Original Research Paper

Volume-9 | Issue-7 | July - 2019 | PRINT ISSN No. 2249 - 555X

Biological Science

# *IN-VITRO* EXPERIMENTAL MODELS TO STUDY OXIDATIVE STRESS-INDUCED CELL MEDIATED DNA DAMAGE AND MITOCHONDRIAL DYSFUNCTION TO TREAT VARIOUS DISEASES.

**Rajaguru Arivuselvam**

Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, Rocklands, Ooty, Pincode-643001, Tamilnadu,

**ABSTRACT** Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by numerous endogenous and exogenous processes. In these studies free radicals that can involve in normal biological function and start disturbing. It may be leading to a variety of diseases like cancer, diabetes, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, atherosclerosis, etc. Free radical toxicity that causes damage to proteins and DNA injury, inflammation, tissue damage, and subsequent cellular apoptosis, mitochondrial dysfunction. However, in the Current scenario, the oxidative stress increases the production of free radicals that interact with diseased cell also (cell biological function) and it starts disturbs. ROS generated by oxidative stress that interacts with the diseased cells and that leads to causing DNA damage and altering the mitochondria function. Finally, that causes cell death. Researchers still remain a challenge to analysis in-vitro the strategy of a drug that induced oxidative stress to target various diseases. In this article, we briefly discussed in-vitro models to evaluate the induction of oxidative stress to treat disease.

**KEYWORDS :** oxidative stress, free radicals, treat disease.

## 1. INTRODUCTION

The stage Oxidative stress originating from altering the balance (it means higher or lower than the normal) between the production of free radicals and antioxidant defenses [1].It has negative benefits it was oxygen free radicals highly involve in normal biological function and it disturbs the macromolecules such as lipids, proteins, and DNA[2]. So, it may lead to cause many chronic diseases such as cancer, diabetics, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, atherosclerosis, etc.[3].These reactive oxygen species basically free radicals are most commonly involved in cellular function. There are Superoxide radicals (O2−), hydrogen peroxide (H2O2), hydroxyl radicals (OH), and singlet oxygen[4]. Reactive oxygen species (ROS) are an extremely reactive molecule that starts from the normal cellular process and it involves increases the number of cellular sources. In that case one of the cellular sources is mitochondria[5]. Mitochondria play an important role powerhouse of the cell and it produces ATP followed by the glucose breakdown into pyruvate derived from glycolysis. They are also playing the one way for producing reactive oxygen species (ROS), which have the role to regulate cell death and proliferation. However, in when the diseased cell contains mitochondrial DNA it undergoes mutation through the oxidative stress especially ROS (reactive oxygen species) that interact with the diseased cells and that oxidative stress helps to alter the mitochondria it causes cell death and also prevents from several diseases[6].

## 2. METHODS

### 2.1. Preliminary Radical scavenging assay

This assay basically performed in order to check the sample having the property of the radical scavenging effect (in other words, the sample shows the effect of antioxidant or pro-oxidant). Accordingly, the free radical theory shows the antioxidants are the system involves reducing the oxidative stress and Pro-oxidant defined as the xenobiotic that causes oxidative stress either by the production of ROS or by obstructing antioxidant systems [7].

These are the methods involved in preliminary radical scavenging assay

- Deoxyribose degradation assay[8,9].
- Diphenyl Picryl Hydrazyl (DPPH) radical scavenging assay [10,11]
- Membrane Lipid Peroxidation Assay [12,13]
- Hydrogen peroxide (H2O2) inhibition assay[12]

### 2.1.1. Deoxyribose degradation assay [8,9]

**Principle:** The principle behind the deoxyribose degradation assay interaction between the hydrogen peroxide and ions in the biological system it causes the formation of highly reactive tissue-damaging species that was believed to be hydroxyl radical.

**Method:** Various concentrations of drug or isolated components have to prepare. Then the samples have to add with a reaction mixture containing 0.2ml (3mm) Deoxyribose, 0.2ml (0.1mm) Ferric chloride, 0.2ml (0.1mm) EDTA, 0.2ml (0.1mm) Ascorbic acid and 0.2ml (2mm) H2O2 in phosphate buffer (pH 7.4). When these mixtures were kept for incubation for 1hour in at 37°C. After incubation 0.2ml, 15%w/w, trichloroacetic acid and 0.2ml, 1%w/v, thiobarbituric acid in 0.25N HCl were added. Then this was heated for 30min and absorbance was measured at 532nm.

### 2.1.2. Diphenyl Picryl Hydrazyl (DPPH) radical scavenging assay[10,11]

**Principle:** The principle behind in the assay measure the scavenging ability of antioxidants present in the drug or isolated components to neutralize the stable radical DPPH. First free stable radical when it reacts with hydrogen donor is reduced into hydrazine, then the ability of antioxidant was confirmed by decolorization, which evaluates the absorbance decrease at 515–530 nm produced by the addition of the antioxidant to a DPPH solution in ethanol or methanol.

**Preparation of test solutions**:

- DPPH, (100 mM). 22 mg of DPPH is accurately weighed and dissolves in 100 ml of methanol. From this stock solution, 10 ml have to take and diluted to 100 ml using methanol to obtain 100 mM DPPH solution.
- 21 mg of each of the sample has to weigh and it dissolves in distilled 1ml DMSO separately to obtain a solution of 21 mg/ml concentration. Take 1ml of prepared solution dissolved in 9ml of DMSO the final concentration ranging from 1, 000 µg/ml to 0.9765 µg/ml.

**Method:** The assay was carried out in a 96 well microtiter plate. To 200µl of DPPH solution, 10µl of each of the test sample or the standard solution was separately added into the wells of the microtiter plate. The 96 well plates have to be incubating at 37°C for 30 minutes and the absorbance have to measure at 530 nm, using ELISA reader against the corresponding test and standard blanks and the remaining DPPH was calculated. IC50 (Inhibitory Concentration) is the concentration of the sample required to scavenge 50% of DPPH free radicals.

$$\%\ of\ inh\ ibition = (Control - Sample \mid Sample)\ X\ 100$$

### 2.1.3. Lipid peroxidation (LPO) assay [12]

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**:[13]

Eggs yolks have to separate from the egg and collect the egg yolk into

the 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 drug or isolated components (100 µl) of different concentrations separately added into 1 ml of egg lecithin mixture, control without a drug or isolated components. Lipid peroxidation has to induce by adding 5 µl FeCl3 (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 HCl 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 530nm.

**2.1.4. Hydrogen peroxide ($H_2O_2$) inhibition assay**[12]

**Principle:** hydrogen peroxide generated by enzymatic reactions. These are the enzymes are found in microsomes, peroxysomes, and mitochondria. Even in normal conditions, the hydrogen peroxide production in relatively significant and 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 O2 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:** concentrations of 1 ml of the drug or isolated components 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 drug or isolated components in PBS without $H_2O_2$.

**2.2. Determination of Oxidative stress on cells [14]**

DCFH-DA assay mainly responsible for the treatment with drugs or isolated component attenuated the production of intracellular ROS. Apoptosis induced by drug or isolated components was not inhibited by the use of caspase-inhibitor Z-VAD-FMK, proposing that cell death is caspase-independent. The use of JC-1 dye that drug or isolated components are used that may be it can disturb the mitochondrial membrane potential.

**2.2.1. Evaluation of the induction of oxidative stress in animal cells [14]**

**Method:** The animal cells were seeded at a 96 well plate.in the density of 5,000 cells per well and it incubates at a co2 incubator for overnight for cell attachment. Cells were pre-treated with 0.1 mm ascorbic acid for 6 h before treatment with drug or isolated components. Untreated is 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. A supernatant layer will be removed and 100µl of DMSO is added to solubilize the purple-blue formazan. The absorbance has to measure with the help of Microplate Reader at a wavelength of 570 nm, and 630 nm as the reference wavelength. A graph of the percentage of cell viability versus various concentrations of extracts was plotted, and the IC50 was determined**.**

**2.2.2. Determination of the involvement of ROS in extracts induced oxidative stress[14]**

**Method:** The dichloro dihydro fluorescein diacetate (DCFH-DA) assay was used to the determined level of intracellular reactive oxygen species (ROS). The animal cells were seeded at a 96 well plate.in the density of 5,000 cells per well and it incubates at the 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 the co2 incubator. Subsequently, the cell was washed with PBS and treated with various concentrations of drug or isolated components, 50 µM of $H_2O_2$ solution (positive control) for 3 h. Fluorescence intensity was measured at a wavelength of 485 nm and an emission wavelength of 535 nm.

**2.3 Evaluation of DNA Damage**

**2.3.1. Evaluation of DNA Damage by Fenton's reagent**[15,16]

**Principle**

The principle behind the assay was hydroxyl radicals produced by the Fenton's reagent that cause DNA damage. That the study examined by whether the addition of drug or isolated componentsalong with the Fenton's reagent that can able to protects DNA from damage induced by scavenging hydroxyl radical that can clearly examine using by gel electrophoresis method.

**Standard Preparation**: The Fenton's reagent made up of 30mm (Hydrogen peroxide, 50mm of ascorbic acid, and 80mm of ferric chloride) was prepared.

**METHOD**: In this DNA nicking assay was carried out using 5 µL plasmid DNA, 5µLof Fenton reagent, 10 µL of different concentration of drug or isolated compounds,4 µL of loading dye ( bromophenol blue dye (0.25% in 50% glycerol) was added to form a mixture. 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.

**2.3.2. Evaluation of DNA Damage by UV radiation [17,18]**

**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 drug or isolated components along with the DNA that can able to protects DNA from damage induced by UV radiation that can clearly be examined using by gel electrophoresis method. Finally compared with the control and test concentration we can clearly say the drug or isolated components protect the mutation.

**METHOD**: In this DNA nicking assay was carried out using 10µL plasmid DNA, 10µL of different concentration of drug or isolated components,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

**2.3.3. Evaluation of DNA damage induced on animal cells** [19]

**Principle**: Generally, cell death occurs [19]in three different types. There are apoptosis, autophagic cell death, and necrosis. This method used to distinguish between apoptotic and necrotic cell death that can be visualized 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 each well of a 96-well plate (5000 µl/well), 10µL of different concentration of drug or isolated components 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

**2.4. ANALYSIS OF CELL DEATH**

**2.4.1. Direct fluorescence microscopic analysis for apoptosis induction by AO/EtBr [20]**

**Principle:** Both live and dead cells are stained by acridine orange. Cells that have lost membrane integrity are stained by ethidium bromide Live cells will look like uniformly green. Primary apoptotic cells will look like green but it contains bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Secondary apoptotic cells will also fit in ethidium bromide and therefore stain orange, but it shows necrotic cells, the

secondary apoptotic cells will show condensed and often fragmented nuclei. [21]

**Method**: The animal cell will be added in each well of a 6-well plate (5000 µl/well) and incubated. Then the highest concentration of drug or isolated components will be added in 6-well. the cells will be incubated for different time intervals 24 h, 48 h, and 4 d. Dual fluorescent staining solution (1 µl) containing 100 µg/ml AO and 100 µg/ml EB will be added to each suspension and then cover with the help of coverslip. The morphology of apoptotic cells is examined and cells will be counted within 20 min using a fluorescent microscope. Dual acridine orange/ethidium bromide (AO/EB) staining method will be repeated 3 times at least.

### 2.4.2. Analysis of mitochondrial membrane potential loss [22]

**Principle:** This assay based on the principle of oscillations of mitochondrial potential are associated with mitochondrial. The mitochondrial into the cytosol arises from the compartment redistribution of lipophilic dyes. This redistribution decreases the mean pixel intensity in that particular cell. From this assay clearly, absorb the mitochondrial membrane potential loss.

**Method** The animal cell will be added in each well of a 6-well plate (5000 µl/well) and incubated. The highest concentration of drug or isolated components will is added in 6-well. the cells will be incubated for different time intervals 24 h, 48 h, and 4d. Then remove the media from cells and Rinse the cells three times with the help of PBS. Then Rhodamine 123 solution will be added in the solution and incubate it at 37°C for 15 minutes. Remove the solution and rinse the cells three times with PBS then Image cells in PBS under a fluorescence microscope.

### 2.4.3. HOECHST 33342 staining for nuclear apoptosis [23]

**Principle**: the principle behind the Hoechst 33342 staining is particularly staining the nuclei of living or fixed cells and tissues. This stain is normally used in mixture with 5-bromo-2'-deoxyuridine (BrdU) labeling to find out compact chromatin of apoptotic nuclei, to identify replicating cells and to sort cells based on their DNA content. Hoechst 33342 and propidium iodide are commonly used together because of simultaneous flow cytometric and fluorescence imaging analysis of the stages of apoptosis and cell-cycle distribution.

**Method** The animal cell will be added in each well of a 6-well plate (5000 µl/well) and incubated. The highest concentration of drug or isolated components will be added in 6-well. then the cells will be incubated. Add Hoechst stain using the concentrations between 0.5 and 5 µM and incubate it for 15 to 60 minutes. In initial experiments, it may be best to try several dye concentrations over the entire suggested range to determine the concentration that yields optimal staining.

## CONCLUSION

From this above method can conclude the *in-vitro* analysis of drug or isolated compounds that can produce oxidative stress-mediated DNA damage and altering the mitochondrial function. This study used to treat the various diseases like cancer, diabetics, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, atherosclerosis. It includes four stages preliminary radical scavenging assay, induction of oxidative stress, evaluation of DNA damage, analysis of cell death. In these four stages can calculate the ability of drug or isolated components that can scavenge the free radicals, cell-killing property, and induction of oxidative stress, the ability to damage the DNA, nuclear apoptosis, and mitochondrial membrane potential damage. Finally, these methods provide a scope of future studies on isolated compounds and drug development for the treatment of various diseases.

## ABBREVIATIONS

DNA: Deoxyribonucleic acid, PBS: Phosphate-buffered saline, AO/EtBr: Acridine orange/ Ethidium bromide, TBE buffer: Tris-acetate buffer, TCA: Trichloroacetic acid, TBA: tert-Butyl alcohol, LPO: LIPID PEROXIDATION, DCFH-DA: Dichloro-dihydro-fluorescein diacetate, DMSO: Dimethyl sulfoxide, HCL: hydrochloric acid, MTT DYE: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

## REFERENCES


1. Hospital, M. What Is Oxidative Stress? 2000, 3–8.
2. What Is Oxidative Stress ? 2002, 124, 271–276.
3. Uttara, B.; Singh, A. V; Zamboni, P.; Mahajan, R.T. Oxidative Stress and Neurodegenerative Diseases : A Review of Upstream and Downstream Antioxidant Therapeutic Options. 2009, 65–74.
4. Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D.; Bitto, A. Review Article Oxidative Stress : Harms and Benefits for Human Health. 2017, 2017.
5. Ill, J.E.G.G.; Iskounova, E.L.P.; Orrison, S.E.A.N.J.M.; Al, G.E.T. Cancer , Oxidative Stress , and Metastasis. 2016, LXXXI.
6. Chen, E.I. Mitochondrial dysfunction and cancer metastasis. 2012, 619–622.
7. Rahal, A.; Kumar, A.; Singh, V.; Yadav, B.; Tiwari, R.; Chakraborty, S.; Dhama, K. Oxidative Stress , Prooxidants , and Antioxidants : The Interplay. 2014, 2014.
8. Campus, S.; Standards, B.; Hill, H. The Deoxyribose Method : A Simple " Test-Tube " Assay for Determination of Rate Constants for Reactions of Hydroxyl Radicals. 1987, 19.
9. Oh, T.; Tadolini, B.; Cabrini, L. The deoxyribose assay : an assay both for ' free ' hydroxyl radical and for site-specific hydroxyl radical production. 1987, 932–933.
10. Systems, B. Food Science and Technology International Review : Methods Used to Evaluate the Free Radical Scavenging. 2002.
11. Himamura, T.S.; Umikura, Y.S.; Amazaki, T.Y.; Ada, A.T.; Ashiwagi, T.K.; Shikawa, H.I.; Atsui, T.M.; Ugimoto, N.S.; Kiyama, H.A.; Keda, H.U. Applicability of the DPPH Assay for Evaluating the Antioxidant Capacity of Food Additives – Inter-laboratory Evaluation Study –. 2014, 30.
12. Srinivasan, R.; Chandrasekar, M.J.N.; Nanjan, M.J.; Suresh, B. Antioxidant activity of Caesalpinia digyna root. 2007, 113, 284–291.
13. Pangborn, C. A simplified. 1950, 471–477.
14. Tor, Y.S.; Yazan, L.S.; Foo, J.B.; Wibowo, A. Induction of Apoptosis in MCF-7 Cells via Oxidative Stress Generation , Mitochondria- Dependent and Caspase-Independent Pathway by Ethyl Acetate Extract of Dillenia suffruticosa and Its Chemical Profile. 2015, 1–25.
15. Kumar, R.; Arora, R.; Mahajan, J.; Mahey, S.; Arora, S. Polyphenols From Cutch Tree ( Acacia catechu Willd .): Normalize In Vitro Oxidative Stress and Exerts Antiproliferative Activity. 2017, 60, 1–13.
16. Golla, U.; Sunder, S.; Bhimathati, R. Evaluation of Antioxidant and DNA Damage Protection Activity of the Hydroalcoholic Extract of Desmostachya bipinnata L . Stapf. 2014, 2014.
17. Ahsan, H.; Parveen, N.; Khan, N.U. Pro-oxidant , anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. 1999, 121, 161–175.
18. Rastogi, R.P.; Kumar, A.; Tyagi, M.B.; Sinha, R.P. Molecular Mechanisms of Ultraviolet Radiation-Induced DNA Damage and Repair. 2010, 2010.
19. Benhusein, G.M.; Mutch, E.; Aburawi, S.; Williams, F.M. Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay. 2010, 1, 1–6.
20. Dual AO / EB Staining to Detect Apoptosis in Osteosarcoma Cells Compared with Flow Cytometry. 2015, 15–20.
21. Kasibhatla, S.; Finucane, D.; Brunner, T. Acridine Orange / Ethidium Bromide ( AO / EB ) Staining to Detect Apoptosis. 2006, 4493.
22. Toescu, E.C.; Verkhratsky, A. Assessment of mitochondrial polarization status in living cells based on analysis of the spatial heterogeneity of rhodamine 123 fluorescence staining. 2000, 941–947.
23. Excitation, A.F. Hoechst Stains. 2005, 9–12.