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Inhibitory effect of milk thistle seed extract on cadmium chloride induced DNA damage in liver cells

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Abstract: Cadmium (Cd) is a biologically non-essential but economically valuable metal. Its increase in the environment has been linked to various diseases such as diabetes, organs failure, bone damage, endocrine hormone imbalance and cancer in humans and animals. Cd mainly accumulates in the liver and lead to its malfunction. In the present paper, we investigated the inhibitory effect of milk thistle (*Silybum marianum*) seed (MTS) extract on cell death and Deoxyribonucleic acid (DNA) damage induced by cadmium chloride ($CdCl_2$) in rat liver cells. The alkaline and neutral comet assays were used to evaluate double and single breaks in DNA. The treatment of the cells with $CdCl_2$ alone resulted in dose dependent decrease in cell viability compared to control cells; while cells co-treated with MTS extract resulted in increased cell viability. Furthermore, treatment of the cells with $CdCl_2$ alone caused increase in DNA damage as shown by the % DNA in the tail and olive tail moment in both alkaline and neutral comet assays. Co-treatment with MTS extract inhibited the Cd-induced DNA damage as shown by the decreased % DNA in the tail and olive tail moment. Our results clearly showed the inhibitory effect of MTS extract on Cd-induced DNA damage in rat liver cells.

Keywords: Cadmium, Comet assay, DNA damage, Inhibition, Milk thistle.

Citation: Camille A.M.Webster et.al. (2018) Inhibitory effect of Milk thistle seed extract on cadmium chloride induced DNA damage in liver cells. Journal of PeerScientist 1(2): e1000007.

Received May 24, 2018; **Accepted** November 06, 2018; **Published** December 29, 2018.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing Interests: The authors have declared that no competing interests exist.

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I. INTRODUCTION

Cadmium (Cd) is a toxic heavy metal that accumulates for long time in different parts of the human body due to its long half-life (15-20 years) and poor excretion. Cd occurs naturally and its level in the environment is elevated via anthropogenic activities. Natural sources of Cd can result from volcanic eruptions and forest fires. Cd and its compounds are economically valuable and are used in the manufacturing of NiCd batteries which has dominated the market due to their use in power tools, household appliances and other communication devices. Furthermore, it is also used in manufacturing of paints, plastics, and phosphate fertilizers [1]. One of the main anthropogenic sources of Cd is airborne from smelters, iron and steel production facilities and burning of coal, oil, and municipal waste [2]. There are three routes of exposure to Cd: inhalation of polluted air, ingestion of contaminated food and water, and dermal exposure from lack of unprotected coverage at work. Of these routes, ingestion through certain foods grown on contaminated soil, and inhalation of cigarette smoking is the major ways of Cd exposure to humans [3-4]. The increasing potential of Cd toxicity has become a global concern. Cd is present in many items in every house and causes irreversible damage to body organs even at low concentration (1-3 μg). It has been classified by the IARC (International Agency for Research and Cancer) as a human carcinogen which causes tumors of the liver, prostate, lung, and other tissues [5]. The major proposed mechanisms by which Cd causes toxicity are: inhibition of DNA damage repair, aberrant gene expression, apoptosis, and the induction of oxidative stress [6-9].

There are a few well-studied internal and external protective agents against Cd toxicity. One of the internal protective agents is small molecular weight protein called metallothionine [10], while external protective agents may be synthetic or natural compounds including metals such as Zn and Se that acts as antioxidants by binding to free oxygen radicals that are induced by Cd [11-12]. Other external protective agents that acts as chelators

such as monoisoamyl 2, 3-dimercaptosuccinate [13-14] and N-Acetyl Cysteine [15-16] binds with Cd and makes it unfree to bind to biomolecules.

As our team continuous studies to investigate various ameliorative compounds against Cd toxicity, we tested milk thistle seed (MTS) extract in this report. MT (*Silybum marianum*) is a plant that belongs to Asteraceae/Compositae family. The seeds of MT (MTS) have been used to treat liver and gallbladder disorders [17]. It is one of the most commonly used non-traditional medicines in Germany [18]. MTS powder is commonly used as a dietary supplement in USA for liver detoxification. Given its protective effects on liver and knowing that Cd accumulate mostly in the liver, we hypothesized that MTS extract may play an important role against Cd induced toxicity. In the current study, we evaluated the inhibitory effect of MTS extract on Cd-induced DNA damage through alkaline and neutral comet assays which detect single strand and double strand breaks in $CdCl_2$ treated rat liver cells.

II. Results and Discussion

Cd accumulates mostly in the liver due to its role in metal homeostasis and detoxification via the first bypass. It was evidenced by the analyses of mammalian organs showing more Cd accumulation in liver and kidney than in other parts of the body [19-20]. The liver injury was characterized by the increased serum levels of hepatic transaminases and massive necrosis of hepatocytes [21]. It is reported that environmental Cd exposure is associated with hepatic necro-inflammation in both men and women with increased risk of liver disease mortality [22]. In the present study, we treated the cells with 50, 75 and 100 µM $CdCl_2$ based on our previous report [8]. The Milk thistle seed (MTS) extract is commonly used as liver protective agent in US. In this study, the inhibitory effects of MTS extract on cell death and DNA damage through viability and comet assays were evaluated in $CdCl_2$ treated rat liver cells.

The viability data showed that $CdCl_2$ caused significant (p<0.05) dose-dependent decrease in viability of liver cells in comparison to untreated control cells (Fig. 1). This data was supported by our previous report where it was shown that $CdCl_2$ caused dose-dependent decrease in viability of same liver cell line [8]. However, more toxicity was observed in the current study due to different factors. One of the factors may be the passage number of liver cells used in the experiments. The MTS extract showed significant (p<0.05) maximum protection against Cd-induced toxicity at 5 µg/ml concentration (Fig. 1, lane 5). Hence, we used this MTS concentration for further studies in liver cells.

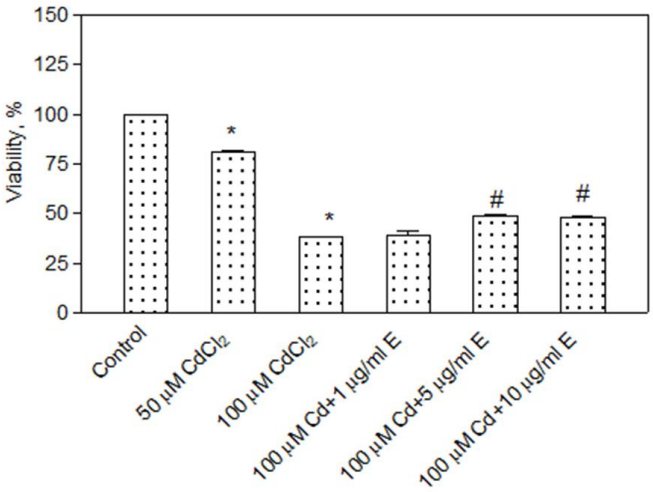

Figure 1: The effect of MTS extract on the viability in $CdCl_2$ treated normal rat liver cells. Data represented as means ± SD (n=3). *p<0.05 statistically significant compared to control; #p<0.05 compared to $CdCl_2$ alone. Analyzed by One way ANOVA, Bonferroni multiple comparison tests of Prizm software 3.0.

The inhibitory effect of MTS extract on DNA damage in $CdCl_2$ treated normal rat liver cells was evaluated by alkaline and neutral comet assays. The control cells of alkaline comet assay showed round DNA without any comet tail (Fig. 2a). In the cells treated with 50 and 100 µM $CdCl_2$ alone, the comet tail was observed with dose dependent increase in length (Fig. 2b-c). However, in co-treated cells with MTS extract, the comet tail was decreased in comparison to $CdCl_2$ alone treated cells (Fig. 2d).

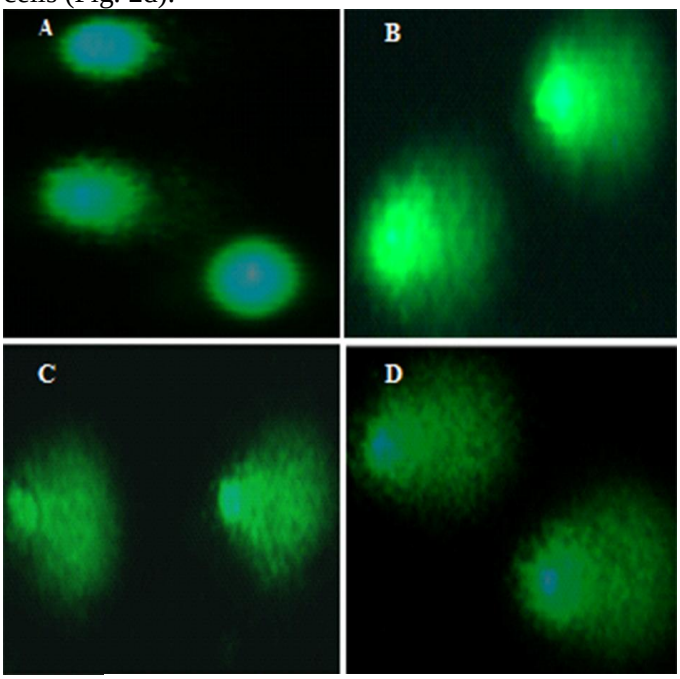

Figure 2: Comet images of alkaline gel electrophoresis assay showing single strand DNA breaks in normal rat liver cells treated with $CdCl_2$ alone or co-treated with MTS extract. Control (A), 50 µM $CdCl_2$(B), 100 µM $CdCl_2$ (C), and 100 µM $CdCl_2$ + 5 µg/ml MTS extract (D).

The % DNA in the tail is the ratio of total intensity of the tail and total intensity of the comet (head and tail together). The results of alkaline comet assay showed that in the cells treated with 50 and 100 µM $CdCl_2$, the % DNA in the tail was increased significantly ($p< 0.05$) to 20.65 ± 4.0 and 83.44 ± 3.8 (Fig. 3a, lanes 2-3) respectively in comparison to the control (2.82 ± 1.1; Fig. 3a, lane 1). However, in the cells co-treated with 5 µg/ml MTS extract and 100 µM $CdCl_2$, the % DNA in the tail significantly ($p<0.001$) decreased to 43.62 ± 1.9 (Fig. 3a, lane 4) in comparison to 100 µM $CdCl_2$ alone (83.44 ± 3.8, Fig. 3a, lane 3). The olive tail moment is the product of tail length and the percentage DNA in tail. It was observed that in the cells treated with 50 and 100 µM $CdCl_2$ alone, there was a significant ($p<0.05$) increase in olive tail moment to 9.12 ± 4.8 and 31.72 ± 4.4 px respectively (Fig. 3b, lanes 2-3) in comparison to the control (0.5 ± 0.2 px, Fig. 3b, lane 1). However, in the cells co-treated with 5 µg/ml MTS extract and 100 µM $CdCl_2$, the olive tail movement significantly ($p<0.001$) decreased to 14.25 ± 0.6 px (Fig. 3b, lane 4) in comparison to 100 µM $CdCl_2$ alone (31.72 ± 4.4; Fig. 3b, lane 3).

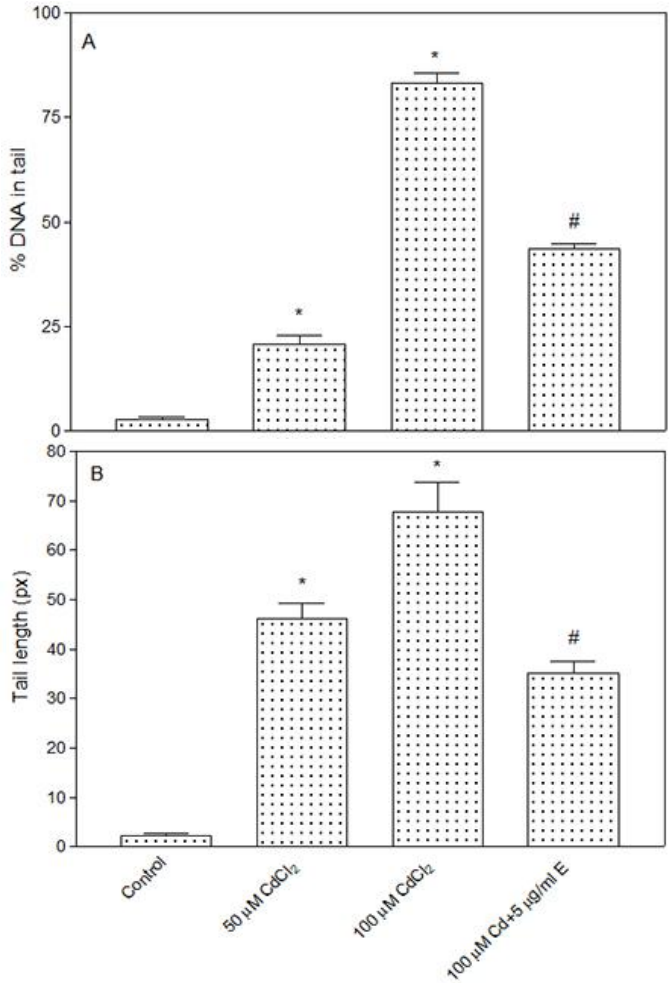

Figure 3: Quantitative analysis of alkaline gel electrophoresis assay showing single strand DNA breaks in normal rat liver cells treated with $CdCl_2$ alone or co-treated with MTS extract. Data represented as means ± SD (n=3). *$p<0.05$ statistically significant compared to control; #$p<0.001$ compared to $CdCl_2$alone. Analyzed by one way ANOVA, Bonferroni multiple comparison tests of Prizm software 3.0.

The control cells of neutral gel comet assay showed round DNA without any damage (Fig. 4a). In the cells treated with 50 and 100 µM $CdCl_2$, the comet tail was observed with dose-dependent increase in length (Fig. 4b-c). However, in co-treated cells with 5 µg/ml MTS extract, the comet length was decreased (Fig. 4d) in comparison to $CdCl_2$ alone treated cells.

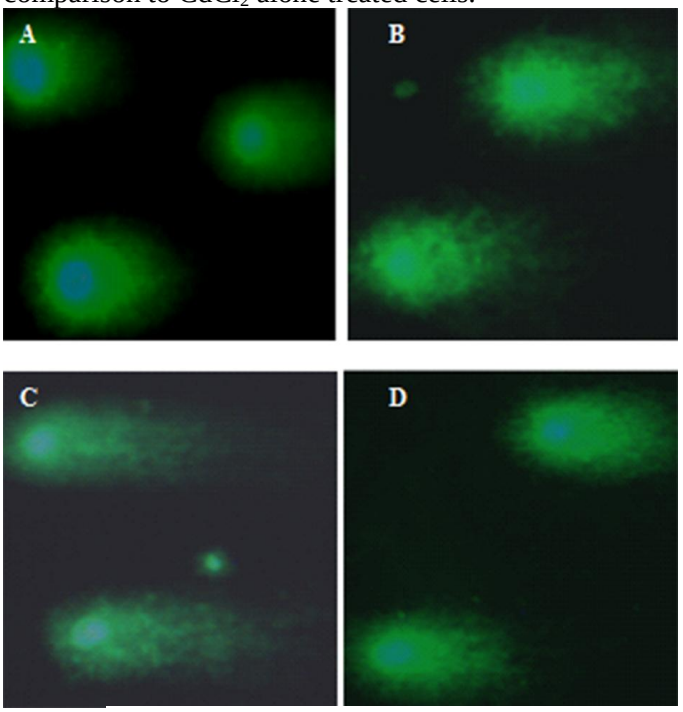

Figure 4: Comet images of neutral gel electrophoresis assay showing double strand DNA breaks in normal rat liver cells treated with $CdCl_2$ alone or co-treated with MTS extract. Control (A), 50 µM $CdCl_2$(B), 100 µM $CdCl_2$ (C), and 100 µM $CdCl_2$ + 5 µg/ml MTS extract (D).

The quantitative results of neutral comet assay showed that in the cells treated with 50 and 100 µM $CdCl_2$ alone, the % DNA in the tail significantly ($p<0.05$) increased to 18.85 ± 1.4px, and 54.38 ± 5.1px respectively (Fig. 5a, lanes 2-3) in comparison to the control (5.82 ± 1.7px; Fig. 5a, lane 1). However, in the cells co-treated with 5 µg/ml MTS extract and 100 µM $CdCl_2$, the % DNA in the tail significantly ($p< 0.001$) decreased to 14.63 ± 4.6 (Fig. 5a, lane 4) in comparison to 100 µM $CdCl_2$ alone (54.38 ± 5.1 px; Fig. 5a, lane 3). In the cells treated with 50 and 100 µM $CdCl_2$ alone, the olive tail moment significantly ($p<0.05$) increased to 8.29 ± 1.3 and 15.05 ± 3.8px (Fig. 5b, lanes 2-3) respectively in comparison to the control (2.09 ± 0.5px; Fig. 5b, lane 1). However, in the cells co-treated with 5 µg/ml MTS extract and 100 µM $CdCl_2$, the olive tail moment significantly ($p<0.001$) decreased to 2.29 ± 0.8 px

(Fig.5b, lane 4) in comparison to 100 µM $CdCl_2$ alone (15.05 ± 3.8px; Fig. 5b, lane 3).

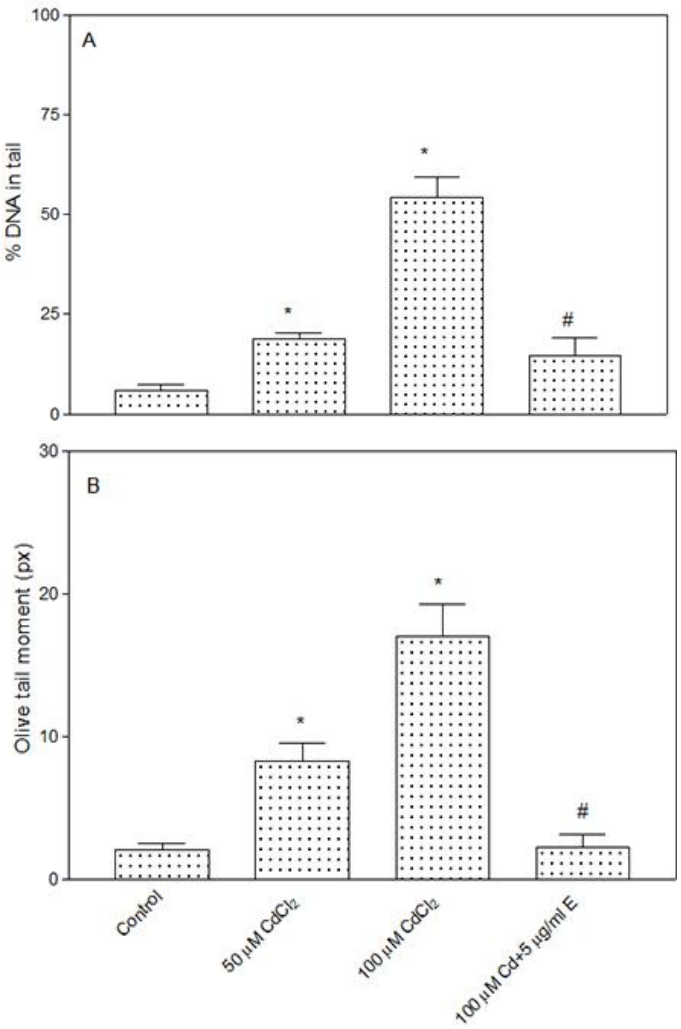

Figure 5: Quantitative analysis of neutral gel electrophoresis assay showing double strand DNA breaks in normal rat liver cells treated with $CdCl_2$ alone or co-treated with MTS extract. Data represented as means ± SD (n=3). *p<0.05 statistically significant compared to control; #p<0.001 compared to $CdCl_2$alone. Analyzed by One way ANOVA, Bonferroni multiple comparison tests of Prizm software 3.0.

The alkaline comet assay showed the single-strand breaks (SSB); while neutral comet assay showed the double-strand breaks (DSB). The level of DNA damage of Cd-treated liver cells is dose dependent (Figs. 2-5). This may be due to the liver cells becoming more sensitive to Cd due to increased oxidative stress and the production of lipoxygenase which destroys the liver cells [23]. It was noticed that the level of DNA damage was higher in single-strand breaks than double strand breaks (Figs. 2-5). This may be because the reaction sites on the single stranded DNA are more accessible by the damaging agents ($CdCl_2$), hence inducing more damage; while the tightly bound proteins on double stranded DNA may inhibit the accessibility by the damaging agents [24]. MTS extract co-treatment significantly increased the viability of Cd treated liver cells (Fig. 1). The MTS extract contains polyphenolic antioxidant flavonoid complex containing several types of flavonoids [25]. The main active ingredient in MTS extract is silymarin. In previous reports, it was shown that silymarin acts on the outer cell membrane and prevent hepatotoxic substances from entering the cell [26-27]. It was also reported that silymarin inhibits lipoxygenase, an enzyme that destroys liver cells [28] and increases the levels of glutathione, a potent antioxidant [25]. Due to the protective nature of silymarin in MTS extract, less Cd might has entered the cells co-treated with MTS extract which was reflected in increase cell viability compared to $CdCl_2$ alone treated cells (Fig. 1, lanes 3-6). The alkaline and neutral comet assays data also showed that the co-treatment of cells with 5 µg/ml of MTS extract and 100 µM $CdCl_2$ showed a decrease in DNA damage (Figs. 2-5). The decrease in DNA damage in the co-treatment group may be due to the chelation of Cd or free radicals by MTS extract leading to less availability of free Cd or free radicals inside the cell that cause directly or indirectly the DNA damage.

III. Conclusion

In summary, MTS extract showed protective effect against Cd toxicity in rat liver cells. Co-treatment with MTS extract increased the viability of Cd treated liver cells. MTS extract exhibited the inhibition of Cd-induced DNA damage that was observed in both alkaline and neutral comet assays in normal liver cells.

IV. Material and Methods:

Amphotericin antifungal solution (1000X), Boric Acid, Cadmium Chloride ($CdCl_2$), Crystal violet dye, Dimethyl Sulfoxide (DMSO), 0.5 mM EDTA, 95% Ethanol, Fetal bovine serum (FBS), F12K medium (1X), 50% Glutaraldehyde, Penicillin-streptomycin antibiotic solution (100X), Phosphate buffer solution (PBS), Sodium Dihydrogen Phosphate, Sodium hydroxide, and Tris Base were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Milk thistle seed Extract was purchased from the local New leaf Market Store (Tallahassee, FL, USA). The CometAssay™ HT kit was purchased from Trevigen Inc. (Gaithersburg, MA, USA).

a. Maintenance of rat liver cell line

Rat normal liver cell line (CRL 1439) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured according to the guidelines supplied by ATCC. The cells were maintained in F12K medium containing 100 units of penicillin/ml, 100 µg of streptomycin/ml, 2 mM L-glutamine and 10%

fetal bovine serum in T-75 cm^2 flasks at 37° C in a 5% CO_2 incubator. When the cells reached approximately 90% confluence, they were sub-cultured using 0.25% trypsin-EDTA (1mM) solution. The number of viable cells in the suspension were counted using trypan blue dye exclusion test in a haemocytometer.

b. Preparation of MTS extract and $CdCl_2$

MTS was prepared by weighing out 0.1g of MTS powder and dissolved in DMSO by bringing the volume to 100 µl. The mixture was vortexed until the MTS was completely dissolved. F12K complete medium was added to prepare the working concentration to be used for testing the ameliorative effect against Cd toxicity. The 10 mM $CdCl_2$ stock solution was prepared with sterile water and used for testing for the Cd toxicity on the cells.

c. Treatment of the cells for viability assay

The inhibitory effect of MTS against Cd toxicity on rat liver cells was carried out in polystyrene, flat bottom 24-well culture plates. The liver cells were plated at an initial density of 1 x 105 cells/well in a final volume of 900 µl complete medium and allowed to stabilize overnight in a 5% CO2 incubator at 37° C. Following this, the cells were treated with 0, 50, and 100 µM $CdCl_2$ alone or co-treated with 1, 5 and 10 µg/ml of MTS and 100 µM $CdCl_2$ in triplicate wells. All treatments were repeated at least twice. Cells were also treated with DMSO (0.1% final concentration) since MTS was dissolved in DMSO. The cell viability was evaluated by dye uptake assay using crystal violet according to our previous report [8].

d. Treatment of the cells for comet assay

The cells were plated at a density of 1.3 x 10^6 cells in each T-25 flask in a final volume of 4.5 ml of complete medium. The cells were allowed to stabilize overnight in a 5% CO2 incubator at 37° C. The cells were treated with 0, 50, or 100 µM of $CdCl_2$ alone or co-treated with 5 µg/ml MTS extract and 100 µM $CdCl_2$ for 24 h in triplicate flasks. At the end of the incubation, the treated cells were harvested by trypsinization and cryopreserved in liquid nitrogen until they were used for comet assay.

e. Preparation of slides for comet assay

The comet assay was conducted according to the protocol by Trevigen's CometAssay™ with minor modifications. The entire experiment was carried out in dimmed light. Cryo preserved control and treated cells were thawed by submerging in 37° C water bath. The cells were washed with 500 µl of ice cold 1X PBS (Ca^{2+} and Mg^{2+} free) solution and centrifuged at 1000 rpm for 5 min. The supernatant (450 µl) was discarded and the cell pellet was resuspended in the remaining 50 µl of 1X PBS solution. The cells were mixed with 500 µl of low melting point agarose gently and 50 µl of cells-agarose suspension per well was spread on 2-well Comet Slide (Trevigen™). The slides were incubated on ice for 5 min for solidification and the slides were submerged and incubated in lysis buffer solution provided by Trevigen's CometAssay™ at 4° C (in refrigerator) overnight. Lastly, the slides were removed from the lysis solution and the excess lysis buffer solution was drained from the slides and electophoresed.

f. Alkaline single-cell electrophoresis assay

This assay was used to detect single strand breaks in the DNA. Freshly prepared alkaline electrophoresis buffer solution (1 mM EDTA and 300 mM NaOH) was kept at -20° C for 30 min. The buffer solution (950 ml) was poured into the pre ice-cooled plexiglass comet assay electrophoresis chamber (Trevigen, MD, USA) and the slides were placed in the electrophoresis chamber for 20 min prior to electrophoresis to facilitate DNA unwinding. Electrophoresis was conducted for 20 min at 21V and 300 mA. The slides were then neutralized thrice with Tris buffer (pH 7.5) in drop wise for 5 min. The slides were then fixed with 95% ice cold ethanol for 5 min and air dried.

g. Neutral single-cell electrophoresis assay

This assay was used to detect double strand breaks in the DNA. The protocol carried out before lysis of the cells was similar to the procedures carried out for alkaline. Freshly prepared neutral electrophoresis buffer (1X TBE) was poured in a plexiglass comet assay electrophoresis chamber. Prior to electrophoresis, the slides were treated with 1X TBE buffer in drop wise for 5 min thrice. Electrophoresis was conducted for 10 min at 21V and 300 mA at room temperature. The slides were neutralized thrice with Tris buffer (pH 7.5) for 5 min. The slides were then fixed with 95% ice cold ethanol for 5 min and air dried.

h. Preparation of slides for scoring

The dried fixed slides were stained with 30 µl of SYBR green per well (1µl from the Trevigen's Comet Assay™ Kit in 10 ml of PBS) and the excess dye was drained prior to keeping on the microscope. The comets were viewed and scored in the slides with Leica DML fluorescence microscope. For each treatment, 50

cells/replicate/treatment were randomly selected and scored at a magnification of 200X (total of 150 cells per treatment). The analysis of DNA fragmentation was done using the software TriTek CometScore™ version 1.5. The % tail DNA and olive tail moment were used to analyze the DNA fragmentation. The olive tail moment has been suggested to be an appropriate index of induced DNA damage in considering both the migration of the genetic material as well as the relative amount of DNA in the tail.

i. Statistical analysis

The comet assay results were presented as mean ± standard deviation (SD; n=3). The data were analyzed for significance by one-way ANOVA, followed by Bonferroni multiple comparison tests using GraphPad Prism Software, version 3.00 (San Diego, CA, USA). The test values $p < 0.05$ and $p < 0.01$ were considered significant and highly significant in comparison to the respective untreated control or $CdCl_2$alone.

Authors Contribution: CAMW, COO, VLDB and LML conceived the idea, performed the experiments, interpreted the data and wrote the manuscript.

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