CHAPTER 5

INVITRO ANTIOXIDANT AND NEUROPROTECTIVE ACTIVITY

5.1 INTRODUCTION

Overproduction and accumulation of free radicals leads to chronic diseases like atherosclerosis, cancer, diabetes, rheumatoid arthritis, cardiovascular diseases, inflammation, ageing and other degenerative diseases [300-302]. Oxidative stress is one of the prime causes for cell death and damage especially in the brain cells. Neural cells are highly susceptible to oxidative stress induced injury because of its high metabolic rate and insufficient level of antioxidants and oxidative damage to DNA and biomolecules has been noted during the pathogenesis of ageing and neurodegenerative diseases [303-305]. Though, the biological systems have an internal defense mechanism to fight against intracellular ROS, at a certain point this fails due to the over expression of ROS [306]. Hence identification of alternate source of antioxidants is required for conferring protection to cells and to slow down the process of ageing and neurodegeneration.

Plants are potent source of natural bioactive components of phytomedicine which has been used since time immemorial. The beneficial activity of plants are typically due to the combined action of the secondary constituents, these compounds may be unique in nature and differ from species to species [307]. Antioxidant based drugs and formulation has been raised from past three decades for the prevention and management of complex disease such as stroke, Alzheimer's, diabetes and cancer [308, 309] which has further evoked the research interest globally on natural antioxidants. Majority of active antioxidants compounds derived from plants are flavonoids, isoflavones, anthocyanins, coumarins, lignans, catechins and isocatechins and also vitamins C and E, beta carotene and tocopherol are recognized as potent antioxidants [310].

Utilization of cell line was initiated due to the difficulties in working with live human tissue sample and several studies have demonstrated that cell line are one of the best models to study stress induced cell damage and neurodegeneration [311-313]. IMR32 is a human neuroblastoma cell line-Tumilocwicz et al. established from an abdominal mass occurring in 13 month old Caucasian male. Being a human neural cell line it may be best suited for studying the mechanism of drugs effective in treating stress induced neurodegeneration in humans. Thus, we have hypothized the present study to evaluate the *In vitro* antioxidant and neuroprotective activity of polyherbal formulation using IMR32 human neuroblastoma cell line.

5.2 Methodology

5.2.1 Antioxidant assays

5.2.2 DPPH radical scavenging activity

DPPH is a stable free radical molecule widely used assay to measure the free radical scavenging capacity of plant samples. Assay was initiated by adding 200 µl of 0.004% DPPH methanolic solution into 96-well plate, followed by addition of 20 µl of polyherbal formulation, or solvent or the blank. The mixture was incubated at 30^0C for 1 hr and the absorbance was measured at 515 nm in a microplate reader (Multiskan, Thermo Scientific) [314-315]. The percentage of inhibition was calculated as:

Inhibition% = 100-100 (As ÷ Ao). Where Ao is the absorbance of the blank and As is the absorbance of the sample at 515 nm.

5.2.3 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay was carried following the method of Su et al., [316] with slight modification. 2 ml of 6 mM ferrous sulphate was added to different concentration of 2 ml of polyherbal formulation (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/ml), to which 2 ml of 6 mM hydrogen peroxide was added. The above mixture was incubated for 10 mins, after which 2 ml of 6 mM sodium salicylate was added and incubated at 37^0C for 30 mins. On completion of the incubation period the samples were read for absorbance at 510 nm using spectrophotometer (Lab India) and the percentage of inhibition was calculated as:

Inhibition% = 1-(As- Aw/Ao)*100. Where, As is the absorbance of the sample with sodium salicylate. Aw is the absorbance of the sample without sodium salicylate and Ao is the absorbance of the reagent.

5.2.3 FTC assay

The antioxidant activity of polyherbal formulation on inhibition of lipid peroxidation was determined using ferric thiocyanate assay [317]. A mixture containing the sample 4 ml in absolute ethanol, 2.51% linoleic acid in absolute ethanol (4.1 ml), 0.05 M phosphate buffer pH 7 (8 ml) and distilled water (3.9 ml) was placed in a vial with a screw cap. The vial was placed in an oven at 40°C in the dark. To 0.1 ml of the above solution 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate added. Three minutes after adding 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm every 24 hr until one day after absorbance of the control (without sample) reached maximum. α- tocopherol was used as standard.

5.3 *In vitro* neuroprotective activity

5.3.1 Cell line maintenance

IMR-32 human neuroblastoma cell line was obtained from NCCS, Pune, India and maintained on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with streptomycin (100 U/ml), penicillin (100 µg/ml), 10% FCS (Life Technologies) at 37ºC and 5% CO_2. The H_2O_2 dose (IC_{50}) for ROS neuroprotective studies, was identified by treating cells with different concentrations of H_2O_2 at 50% confluency for 24 hours in serum free medium and the viability was measured using MTT reduction assay.

5.3.2 Cell viability assay

5.3.2.1 MTT assay

MTT assay is a colorimetric assay for finding viable cell population. NAD(P)H-dependent cellular oxido-reductase enzyme under defined conditions, reflect the number of viable cells present. These enzymes present in live cells are capable of reducing the yellow tetrazolium salt that enter live cells and convert into insoluble formazan form, which is purple in color and trapped inside the live cell. The trapped MTT-formazan dye is

solubilized with either dimethyl sulfoxide (DMSO) or an acidified ethanol solution. The resultant colored solution has an absorbance between 500 and 600 nm which is used for quantification of the viable cell population [318].

1×10^4 cells/well were seeded on to 96-well plates and incubated for 24 hrs. The cells were then treated with different concentration of polyherbal formulation (0-100 ug/ml) for 24 hrs, after which the cells were exposed to the toxic stress inducer H_2O_2 for further 24 hrs. Finally, the cells were treated with MTT and then digested using DMSO. The colored solution was then analyzed using a MULTISKAN plate reader at 540 nm.

5.3.2.2 Propidium iodide Assay

Propidium Iodide (PI) is an intercalating agent and is used to stain cellular DNA for both flowcytometry and fluorescent microscopy experiments, to evaluate cell viability, DNA content, cell cycle analysis and DNA damage. It can be used to differentiate between necrotic, apoptotic and live cells. PI is membrane impermeant and is generally unable to enter live cells. However, cells with membrane damage take up PI, which then intercalates with the cellular DNA. Thus PI is commonly used for identifying dead and damaged cells. When PI is bound to nucleic acids, the fluorescence excitation maximum of PI is 535 nm and the emission maximum is 617nm [319].

1×10^4 cells/well were grown in 96-well plates and incubated for 24 hrs. The cells were then treated with different concentration of polyherbal formulation (0-100 ug/ml) for 24 hrs, before its exposure to the toxic stress inducer H_2O_2 for 24 hrs. The cells were then stained with PI, and the no of dead cells was analyzed using flowcytometer (ex 535/em 617).

5.3.2.3 DCFDA assay

DCFDA, a fluorogenic dye is used to measures intracellular ROS activity using flowcytometry or take images using fluorescence microscope. DCFDA diffuses into live cell, where it is deacetylated by cellular esterases into a non-fluorescent compound, this compound is prone to intracellular ROS activity, which converts it into 2', 7'–dichlorofluorescin (DCF). DCF is a highly fluorescent compound (ex 495nm/em 529nm) which can be detected both by flowcytometry or fluorescence spectroscopy [321, 322].

1×10^4 cells were grown in 96-well plates and incubated for 24 hrs. The cells were then treated with different concentration of polyherbal formulation (0-100 ug/ml) for 24 hrs, before its exposure to the toxic stress inducer H_2O_2 for further 24 hrs. The cells were then stained with DCFDA for 1 hr, after which the level of ROS activity was measured by flowcytometer.

5.4 RESULTS

5.4.1 Free radical scavenging activity of polyherbal formulation

DPPH, OH radical scavenging and FTC assays were performed to evaluate the free radical scavenging activity of the polyherbal formulation using ascorbic acid and α-tocopherol as standards. The results confirmed that the polyherbal formulation showed a concentration dependent radical scavenging activity. The IC_{50} value for DPPH assay was found to be 40.91 μg/ml as seen in (Figure 5.1a). The sample was also found show considerable OH radical scavenging activity, the highest scavenging effect was found to be 83.24% at 1.4 mg/ml, with IC_{50} value being 0.39 mg/ml (Figure 5.1b). As OH radical scavenging activity is an important factor for evaluating antioxidant property the present study shows that the polyherbal formulation is an efficient antioxidant. FTC results portrayed that the sample possess comparable lipid peroxidation activity with that of standard (Figure 5.1c).

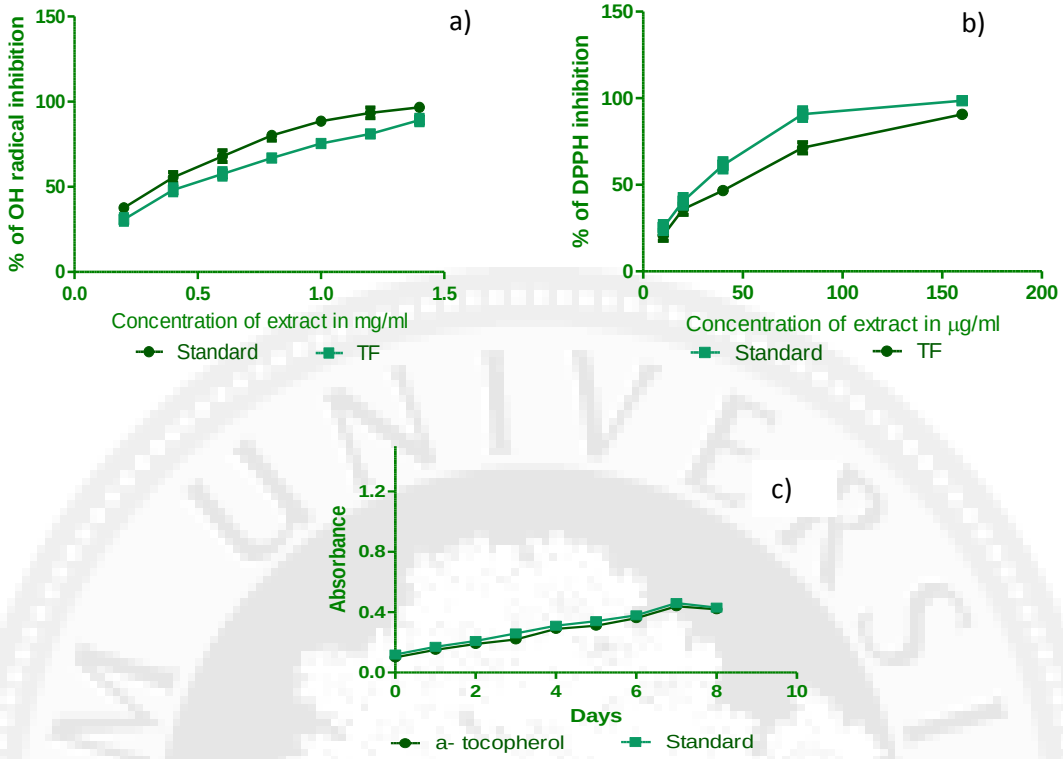

Figure 5.1 Free radical scavenging activity of polyherbal formulation a) DPPH assay, b) OH radical scavenging activity, c) Lipid peroxidation activity.

5.4.2 Effect of the polyherbal formulation sample on IMR32 cell line

Cytotoxic effect of the polyherbal formulation on human neural cell line-IMR32 was evaluated by incubating it with various concentration of the polyherbal formulation sample (1-1000 µg/ml) as seen in Figure 5.2. The toxicity results revealed a decrease in percentage of cell viability at higher concentrations of formulation and the IC_{50} value was found to be 279.73±2.6 µg/ml. The viability of cells were least affected at 100 µg/ml hence, it was chosen as the highest safe dose for evaluating efficacy of the formulation.

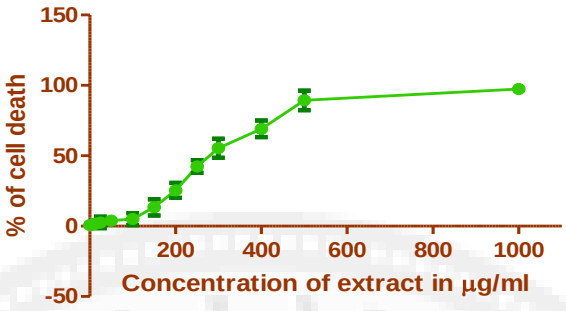

Figure 5.2 Concentration dependent effect of polyherbal formulation on Human neural cell line- IMR32. Note- Values represented as mean ± SD.

5.4.3 Neuroprotective effect of the polyherbal formulation on H_2O_2 cytotoxicity

Neuroprotective activity of the polyherbal formulation was assessed by subjecting IMR32 cells to 24 hrs H_2O_2 (250µM) challenge, which were pretreated with different concentrations of the formulation for 24 hrs (3.2-100 µg/ml). The cell viability was determined using propidium iodide and MTT assays. An increase in cell viability was observed in treated cells compared to H_2O_2 control. The viability was seen in a dose dependent manner as illustrated in Figure 5.3 100 µg/ml was found to be the effective efficacy concentration; approximately similar results were obtained from both the assay MTT assay.

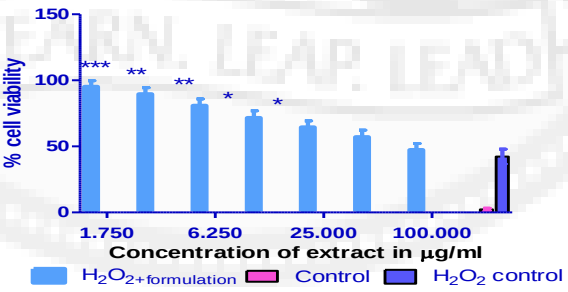

Figure 5.3 Neuroprotective effect of polyherbal formulation H_2O_2 induced cell cytotoxicity. Note- Values represented as mean ± SD. Toxicity results of IMR32 cell line induced with 250 µM of H_2O_2 and pre incubated with different concentrations of polyherbal formulation.

5.4.4 Effect of the polyherbal formulation on intracellular ROS activity

DCFDA assay was used to measure the amount of ROS generated in IMR32 cells during oxidative stress injury. The amount of fluorescence liberated was directly proportional to the ROS generated in the cells. Incubation of IMR32 cells with 250 µM of H_2O_2 induced intracellular ROS accumulation along with other peroxides which were detected with DCF stain. ROS activity was found to be decreased in pretreated cells as seen in Figure 5.4. The amount of fluorescence is directly proportional to the ROS liberated, it was observed that the polyherbal formulation significantly reduced the relative fluorescence by 60-70% when compared to un-treated cells, the level of significance in treated cells at 50 and 100 µg/ml concentration were $P<0.05$ and $P<0.01$ respectively.

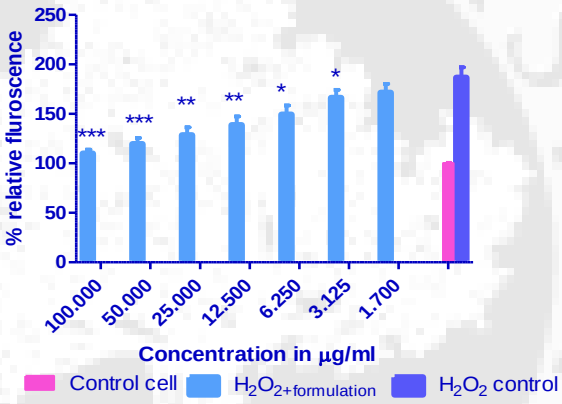

Figure 5.4 Effect of polyherbal formulation on intracellular ROS production. Note- Values represented as mean ± SD. Intracellular ROS activity results for IMR32 cell line induced with 250 µM of H_2O_2 and pre incubated with different concentrations of polyherbal formulation.

5.5 DISCUSSION

The present study provides information on the antioxidant and neuroprotective activities of polyherbal formulation against exogenous stress induced cytotoxicity.

Oxidative stress is one of the prime causes for cell death and damage especially in the brain cells. Though, the biological systems have an internal defense mechanism to fight against intracellular ROS, at a certain point this fails due to the over expression of ROS. Excess accumulation of ROS leads to cellular damage and inflammation of the tissues [322]. ROS plays a major role in cellular senescence paving way to neural cell death; therefore, there is an urgent need for potential therapeutics that may prevent oxidative stress induced neurodegeneration. Hence, identification of alternate source of antioxidants is required for conferring protection to cells and to slow down the process of ageing and neurodegeneration.

With the above context and the side effects of conventional drugs have evoked researcher's focus on natural antioxidants especially from plants, as they possesses strong free radical scavenging activity, and prevents oxidant–induced cellular damage [323-327].

Literatures quote that the three plants constituents used from the preparation of the polyherbal formulation are potent antioxidants, which provoked to evaluate its neuroprotective activity and thus we have initiated the study by screening the polyherbal formulation for its antioxidant property.

The antioxidant activity of test formulation was studied using DPPH, hydroxyl radical scavenging and FTC assays, these assays are accurate and best methods employed for screening antioxidants. The results confirmed that the polyherbal formulation effectively scavenged the free radicals liberated in each of the above mentioned assays which were comparable with the standards.

The most common method applied for studying the *in vitro* neuroprotective activity of antioxidants is H_2O_2 induced cytotoxicity [328-330], hence; this method was employed to study the neuroprotective effect of polyherbal formulation. It was observed that human neural cell line (IMR32) was protected against H_2O_2 challenge by pre-treating the

cells with different concentrations of polyherbal formulation. A significant increase in cell viability was found in polyherbal formulation treated cells compared to H_2O_2 control and the increase in cell viability was dose dependent. This increase could be because of the ability of compounds present in formulation to respond to cellular stress and protect the macromolecules from toxicity [331].

The polyherbal formulation was found to act on peroxides and other free radicals which were evident through the DCFDA results. Decrease in the level of fluorescence was observed in cells treated with test formulation. The antioxidant and neuroprotective activity of formulation would be the result of its rich bioactive contents like vitamins A, C, E, and F, riboflavin, folic acid, carotenoids, phytosterols, organic acids, polyunsaturated fatty acids and essential amino acids [332-334].

The individual plants present in the formulation have shown antioxidant activity and it scavenges free radicals such as peroxides and hydroxyl ions [335, 336]. Researchers have found pretreatment with *B. monnieri* may decrease lipid peroxidase, a marker of oxidative stress [337]. The present result confirms the results of previous studies that *Dioscorea sp* has the ability to scavenge DPPH and superoxide radicals [338]. Also the high antioxidant content of *H. rhamnoides* could have together contributed to the *in vitro* antioxidant property and thus acted effective in protecting the neural cell against exogenous induced oxidative stress.

5.6 CONCLUSION

The present study reports the neuroprotective and antioxidant effect of polyherbal formulation. It was found that polyherbal formulation protects the neural cells form oxidative stress induced damage through its antioxidant property. Hence, the formulation may be advocated for neurodegenerative disorder induced by oxidative stress. It has to be further validated with advanced molecular mechanism and gene regulation studies.