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***Research article***

# NEPHRO-PROTECTIVE ACTIVITY OF ETHANOLIC EXTRACT OF *MELIA AZADIRACHTA* AGAINST $H_2O_2$ INDUCED TOXICITY IN VERO CELL LINE

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**ABSTRACT:** Renal disorders have become very common nowadays, which may also lead to kidney failure. The disorder may be caused by the commonly used chemicals such as acetaminophen, $CCl_4$, streptromycin, $H_2O_2$ etc. The objective of this work is to determine the nephroprotective potential of ethanolic extract against $H_2O_2$ induced toxicity in VERO cell line. Ethanolic extract is known for its antioxidant, anti-inflammatory and anti-microbial effects, which make it a most sought for herbal medicine. Its characteristic features have identified this compound as a potential hepatoprotective and nephroprotective agent. VERO cells are cells taken from the kidney of an African green monkey, which are used in our study. The matured leaves of *Melia Azadirachta* were used to prepare ethanolic extract and the same was used to test for its inhibitory effect in 96 micro plate formats against in VERO cell lines. To study the cytotoxic properties of ethanolic extract against VERO cell line, we have tested the MTT assay with different concentrations in the range of 1000 to 62.5 µg/ml. From the performed assay, the effect of ethanolic extract drug reveals an enhanced activity on in VERO cell lines and that infers *Melia Azadirachta,* can be used as nephroprotective agent.

**Key words:** *Melia Azadirachta,* VERO cell line, $H_2O_2$, nephroprotective, Ethanolic extract.

## Introduction:

Several million people get diagnosed with kidney disorders every year around the world. Acute kidney disorders are most often due to synthetic drugs and are called Drug-induced nephrotoxicity (DIN). Nephrotoxicity is a serious consequence of $H_2O_2$ (Wink, David A et al, 1996), which is a diffusible reactive oxygen metabolite and a commonly used liquid to fight germs. Several parameters in blood enable the easy diagnosis of renal failures. Such failures require immediate medical attention or otherwise may often lead to death.

The extracts of various medicinal plants are generally used in curing hepato and nephrotoxicity. One of such traditional medicinal herb is Melia Azadirachta. This plant belongs to Meliaceae species and is from west Asia. The extract of *Melia Azadirachta* have phyto-constituents such as Squalene, Oleic Acid, Dodecanoic acid, 1,2,3-propanetriyl ester etc., help as to cure from nephrotoxicity.

The objective of this paper is to determine of Nephroprotective potential of Melia Azadirachta against $H_2O_2$ induced toxicity in VERO cell line. This paper is organized as follows: section II presents the literature survey, section III provides materials and methods used in this study. Results and discussion is given in section IV followed by conclusion in section V.

## Literature Review

(Custódio L et al, 2009) Examined in vitro inhibition of tumor cell growth of carob tree (Ceratonia siliqua). The methanolic extracts of the carob tree has shown considerable radical scavenging activity and a notable ability to resist tumor cell proliferation.

Vero cells are the cells that are derived from the kidney of an African green monkey, and are one of the most commonly used mammalian continuous cell lines in microbiology, and molecular and cell biology research (Sheets, (Rebecca 2000). Measurement of cell viability and proliferation are the prominent for various in vitro assays of a cell population's response to external factors. The MTT Assay (Mosmann, Tim. 1983) is a most responsive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay in living cells will be transformed from the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a purple formazan product that is insoluble in water. Precipitates of MTT in the cellular cytosol can be dissolved by after cell lysis in viable cells, while the dead cells will not transform the MTT. This is directly proportional to the number of viable cells and inversely proportional to the degree of Cytotoxicity. MTT can be reduced through the mediation by NADH or NADPH within the cells and out of mitochondria (Berridge, Michael V., and An S. Tan. 1992, Fotakis, George, and John A. Timbrell. 2006).

The ethanolic extract of the plant of *Solanum nigrum* was assessed in vitro for its cytoprotection. Cytotoxicity was induced by gentamicin on Vero cells. In this study (V. Kumar et al, 2001), cytotoxicity was significantly inhibited by the Trypan blue exclusion assay and mitochondrial dehydrogenase activity (MTT) assay. The authors have found that test extract has exhibited significant hydroxyl radical scavenging effect and hence suggested it as a potential mechanism for cytoprotection.

Increasing evidences have suggested that (Dias, E et al, 2009), Microcystin-LR (MCLR) can induce renal disorders. Their study evaluated the cytotoxicity induced by MCLR on a kidney cell line. Cell viability assays namely, MTT, Neutral Red and LDH were used to study the cytotoxic effects. They suggested that Vero-E6 cell line might constitute a cell model to evaluate the nephrotoxicity of microcystins.

In (Senthilraja P and K. Kathiresanb. 2015) the authors have evaluated the cytotoxic activity of three different extracts of the marine yeasts *Candida albicans, Kuraishia capsulate* and *Sacchromyces cerevisae* against African Green Monkey kidney cell lines (VERO) along with human breast carcinoma cells (MCF7), human hepatocarcinoma (HepG2). Cell viability was determined by the MTT assay. They found that the extracts were able to inhibit the proliferation of the cancer cells (MCF-7, HepG2) than the normal Vero cell viability. In (Pavan Kumar Bellamakondi et al, 2014) the nephroprotective effect of carob pods and leaves were studied, where nephrotoxicity was induced by cisplatin to induce oxidative renal damage in mice. The study shown that the ethanolic extract of carob leaves possessed strong radical scavenging activity in vitro as measured by DPPH assay. They also concluded that carob pods and leaves may be effective to protect from oxidative renal damage and suggested that the leaves are the better nephroprotective agent than pods.

(Ahmed, Mahgoub M. 2010) has investigated the ethanolic and aqueous extract of root of Bauhinia variegata Linn for its antioxidant and nephroprotective effect, where nephrotoxicity was induced by gentamicin in rats. This study has proved that due to the presence of phytochemicals and antioxidants, both the extracts have exhibited significant nephroprotective activity which was carried out in vitro models.

The Plant, Melia Azadirachta is known to possess cytotoxic, anti-inflamatory, anti cancerous, anti bacterial, antimicrobial, anti malarial, anti cholestrol and antioxidant actions (Chiffelle I et al, 2009). Following are the Nephroprotective agents that are reported in literature, Phytolacca roots (Rajagopal, P. L et al, 2013), Rheum species, Cordyceps sinensis, Punarnava, Achyrocline satureioides, Angelica radix root, Clerodendron trichotomum, Camellia sinensis, Cocos nucifera.

(Srinivasan, V. et al, 2014) Studied nephroprotective activity of ethanolic extract taken from Melia azadirachta leaves, against APAP (Acetaminophen) induced nephrotoxicity. Significant changes were noticed in biochemical parameters in APAP induced male albino Wister rats, which were restored towards normalization in Melia Azadirachta treated animals. (Nagyova, A et al 1994) Has suggested that chronic cadmium Cd-induced renal changes are irreversible. They studied the nephroprotective effects of ascorbic acid. Male guinea pigs were administered with chronic cadmium and results observed that ascorbic acid can be effective in the protection of Cd-induced nephrotoxicity. Spirulina fusiformis (Sharma, M. K et al, 2007) was studied for its role to protect against renal damage induced by mercury. This study was done on Swiss albino mice and found that Spirulina fusiformis enhance the immune system.

(Hong, S. K et al, 2002) investigated the direct nephrotoxic effects of nitrobenzene and ten chlorinated nitrobenzene derivatives using rat renal cortical slices as the in vitro model. They demonstrated that chloronitrobenzenes are directly nephrotoxic in vitro and also verified that whenever the number of chloro groups increases nephrotoxic potential also increases. (Tanihara, Y et al, 2009) examined the nephroprotective nature of imatinib, a cationic anticancer agent over cisplatin induced nephrotoxicity. This study was carried out on rats which were administered with the 5 mg/kg of cisplatin and observed the nephrotoxic potential of the given drug. They suggested that, the concomitant administration of imatinib, significantly prevented cisplatin-induced nephrotoxicity.

Gentamicin is known for its morphological, metabolic and functional alterations in the kidney. Accumulation of gentamicin is related to renal proximal convoluted tubules leading to tubular necrosis. (Khan, S. A et al, 2009) has assessed the effect of green tea extract as nephroprotective agent due to the presence of antioxidative properties in it. This study was done on Wistar rats and observed that green tea ameliorated gentamicin induced nephrotoxicity and oxidative damage by improving antioxidant defense, tissue integrity and energy metabolism. They Khan, S. A et al, 2009) also examined the effect of green tea over cisplatin, an anti cancer drug induced nephrotoxicity.

(Pedraza-Chaverri, J et al, 2008) has examined the effect of garlic powder, a recognized antioxidant as a nephroprotective material. This was tested on Wistar rats which were administered with Potassium dichromate ($K_2Cr_2O_7$) to induce nephrotoxicity, oxidative and nitrosative stress. They noticed that the garlic powder has got the ability tackle against $K_2Cr_2O_7$ induced renal damage and suggested the test drug as a potential nephroprotective agent. (Knouzy, B et al, 2010) have examined the role of chloroacetaldehyde (25 to 75 μM) as nephrotoxic material. This was tested on Male Wistar rats and clinically proved that chloroacetaldehyde posses the nephrotoxic effect.

## MATERIALS AND METHODS

### Chemicals

The chemicals namely, 3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Modified Eagle's Medium (MEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. Antibiotics from Hi-Media Laboratories Ltd, Mumbai. Dimethyl Sulfoxide (DMSO), Hydrogen Peroxide ($H_2O_2$) and Propanol from E.Merck Ltd., Mumbai, India. All the chemicals and reagents used in our work are of analytic grade.

### Cell lines and Culture medium

VERO (African Green Monkey Kidney) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of VERO were cultured in MEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% $CO_2$ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 $cm^2$ culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

### Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with MEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out nephroprotective studies.

### Determination of cell viability by MTT Assay

**Principle*:*** The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used (Pedraza-Chaverri, J et al, 2008).

**Procedure*:*** The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x $10^5$ cells/ml using MEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at $37^o$ C for 3 days in 5% $CO_2$ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval.

After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% $CO_2$ atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% ($CTC_{50}$) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)$$

**Determination Nephroprotective activity**

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x $10^5$ cells/ml using MEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium. 50 µl of MEM with nontoxic concentration (1mM $H_2O_2$) of toxicant and 50 µl of different non-toxic test concentrations of test drugs were added. The plates were then incubated at 37° C for 24 h in 5% $CO_2$ atmosphere. After 24 h, the cell supernatants were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% $CO_2$ atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage cell viability was determined, based on which the percentage protection offered by test and standard drugs was calculated over the $H_2O_2$control.

## RESULTS AND DISCUSSION

In vitro confirmation of Melia Azadirachta toxicity in Vero cell lines was conducted. Trypan blue dye exclusion technique was used to determine the percentage of cell viability. The cytotoxicity activity was carried out by using MTT assay. The effect of ethanolic extract of Melia Azadirachta (test) and $H_2O_2$ (control) on the growth of Vero cell line was examined by MTT assay. The susceptibility of cells to the extract exposure was characterized by CTC50 values. Results are tabulated in Tables 1, 2 and 3.

**Cytotoxicity activity**

The cytotoxicity study was carried out from the plant extract of Melia Azadirachta (MA). Its extract was screened for its cytotoxicity against Vero cell lines at different concentrations to determine the CTC50 (50% growth inhibition) by MTT assay.

**Table 1: Cytotoxic properties of test drugs against VERO cell line**

| S. No | Name of Test sample | Test Conc. ( µg/ml) | % Cytotoxicity | $CTC_{50}$ ( µg/ml) |
|---|---|---|---|---|
| 1 | MA | 1000 | 48.43±4.5 | >1000 |
| | | 500 | 35.39±1.9 | |
| | | 250 | 17.25±0.7 | |
| | | 125 | 14.85±5.3 | |
| | | 62.5 | 4.59±0.9 | |

**Table 2: Nephroprotective study (N1) of in VERO cell line.**

| S. No | Test drug | Test Conc. (µg/ml) | % Protection offered over toxicant control |
|---|---|---|---|
| 1 | MA | 250 | 10.06±5.7 |
| | | 125 | 0.85±1.0 |

**Table 3: Nephroprotective study (N2) of in VERO cell line.**

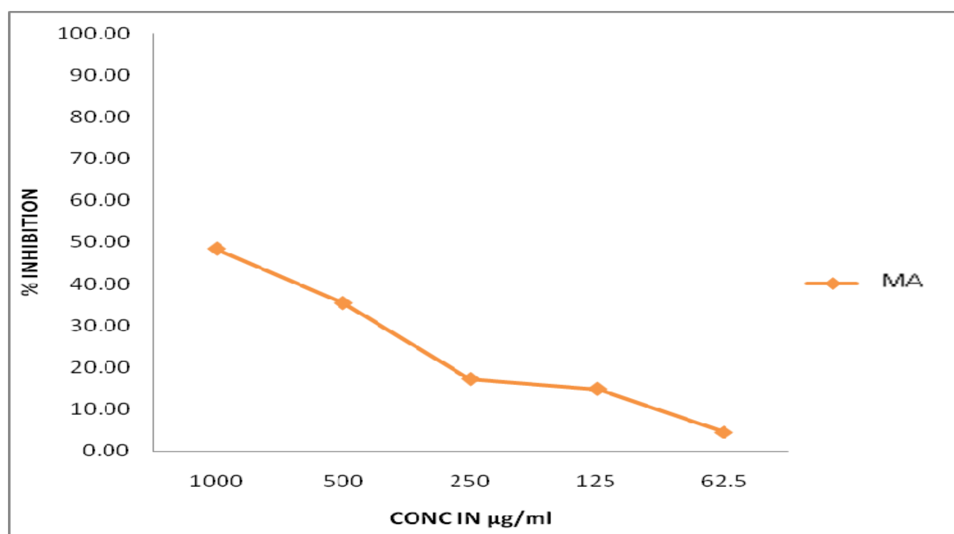
| S. No | Test drug | Test Conc. (µg/ml) | % Protection offered over toxicant control |
|---|---|---|---|
| 1 | MA | 250 | 9.07±2.2 |
| | | 125 | 1.45±0.8 |

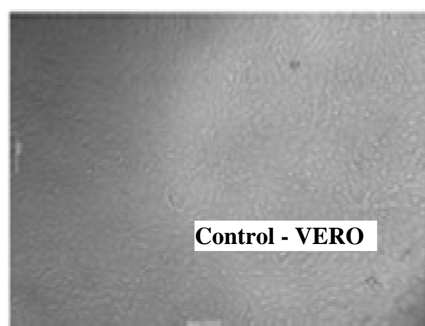
**Cytotoxic effect of the sample MA on VERO Cell line.**

**Inference**

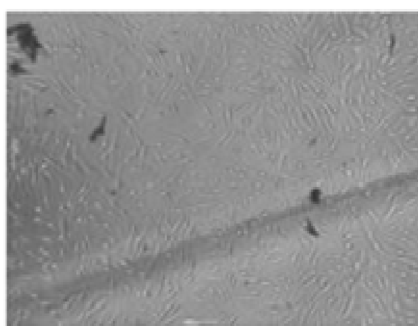
Test Substance showed moderate protection with 10.06±5.7% over toxicant control at 250 µg/ml and 0.85±1.0% over toxicant control at 125 µg/ml (N1) in Table 2, 9.07±2.2% over toxicant control at 250 µg/ml and 1.45±0.8% over toxicant control at 125 µg/ml (N2) in Table 3, which showed dose dependent protection.

Dose response curve constructed between the range 1000 µg/ml and 62.5µg/ml for *Melia Azadirachta* in Fig. 1, shows decreasing number of viable cells with increasing concentration of ethanolic extract.

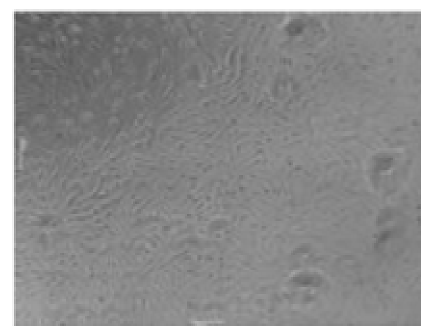



Control - VERO



MA 1000 µg/ml



MA 500 µg/ml

## CONCLUSION

In this paper, nephrotoxicity was induced by $H_2O_2$ in vero cell lines of African green monkey. *Melia Azadirachta* has been evaluated for its nephroprotectiveness. The presence of phytoconstituents in *Melia Azadirachta* has inhibited the induced nephrotoxicity. This has been revealed through the results, that shown moderate protection of about 10 % over toxicant control during two test trials with different concentrations of test sample.

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