ZnC4H6O4* *Pb (C2H3O2)2*

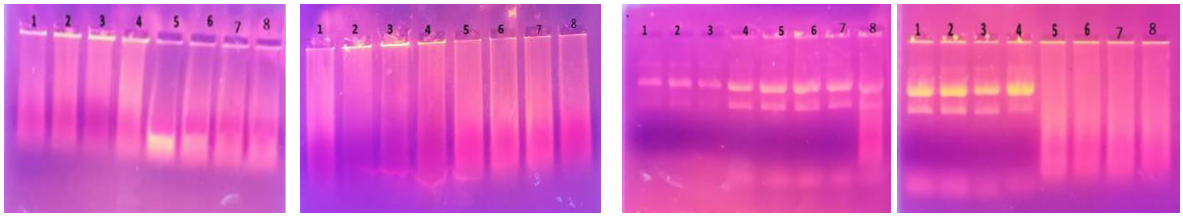


MgSO4 *Standard*

Figure 3

3.4. Evaluation of DNA Damage by UV radiation

Hence we investigated the evolution of DNA damage by UV radiation. In this study, the following figures represent the DNA damage caused by heavy metals whether it exposed to UV radiation or Non UV radiation. Form the following (figure 5 (A- D)) Lane 1 to 4 DNA treated with heavy metals and it also exposed to UV radiation (The concentrations starts with a lower concentration to higher concentrations). The Lane 4 to 8 also treated with heavy metals but it won't be exposed to UV radiation and it also indicates Non UV treated (the concentrations starts with a lower concentration to higher concentrations). Figure A & B represents the lowest concentration of heavy metal ($CuSO_4$ & $ZnC_4H_6O_4$) induced oxidative stress that caused DNA damage by both (UV treated or Non-UV treated). Figure C also clearly represents the heavy metals ($MgSO_4$) caused DNA damage by producing more fragments in both UV treated and Non-UV treated. So we concluded that particular heavy metal (MgSO4) having the property of producing the highest number of oxidative stress at a lower concentration. Figure 4 also clearly represents heavy metals (Pb $(C_2H_3O_2)_2$) caused DNA damage by producing more fragments in UV treated. From this figure we concluded that particular heavy metals (Pb $(C_2H_3O_2)_2$ producing lowest number oxidative stress caused DNA damage in Non-UV radiation but when the heavy metals (Pb $(C_2H_3O_2)_2$ exposed to UV radiation it tigers to produce oxidative stress. (Figure 4)



A *B* *C* *D*

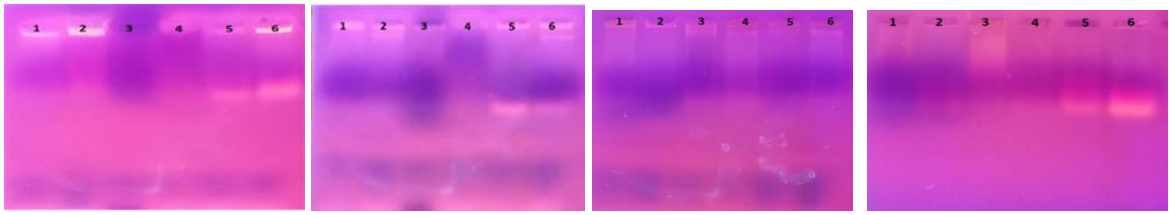
Figure 4 (A – D) Evaluation of UV induced DNA damage

A.CuSO4, B. ZnC4H6O4, C. MgSO4, D Pb(C2H3O2)2

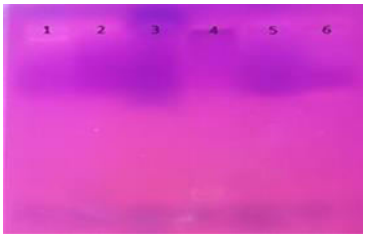
Lane 1 = UV+12.5µl of drug+10µl pBR322 DNA, Lane 2 = UV +25µl of drug+10µl pBR322 DNA, Lane 3 = UV +50µl of drug+10µl pBR322 DNA, Lane 4 = UV +100µl of drug+10µl pBR322 DNA, Lane 5 = NON UV+12.5µl of drug+10µl pBR322 DNA, Lane 6 = NON UV +25µl of drug+10µl pBR322 DNA, Lane 7=NON UV +50µl of drug+10µl pBR322 DNA, Lane 8 =NON UV +100µl of drug+10µl pBR322 DNA.

3.5. Evaluation of DNA Damage on Microorganism by H_2O_2

This assay was based on the ability of heavy metals to protect the DNA against damage caused by hydroxyl (•OH) radicals. Hydroxyl radicals generated by the hydrogen peroxide are known to cause oxidative stress-induced breaks in DNA strands to yield its open circular or relaxed forms. Exposure of microorganism to hydrogen peroxide ultimately results in strand breaks the cell membrane and cause DNA damage, mainly due to the generation of reactive species-hydroxyl radical and the subsequent free radical-induced reaction on cells. Hydroxyl radicals react with nitrogenous bases of DNA producing base radicals and sugar radicals. The base radicals, in turn, react with the sugar moiety causing breakage of the sugar-phosphate backbone of nucleic acid, resulting in strand break. The scavenging effect of heavy metals was evaluated in DNA Damage study. The following Figure depicts the ability of the compounds to reduce -dependent on cell-mediated DNA damage. From the figure represents When the heavy metals interact with microorganism it may produce oxidative stress leads to breaks the cell membrane and it causes cell-mediated DNA damage but when the microorganism interact with ascorbic acid it don't produce DNA damage. (Figure 5 (A-E))



A *B* *C* *D*



E

Figure 5 (A-E) to induction of oxidative stress induced DNA damage using H2O2

A. Escherichia coli, B. Klebsiella pneumoniae, C. Pseudomonas aeruginosa, D. Staphylococcus aureus,

E. Mycobacterium phlei.

Lane 1 = Microbial cell + hydrogen peroxide, Lane 2 = Microbial cell + CuSO4, Lane 3= Microbial cell + ascorbic acid, Lane 4 = Microbial cell + MgSO4, Lane 5 = Microbial cell + ZnC4H6O4, Lane 6 = Microbial cell + Pb(C2H3O2)2.

3.6. Involvement of ROS induced oxidative stress

The involvement of oxidative stress by compounds was confirmed by pre-treatment of Vero cell with antioxidant before compound treatments. Oxidative stress is the outcome of the excessive amount by free radicals produced inside the cell. The increased oxidative stress leads to cause cell death. In this study, cell viability was measured at 530 nm and 630 nm. The results found that ($Pb(CH_3COO)_2$) showed less viability (43%) at 530 nm and 41% at 630 nm indicates that the production of excessive oxidative stress in Vero cell followed by MgSO4 with 61% and 59% at 530 nm and 630 nm respectively. So it is understood that both ($Pb(CH_3COO)_2$) and $MgSO_4$ reduced the viability of the cell drastically that indicates both compounds induces the oxidative stress in Vero cell line upon the days increase. In this assay, ascorbic acid showed more viability (96% at 530 nm and 97% at 630 nm on 72 hours) since the ascorbic acid reduces the oxidative stress inside the cell. However, the results showed less viability by ($Pb(CH_3COO)_2$) and $MgSO_4$. It is deduced that the number of antioxidants in cells was depleting overtime. (Figure 6 & 7).

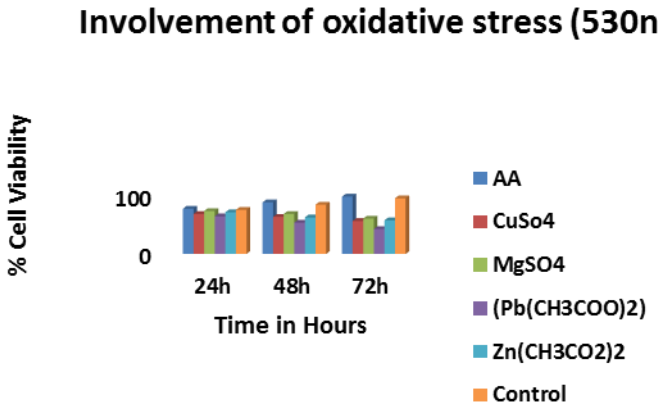


Figure 6

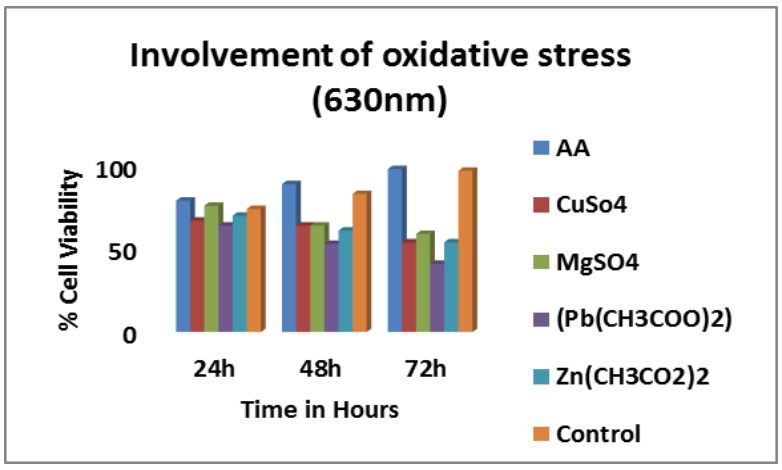


Figure 7

3.7. Determination of the involvement of ROS in heavy metal induced oxidative stress

Fluorescent probe DCFH-DA was used to measure the formation of intracellular ROS in compounds induced cell death.H_2O_2 showed the highest fold change into 6.1. in case of heavy metals, $Zn(CH_3CO_2)_2$ showed highest fold change of 5.6 followed by $MgSO_4$, ($Pb(CH_3COO)_2$) and $CuSO_4$ with a relative fold change of 4.1, 3.6 and 2.7. Ascorbic acid-induced oxidative stress in Vero cells was without the involvement of certain reactive species. This can be explained by DCFH-DA that is not sensitive to other reactive species such as reactive nitrogen species, H_2O_2, lipid peroxides, singlet

O_2. Thus, the induction of oxidative stress by compounds could be due to these reactive species. (Figure 8).

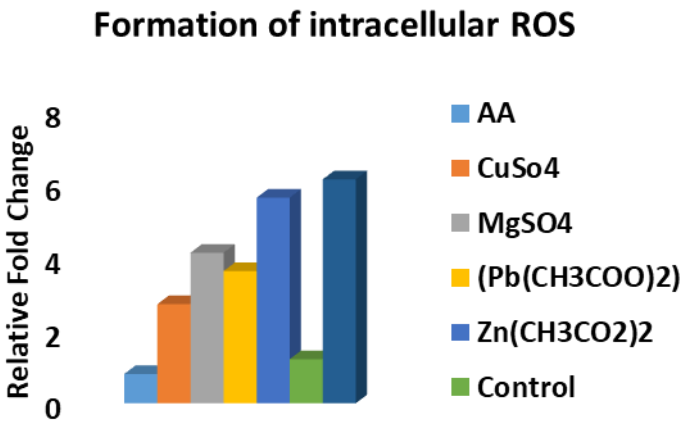
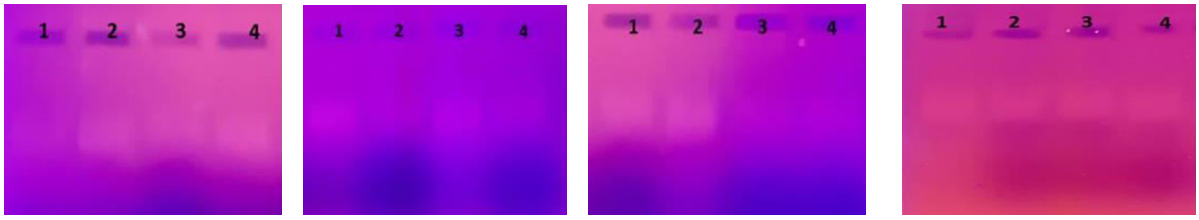


Figure 8

3.8 Mechanism of induction of cell death in Vero cell line

In general, there are three types of cell death occurs in the cell. The apoptosis, autophagic cell death, and necrosis. The various methods of distinguishing between apoptotic and necrotic cell death rely on characteristic features that can be visualized using DNA fragmentation. Apoptosis is accompanied by fragmentation of DNA, necrosis occurs without fragmentation of DNA. The following (figure 10 (A-D)) clearly shows that there was necrosis with no fragmentation happened in case of heavy metals $CuSO_4$, $(Pb(CH_3COO)_2)$. At the same time, apoptosis with the fragmentation of DNA was obtained from two heavy metals namely $MgSO_4$ and Zn $(CH_3CO_2)_2$.



A *B* *C* *D*

Figure 9 (A-D) Mechanism of induction of cell death in Vero cell line

A.CuSO4, B. MgSO4,C. Pb(C2H3O2)2, D. ZnC4H6O4,

Lane 1 = 12.5µl of drug+200µl vero cells, Lane 2 = 25µl of drug+200µl vero cells,

Lane 3 = 50µl of drug+200µl vero cells, Lane 4 = 100µl of drug+200µl vero cells

4. Summary & Conclusion

In hydrogen peroxide radical scavenging assay, ascorbic acid showed a strong free radical scavenging effect. At the same time, other compounds are not able to scavenge the free radicals produced during the reaction. During the assay, Zn $(CH_3CO_2)_2$ and (Pb $(CH_3COO)_2$) showed the highest production radicals.

Lipid peroxidation assay was carried out for all the compounds. Among the compound tested for lipid peroxidation assay, ascorbic acid showed a strong scavenging effect than the heavy metals. All the heavy metals showed a reduction in inhibition according to their concentration.

Microbial Mutagenicity by Ames assay was carried out to know the mutagenicity nature of the heavy metals. In this assay clear that heavy metals are mutant to the histidine gene present in E. coli and

have carcinogenicity. However, all other heavy metals used in this study showed mutation on histidine gene clusters of E. coli. Therefore, it is understood that the mutation effect of the heavy metals may be due to the production of oxidative stress that might induce DNA damage.

DNA damage study was done in both microorganism and UV radiation. It indicates the production of oxidative stress and it causes cell-mediated DNA damage.

Induction of oxidative stress was done by using DCFH-DA and 530nm and 630nm.in this assay among the heavy metals the (Pb $(CH_3COO)_2$) has the property of producing the highest number of oxidative stress.

Finally, mechanism of induction of cell death in Vero cell line was carried out, the results showed that there was necrosis with no fragmentation was observed in case of $CuSO_4$, (Pb $(CH_3COO)_2$). At the same time, apoptosis with the fragmentation of DNA was obtained from $MgSO_4$ and Zn $(CH_3CO_2)_2$

In conclusion, we investigated and demonstrated the efficacy of the heavy metals for their oxidative stress-induced DNA damage via the ability to scavenge free radicals, cell-killing property, induction of oxidative stress, Mutagenicity nature, and the ability to damage the DNA. Finally, these findings provide scope for future studies on heavy metal-based drug development for the treatment of various diseases.

Abbreviations

TCA - Trichloroacetic acid

TBA - Tert-butyl alcohol

FeCl3- Ferric chloride

H2O2-Hydrogen peroxide

DCFH-DA - dichloro-dihydro-fluorescein diacetate

PBS - Phosphate buffer

MTT DYE: 3-(4, 5-dimethylthiazol-2-yl)-2

LPO: Lipid Peroxidation

DNA: Deoxyribonucleic acid

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