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Neuroprotective Effect of *Emblica Officinalis* Extract Against H_2O_2 Induced DNA Damage and Repair in Neuroblastoma Cells

Ramakrishna V*, Preeti Gupta K, Oruganti Setty H, and Anand Kondapi K

Department of Biotechnology, School of Life Sciences, University of Hyderabad, 500 046-Hyderabad, India

Abstract

Emblica officinalis is used in ancient Indian traditional phytomedicine to treat various human diseases including neurodegenerative diseases. The present study was designed to investigate the protective effect of *Emblica officinalis* (EO) extracts against H_2O_2 induced DNA damage and repair in human neuroblastoma cells (SK N SH). Aqueous and methanol extracts of *Emblica officinalis* were prepared and evaluated the effect of extracts on cell viability through MTT assay. DNA damage and protection by EO extracts was measured by comet assay using H_2O_2 as DNA damaging agent and compared with catechin positive control. A significant growth and proliferation of the cells be observed in the presence of EO extracts. Cell viability is markedly decreased with increased DNA damage after a 24 h exposure to H_2O_2. However, cell toxicity was significantly attenuated in a dose-dependent manner when the cells were prior loaded with aqueous or methanol EO extracts (0.1 – 1.0 mg/ml) for 24 h, and then exposed to H_2O_2. Oxidative DNA damage in these EO loaded cells was shown a significant reduction in the intensity of DNA damage as measured through length of comets and also brought to control level. Among EO extracts, methanol extract is more effectively offset the H_2O_2 induced oxidative DNA damage and bring back to normal like in the positive control catechin flavanoid. From this study, it is concluding that the neuroprotective effects of *Emblica officinalis* appeared to be its antioxidant properties to suppress neuronal oxidative DNA damage in cells treated with H_2O_2.

Keywords: Neuroprotection; *Emblica officinalis*; Oxidative stress; DNA damage and repair

Introduction

Neuronal cells, because of their high rate of oxidative metabolism and low levels of antioxidant enzymes, are quite susceptible to damage by ROS. In fact, oxidative DNA damage along with other biomolecules has been described by some authors as an important type of damage that occurs in neuronal cells, playing a critical role in neurodegenerative diseases [1-3]. Even though, the cells are equipped with efficient defense mechanisms to remove this kind of damage by various DNA repair pathways, still the causes of these diseases are not fully prevented, but it is believed that these diseases are affected by multiple factors including over expression of ROS [4]. Thus, identification of agents conferring neuroprotection could potentially lead to therapies slow or ameliorate the progression of neurodegenerative diseases. Therefore intake of antioxidants from natural origin with less toxicity (compared to synthetic antioxidant) in relation to human health is useful. Plants have several phytochemicals/micronutrients with creditable medicinal values useful in ayurveda. Based on this, a holistic approach in neurodegeneration and neuroprotection research seems to be more reasonable.

There are some medicinal plants (*Withania somnifera, Bacopa monniera, Centella asiatica, Convolvulus pluricaulis, Emblica officinalis* and *Ocimum sanctum)* of Indian origin are discussed by Husain et al. [5] which are used in various central nervous system (CNS) disorders since ancient times. The other blockbuster herbal drugs of non-Indian origin like *Ginkgo biloba*, St. John's wort, Kava kava and Valerian are also discussed which have been extensively reviewed elsewhere for their effectiveness in many brain disorders [6,7]. Using cell line as a simple model, a correlation between oxidative stresses induced DNA damage and neurodegeneration has been established by some authors including our previous study [8-11]. However, the studies of neuroprotective effects of plant/herbal extracts on cell line models or animal models are very limited. A growing number of studies suggest that natural extracts and phytochemicals have a positive impact on various diseases, this lead to the study of Indian gooseberry neuroprotection against oxidative stress induced DNA damage.

Emblica officinalis commonly known as Amla or Indian gooseberry have been reported to exhibit significant adaptogenic, anti-stress immunopotentiating and memory-facilitating effects [12,13]. The tannoid principles of *Emblica officinalis* have recently shown an antioxidant activity in chronic stress induced changes in rat brain [14]. It is also nourishes all the body tissues and accelerates the cell regeneration process. *Emblica officinalis* extract contains several antioxidants such as emblicanin A and B, gallic acid, ellagic acid, ascorbic acid that possesses strong antioxidative activity and act in memory enhancement [15-17]. Though, the *Emblica officinalis* and its parts have been routinely and extensively used by tribals for the treatment of various diseases including neurological, but no scientific studies have been undertaken to substantiate these claims of neuroprotective effect both in *in vivo* and *in vitro* models. Thus, the present study was designed to investigate the effects of *Emblica officinalis* extract on H_2O_2 oxidative stress induced neurotoxicity in neuroblastoma cells (SK N SH), with the aim of providing possible therapeutic application for prevention and treatment of neurodegenerative diseases. We screened and identified pro and antiproliferative agents from 10 selected medicinal plants on cell lines and finally observed H_2O_2 induced DNA damage was decreased in the presence of *Emblica officinalis* extracts by doing COMET assay.

***Corresponding author:** Ramakrishna V, Department of Biotechnology & Bioinformatics, Yogi Vemana University, 516003-Kadapa, Andhra Pradesh, India, Tel: 919866016961; E-mail: vrkrishna70@gmail.com

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Materials and Methods

Materials

MTT, PMSF, BSA, protein A, LMP agarose, Triton X-100, DTT, trypsin, catechin and trypsin inhibitor were from Sigma, USA. All the other chemicals and reagents were biochemical grade.

Preparation of the plant extracts

Bacopa monnieri L. (leaves), *Terminalia bellerica Roxb.*(bark), *Ficus benghalensis L.*(bark), *Emblica officinalis L.* (bark), *Ficus racemosa* (bark), *Asparagus racemosus* (aerial part), *Tinospora cordifolia* (stem), *Hemedesmus indicus* (root), *Phyllanthus fraternus* (whole plant), *Terminalia arjuna* (bark) were collected from the university campus during monsoon, air dried at room temperature and used for the preparation of aqueous and methanol extracts. In each case, powdered air-dried plant material was extracted with methanol followed by water. Air dried plant materials were continuously extracted for 48 h with methanol in a Soxhlett apparatus. The extract was filtered and concentrated under vacuum at 60°C to obtain a dry extract through rotovaporator. The marc obtained following the methanol extraction was later extracted with water in the same manner as methanol. The water extract was concentrated and dried in lyophiliser.

Cell culture

SK-N-SH cell line (human neuroblastoma cells) was obtained from National Centre for Cell Sciences, Pune, India. SK-N-SH was cultured in MEM containing 0.5 mM L-glutamine, 0.1 mM sodium pyruvate and 1 mM non-essential amino acids with 10% FBS.

Identification of cytotoxic (pro- or anti-proliferative) effects of plant extracts by MTT assay

Reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Sigma) is chosen as a cell viability measurement optimal endpoint [18]. SK-N-SH cells ($0.2 * 10^6$ cells per well) in 200 µl of corresponding medium with 10% FBS were seeded into 96-well plate. Increasing concentrations of H_2O_2 or plant extracts dissolved in DMSO or both were added to the cells and incubated at 37°C for 24 h in a humidified CO_2 incubator with 5% CO_2. The medium was replaced along with 20 µl of 5 mg/ml MTT (3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazoliumbromide). After incubation for 4 h in humidified atmosphere, the medium was removed and 200 µl of 0.1 N acidic isopropyl alcohol was added to the wells to dissolve the MTT formazan crystals. Absorbance was recorded at 570 nm immediately after the development of purple color. Each experiment was conducted in triplicate. Relative cell viability was evaluated according to the quantity of MTT converted into insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The results were expressed as mean percent of viable cells versus respective control. Experiments were repeated three times and the results represented as averages with standard error.

Trypan blue exclusion assay

After completion of incubation with H_2O_2, cells were stained with 0.4% (w/v) trypan blue solution (400 µl/well, prepared in 0.81% NaCl and 0.06% K_2HPO_4) at room temperature for 10 min. Only dead cells with damaged cell membrane are permeable to trypan blue. The numbers of trypan blue-permeable blue cells and viable white cells were counted in six randomly chosen fields per well under microscope (Olympus, Tokyo, Japan).

Neutral comet assay

Approximately, half a million SK-N-SH cells were seeded in 2 ml MEM medium with 10% FBS into 6 well plates and cultured for 48 h. Increasing concentrations of H_2O_2 or plant extracts or positive control (catechin – flavanoid) were added to the cells and incubated at 37°C for 24 h in humidified incubator with 5% CO_2. The medium was removed and the cells washed with fresh media. At the end of 24 h, half the cells were pelleted and the remaining subjected to a fresh media change and allowed to recover for 72 h. Subsequently, 5000 cells were taken in a micro-centrifuge tube to which 1 ml cold 1x PBS wash was given and processed for neutral comet assay as described by Kent et al. [19]. Briefly, the cells were centrifuged and resuspended in 500 µl of cold 1x PBS and 1.5 ml of 1% LMP agarose was added to each of the samples. The agarose-cell suspension was gently layered on a 1% agarose coated microscopic frosted end glass slide, allowed to solidify on ice for 5 min, and then placed immediately in ice-cold lysis buffer containing 30 mM disodium ethylenediamine tetraacetic acid (EDTA, pH 8.0), 0.5% sodium dodecyl sulfate (SDS) and 0.25 mg/ml proteinase K (Sigma). The samples were lysed for 1 h at 4°C and then kept at 37°C for 12–16 h. After cell lysis and digestion of protein–DNA complexes with proteinase K, the agar slides were equilibrated in 1x TBE (90 mM Tris–HCl, 90 mM boric acid and 2 mM EDTA, pH 8.0) for 2 h, with change of buffer every 15 min. The samples were electrophoresed with 1x TBE buffer for 20 min at 25 V. The DNA was then stained with 20 µg/ml of ethidium bromide (Sigma) for 20 min and the slides washed twice for 5 min in TBE. To ensure random sampling, 50 images/ slide were captured and, in some experiments, the observer was blinded to the conditions. The images were captured on a Confocal Microscope (Lieca) and comet tail length was quantified using Comet-IV software (Perceptive Instruments, UK). The mean comet tail length value of control samples was subtracted from the mean comet tail length for each H_2O_2 dosage. The results shown are the Mean ± SEM value from four independent experiments (50 images for each dose of each independent experiment).

Data analysis

Numerical data was presented as Mean ± SEM. Statistical significance was calculated using ANOVA to determine whether the compared groups are distinct. The level of significance was set at $p < 0.05$.

Results

Evaluation of the effect of plant extracts on neuroblastoma cells

About ten most commonly used plants in Indian traditional medicine, Ayurveda, were selected viz. *Bacopa monnieri L.* (leaves), *Terminalia bellerica Roxb.*(bark), *Ficus benghalensis L.*(bark or root), *Emblica officinalis L.* (bark), *Ficus racemosa* (bark), *Asparagus racemosus* (aerial part), *Tinospora cordifolia* (stem), *Hemedesmus indicus (root), Phyllanthus fraternus* (whole plant), *Terminalia arjuna* (bark), prepared the aqueous and methanol extracts and evaluated their cytotoxic effect (IC_{50}) on human neuroblastoma cells (SK N SH) through MTT assay. The extracts of *Terminalia bellarica, Terminalia arjuna, Asperagus recemoses, Tinospora cardifolia* were shown cytotoxicity at the level of $IC_{50} > 0.01$ mg. Cytotoxicity was not noticed even up to $IC_{50} > 0.2$ mg in the extracts of *Bacopa monnieri*, *Phyllanthus fraternus and Hemedesmus indicus.* However, the extracts of *Emblica officinalis, Ficus recemosa* and *Ficus benghalenses* were stimulated the growth and proliferation of cells with increasing concentration

of extract (1 mg). From these results, we have chosen the widely used traditional medicinal plant *Emblica officinalis* for the study of neuroprotection against H_2O_2 induced oxidative stress. Prior to testing the neuroprotective effect of EO extracts, the extracts (AEO–Aqueous extract of *Emblica officinalis*, MEO - Methanol extract of *Emblica officinalis*) direct effect on cell viability and proliferation of SK N SH cell cultures was evaluated. Cell viability was determined following incubating cells with various concentrations (1–1000 µg/ml) of plant extract in cultured medium for 24 h. The results as shown in Figure 1 demonstrated that cell survival was increased with escalating cell proliferation even in the presence of high concentrations of *Emblica officinalis* extracts. Interestingly, we found that when neuroblastoma cells were treated with 200 µg/ml MEO and AEO extracts for 48 and 72 h, cell viability was increased maximum (40%, 30%) for MEO and comparatively less (28%, 25%) for AEO extracts (Figure 2). This observation suggests that certain compounds present in EO extracts were likely to promote cell survival or delay the natural death of neurons in culture medium.

Neuronal protection of EO extracts against H_2O_2-induced oxidative injury

The neuroprotective activity of EO extracts was evaluated by assessing the viability of cultured cells injured with H_2O_2 in the presence or absence of EO extracts. As estimated by MTT assay, cell viability is markedly decreased after a 24 h exposure to 1.0 mM H_2O_2 (Figure 3). However, when cells were preincubated with EO extract (0.1–1.0 mg/ml) for 24 h, and then exposed to H_2O_2, cell toxicity was significantly attenuated in a dose-dependent manner. Addition of EO extract to the culture medium prior to H_2O_2 led to the increase in the number of survival cells, suggesting that EO extract prevented cell damage mediated by H_2O_2.

H_2O_2 induced DNA damage in neuroblastoma cells

As described in our previous studies [11], DNA damage and recovery was studied using human neuroblastoma cell line, SK-N-SH. The cells were incubated in the presence of increasing concentrations of H_2O_2 (0.5, 1.0 and 2.0 mM) for 24 h followed by re-culturing cells in a fresh medium and cell recovery monitored after 72 h. The DNA damage in the cells was analyzed by single cell neutral comet assay using confocal microscope. The results showed that cellular DNA damage was significant in the presence of H_2O_2 as reflected in terms of increased comet tail length in a dose-dependent manner. A significant decrease in DNA damage was observed when the cells were re-cultured in a fresh medium for 72 h.

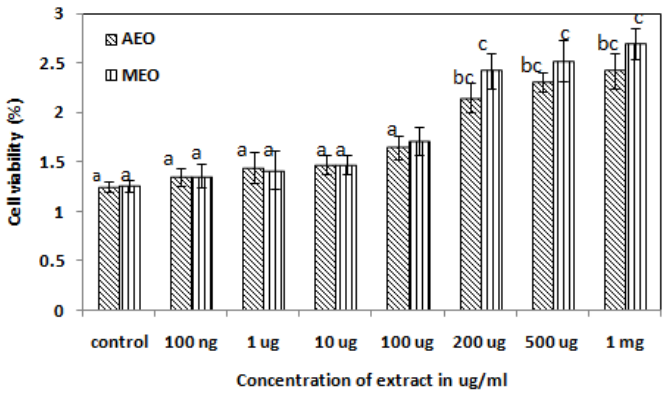


Figure 1: Proliferative effect of EO extract on neuronal cell line, SK-N-SH. Cells were treated with increasing concentrations of EO extract (1-1000 µg/ml media) for 24h. MTT assay was done to assess cell viability. Optical density was measured at 570 nm using spectrophotometer. Each treatment was given in triplicate. The data is represented in terms of % of cell viability. Using ANOVA, increase in cell proliferation was found to be significant at higher concentrations i.e. 200, 500, 1000 µg/ml of extract ($p < 0.05$). Bar charts depict Mean ± SEM of three independent experiments.

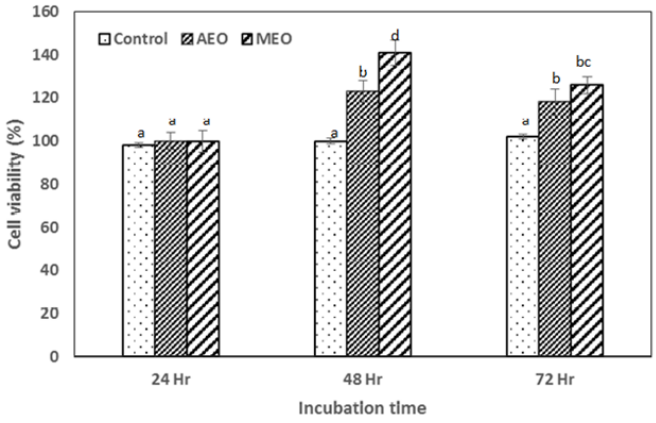


Figure 2: EO extract promotes neuronal cell viability in culture media. SK-N-SH cells were treated with 200 µg/ml of EO extract for 24, 48 and 72 h. Cell viability was determined by MTT assay. Optical density was measured at 570 nm using spectrophotometer. Each treatment was given in triplicate. The data is represented in terms of % of cell viability. Using ANOVA, increase in cell proliferation was found to be significant at 48 and 72h in comparison with 24h ($p < 0.05$). Bar charts depict Mean ± SEM of three independent experiments.

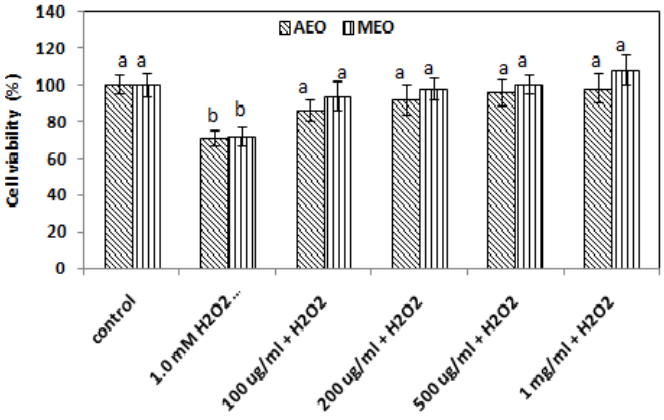
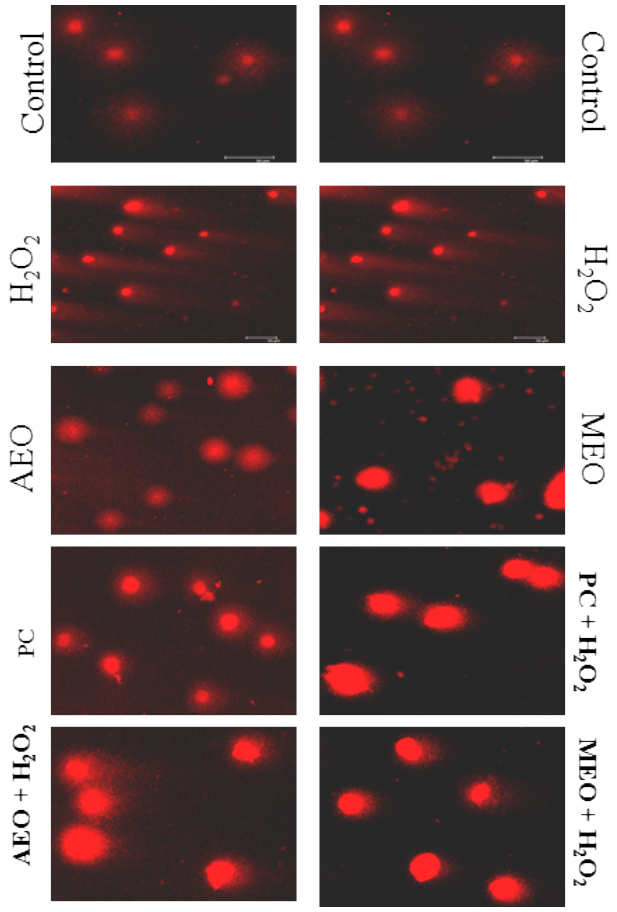


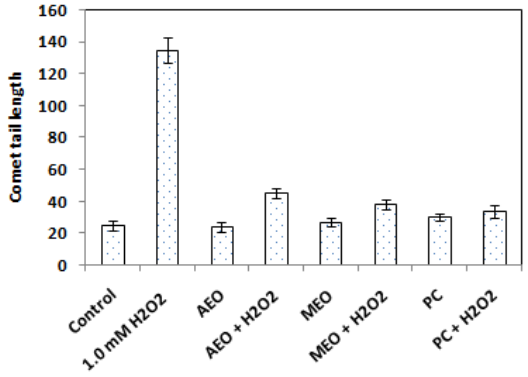
Figure 3: Neuroprotective effect of EO extracts on H_2O_2 induced toxicity. SK-N-SH cells were treated with EO extract for 24 h followed by H_2O_2 for 24h at indicated concentrations. Cell viability was determined by MTT assay. Each treatment was given in triplicate. The data is represented in terms of % of cell viability. Using ANOVA, increase in cell proliferation was found to be significant at pretreatment concentrations of 100, 200, 500 and 1000µg/ml extract ($p < 0.05$). Comparison was done with % of cell viability of H_2O_2 treated SK-N-SH cells. Bar charts depict Mean ± SEM of three independent experiments.

Neuronal protection of EO extracts against H_2O_2-induced DNA damage

Oxidative DNA damage, indicated by the tail lengths and intensity of comets, was increased in the presence of H_2O_2 in SK N SH cell lines. The neuroprotective activity of EO extracts against DNA damage was evaluated by measuring the comets length in the cultured cells injured



Panel A



Panel B

Figure 4: Neuroprotective effect of EO extracts on H_2O_2 induced DNA damage in neuroblastoma cells. SK-N-SH cells were pretreated with of 200µg/ml EO extracts or 20µm/ml catechin (PC - positive control) for 24 h and then with 1.0 mM H_2O_2 for 24h. Panel A represents comets by confocal microscopy and Panel B shows quantified tail length. Corresponding bar charts depict Mean ± SEM. Average of 50 cells was chosen for every experiment (n=3). DNA damage was measured in terms of comet tail length. DNA damage was quantified using Comet-IV software (Perceptive Instruments, UK). There was a concentration dependent production of DNA damage in H_2O_2 treated cells. Using ANOVA, decrease in tail length was found to be highly significant in AEO and MEO pretreated cells when compared with H_2O_2 treatment ($p < 0.001$).

with H_2O_2 in the presence or absence of EO extracts or positive control (catechin) (Figure 4). From the results of Figure 3, 0.2 mg/ml of EO extracts was selected, added to the medium and cultured the cells for 24 hours and then exposed to 1.0 mM of H_2O_2 for 24 hours. Oxidative DNA damage in FB treated cells showed a significant reduction in intensity of DNA damage in terms of comet tail length and also brought the comet pattern to the control level. Methanol EO extract was found to be more effective than aqueous extract in controlling the H_2O_2 induced oxidative DNA damage and bring the cell growth back to the normal comparable to positive control catechin flavanoid. The same was due to the presence of high levels of tannins (5.214 mg/gm) and flavanoids (3.623 mg/gm) in methanol extracts of our preliminary biochemical analysis. Thus, the results showed that EO extracts conferred significant protection from peroxide-mediated DNA damage in neuroblastoma cells.

Discussion

Increasing reports provided evidences implicating oxidative stress as a major pathogenic mechanism in neurodegenerative diseases. Oxidant overproduction leads to oxidative molecular damage of the tissue [4,20]. Therefore, protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment of neurodegenerative disorders caused by oxidative stress. As a result, much attention has been focused on the research of naturally occurring protective antioxidants and on the mechanisms of their action. In line with this, several plant extracts have been found to show strong antioxidant activity and to protect against oxidant-induced damage [5,8,21-23].

In the present study, cytotoxicity was assayed for the extracts of ten selected plants which are used routinely in treatment of various diseases including neurological diseases in Ayurvedic medicine and found the extracts of *Emblica officinalis* were stimulated the growth and proliferation of cells with increasing concentration of extract. Compared with normal cells, cancer cells constitutively generate large but non-lethal amounts of ROS that apparently function as signaling molecules, constantly activating redox sensitive transcription factors and responsive genes. These gene products are involved in the survival and proliferation of cancer cells. Thus, the reduction in oxidative stress may suppress the proliferation of tumor cells [24].

As described in our previous studies [11], DNA damage and recovery was studied using human neuroblastoma cell line, SK-N-SH and observed that cellular DNA damage was significant in the presence of H_2O_2 as reflected in terms of increased comet tail length in a dose-dependent manner. H_2O_2 is a potent genotoxin, able to induce oxidative DNA damage, including DNA-strand breakage and base modification [25]. The mutagenic effects of ROS, in particular H_2O_2, that induces lesions similar to those resulting from ionizing radiation, have been well documented in V79 cells and SK-N-SH cells [11,26,27]. H_2O_2-induced DNA damage in cells has been thought to occur through the Fenton reaction which produces hydroxyl radicals that attack DNA, resulting in damage. Indeed, H_2O_2 treatment was cytotoxic, genotoxic and mutagenic in SK N SH cells. Addition of EO extract to the culture medium prior to H_2O_2 led to the increase in the number of survival cells, suggesting that EO extract prevented cell damage mediated by H_2O_2. Oxidative DNA damage in these EO loaded cells was shown a significant reduction of comet length and intensity of DNA damage and also brought to control level. It is plausible that substances in EO extract (tannins) are able to prolong the lifespan of neurons in culture media somehow by promoting cell survival and/or delaying cell death. The elevation of cell viability could be the result of the ability of some

components in EO to repress cellular oxidative stress. The antioxidant activity was thought to be one mode of action of EO extract regarding its neuroprotective effect. Miranda et al. [21] reported that mate tea could protect against DNA damage and enhance the DNA repair activity. Protection may be afforded by the antioxidant activity of the mate tea's bioactive compounds. The tannoid principles of *Emblica officinalis* have recently shown an antioxidant activity in chronic stress induced changes in rat brain [28]. EO extract have been said to be effective in neurodegenerative conditions associated with aging [5].

According to the cultured cell-based observation from this study, the neuroprotective effects of *Emblica officinalis* appeared to be its antioxidant properties to suppress neuronal oxidative DNA damage in cells treated with H_2O_2. Therefore, treating patients with EO extract may be an alternative direction for ameliorating neurodegenerative disorders. EO extract may be able to suppress oxidative stress of neuronal cells within the brain possibly indicating its neurotonic effects. Taken all these evidences together, EO seems to have a therapeutic potency possibly as alternative therapy for preventing or delaying progression of neurodegenerative diseases. Further *in vivo* experiments needs to be done to make transition from *in vitro* study to preclinical study.

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