

## Protective effect of *Ficus bengalensis* L. extract against H<sub>2</sub>O<sub>2</sub> induced DNA damage and repair in neuroblastoma cells

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### ABSTRACT

**Aim & background:** Neuronal cells, because of their high rate of oxidative metabolism and low levels of antioxidants, are quite susceptible to oxidative DNA damage. *Ficus bengalensis* L. (Banyan tree) is a large tree and possessing medicinal properties. The study was designed to investigate the protective effect of *Ficus bengalensis* (FB) extracts against H<sub>2</sub>O<sub>2</sub> induced DNA damage and repair in human neuroblastoma cells (SK N SH). **Methods:** Aqueous and methanol extracts of *Ficus bengalensis* were prepared and cell viability conferred by these plant extracts was evaluated by MTT assay. DNA damage and protection by FB extracts was measured by comet assay using H<sub>2</sub>O<sub>2</sub> as DNA damaging agent. **Results:** Cell viability markedly decreased with increased DNA damage after a 24 h exposure to H<sub>2</sub>O<sub>2</sub>. However, prior incubation with FB extract (0.1 – 1.0 mg/ml) for 24 h, and then exposed to H<sub>2</sub>O<sub>2</sub>, cellular toxicity was significantly attenuated in a dose-dependent manner. 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 to control level. **Conclusion:** The study indicated *Ficus bengalensis* extract showing potential neuroprotective activity against oxidative environment generated by H<sub>2</sub>O<sub>2</sub> treatment.

**Keywords:** Neurodegenerative diseases - *Ficus bengalensis* extract - DNA damage and repair - Oxidative stress – Neuroprotection.

### INTRODUCTION

Reactive oxygen species (ROS) are produced by all aerobic cells. Neuronal cells, because of their high rate of oxidative metabolism and low levels of antioxidants, are quite susceptible to oxidative DNA damage, which often leads to DNA double strand breaks and mutation and further disease development<sup>1</sup>. Among DNA damage causing disorders, approximately 80% of the damage is caused by ROS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), singlet oxygen, and hydroxyl radical. Furthermore, persistent oxidative DNA damage can alter signaling cascades and gene expression, induce or arrest transcription, and increase replication errors and genomic instability, all of which have been described in the progression of disease<sup>2</sup>. Even though the

cells are equipped with efficient defense mechanisms to remove this kind of damage by various DNA repair pathways, the causes of these diseases are not fully prevented, and it is believed that such diseases are affected by multiple factors including over expression of ROS. This type of damage that occurs in neuronal cells is playing a critical role in neurodegenerative diseases<sup>3,4</sup>. Thus, identification of agents conferring neuroprotection could lead to potential therapies that could slow or ameliorate the progression of neurodegenerative diseases. Therefore, intake of antioxidants of natural origin with less in relation to human health could be useful. For keeping our brains young, in recent years, study of phytomedicine is receiving increasing attention and suggested that some traditional herbs with anti-aging properties are potential candidates in treating chronic and age-related diseases.

*Ficus bengalensis* L. (Banyan tree) is a large tree with aerial roots and is widely distributed throughout India. Information based on ethnomedicinal survey reveals that the herbal preparations of different parts of *Ficus bengalensis* (FB) possess medicinal properties and is considered as an effective, economical and safe agent for curing various

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diseases in Indian traditional system of medicine<sup>5</sup>. Pharmacological investigations of FB indicated the presence of interesting biological activities such as anti-diabetic<sup>6,7</sup>, and anti-bacterial agent<sup>8</sup>. However, information pertaining to the protective properties of FB on neurological disorders is lacking though it has been using traditionally. Therefore, it is important to understand the protective-action of the extracts of FB on H<sub>2</sub>O<sub>2</sub>-induced oxidative damage causing DNA and cell damage, H<sub>2</sub>O<sub>2</sub> being a major mediator of oxidative stress and a potent mutagen.

The present work was carried out to investigate the neuroprotective effect of the aqueous and methanol extracts of the bark of FB against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in neuroblastoma cells (SK-N-SH).

## MATERIALS AND METHODS

### Plant material and extract preparation

*Bacopa monnieri* L. (leaves), *Terminalia bellerica* Roxb.(bark), *Ficus benghalensis* L.(bark), *Embllica 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. All chemicals and reagents used were biochemical grade.

### 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 effects of plant extracts by MTT assay

Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma) is chosen as a cell viability measurement optimal endpoint<sup>9</sup> and methodology has

been described in our previous paper<sup>10</sup>. 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<sub>2</sub>O<sub>2</sub>, cells were stained with 0.4% (w/v) trypan blue solution (400 µl/well, prepared in 0.81% NaCl and 0.06% K<sub>2</sub>HPO<sub>4</sub>) 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). Cells were treated with FB extract prior to the H<sub>2</sub>O<sub>2</sub> treatment.

### 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<sub>2</sub>O<sub>2</sub> or plant extracts or positive control (catechin) were added to the cells and incubated at 37 °C for 24 h in humidified incubator with 5% CO<sub>2</sub>. 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<sup>11</sup>. The images were captured on a Confocal Microscope (Leica) 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<sub>2</sub>O<sub>2</sub> 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 Student t-test to determine whether the compared groups are distinct. The level of significance was set at \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

## RESULT AND DISCUSSION

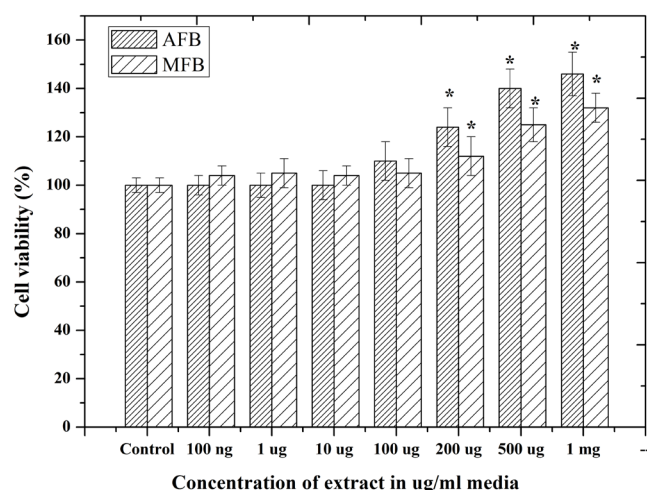
Based on ethnomedical survey, we selected most commonly used ten ayurvedic plants in neurodegenerative diseases viz. *Bacopa monnieri* L. (leaves), *Terminalia bellerica*

*Roxb.*(bark), *Ficus bengalensis* L.(bark), *Emblia officinalis* L. (bark), *Ficus racemosa* (bark), *Asparagus racemosus* (aerial part), *Tinospora cordifolia* (stem), *Hemedesmus indicus* (root), *Phyllanthus fraternus* (whole plant), *Terminalia arjuna* (bark) for screening neuroprotective activity. Aqueous and methanol extracts prepared and evaluated cytotoxic effect ( $IC_{50}$ ) on human neuroblastoma cells (SK N SH) through MTT assay. The extracts of *Terminalia bellarica*, *Terminalia arjuna*, *Asperagus recemosus*, *Tinospora cardifolia* showed 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 *Emblia officinalis*, *Ficus racemosa* and *Ficus bengalensis* at increasing concentrations stimulated growth and proliferation of cells. From these results, we focused on *Ficus bengalensis* for the study of neuroprotection due to its proliferating effect. Prior to testing the neuroprotective effect of FB extracts, the direct effect of extracts (AFB – aqueous extract of *Ficus bengalensis*, MFB – methanol extract of *Ficus bengalensis*) on cell viability and proliferation of SK-N-SH cell culture were evaluated. Cell viability was determined following incubating cells with increasing concentrations (1–1000  $\mu$ g/ml media) of plant extract in cultured medium for 24 h. The results shown in Fig. 1 demonstrate that cell survival significantly enhanced by 40 % suggesting that FB extract stimulated the proliferation of neuroblastoma cells. Interestingly, we also noticed that, when neuroblastoma cells were pretreated with 200  $\mu$ g/ml MFB or AFB extracts for 48

and 72 h, cell proliferation significantly increased by 42 and 30% respectively for AFB and comparatively less (30 and 33%) in the case of MFB extracts (data not shown). This observation suggests that certain compounds present in FB extracts may promote the survival of neuroblastoma cells in the culture medium.

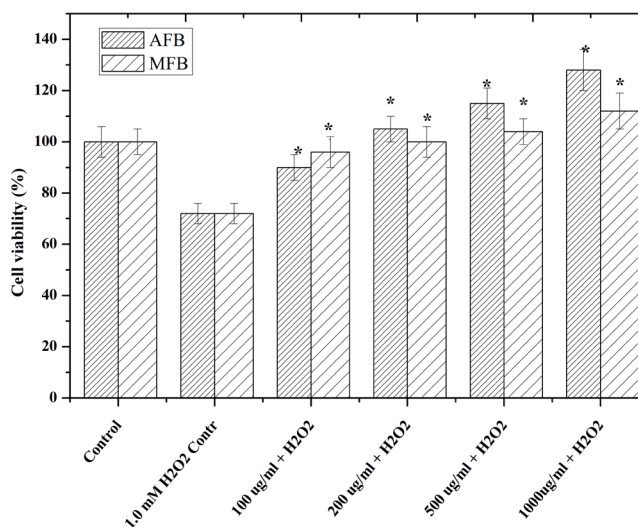
### Neuronal protection of FB extracts against $H_2O_2$ -induced cytotoxicity

$H_2O_2$  is one of the principal reactive products of oxygen metabolism. Accumulation of  $H_2O_2$  can have profound deleterious effects on cells through base modifications and strand breakage in genomic DNA, damage to lysosomal membranes, and the induction of apoptosis<sup>12</sup>. The neuroprotective activity of FB extracts was evaluated by assessing the viability of cultured cells injured with  $H_2O_2$  in the presence or absence of FB extracts. As estimated by MTT assay, cell viability markedly decreased after a 24 h exposure to 1.0 mM  $H_2O_2$ . However, when cells were incubated with FB extract (0.1 – 1.0 mg/ml media) for 24 h, and then exposed to  $H_2O_2$ , cell toxicity significantly attenuated in a dose-dependent manner (Fig. 2). Addition of FB extract to the culture medium prior to  $H_2O_2$  led to an increase in cell viability, suggesting that FB extract prevented  $H_2O_2$  mediated cell damage. Maximal survival of cells was observed in the presence of aqueous extract (AFB). It has been supported by some authors that the constituents present in the extract may decrease the levels



**Figure 1:** Proliferative effect of FB extract on neuronal cell line, SK-N-SH.

Cells were treated with increasing concentrations of FB extracts (1–1000  $\mu$ g/ml media) for 24h (AFB- aqueous extract of *Ficus bengalensis*, MFB- methanol extract of *Ficus bengalensis*). MTT assay was done to assess cell viability. Optical density was measured at 570 nm using spectrophotometer. Each treatment was triplicated. The results are represented in terms of % of cell viability.



**Figure 2:** Neuroprotective effect of FB extracts on  $H_2O_2$  induced toxicity.

SK-N-SH cells were treated with FB extracts for 24 h followed by  $H_2O_2$  for 24 h at indicated concentrations. Cell viability was determined by MTT assay. Each treatment was triplicated. The results are represented in terms of % of cell viability.

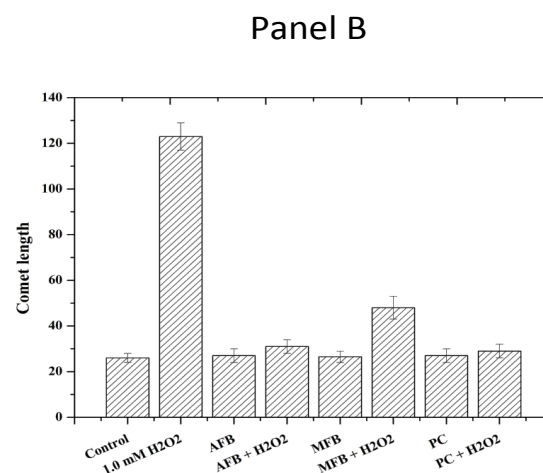
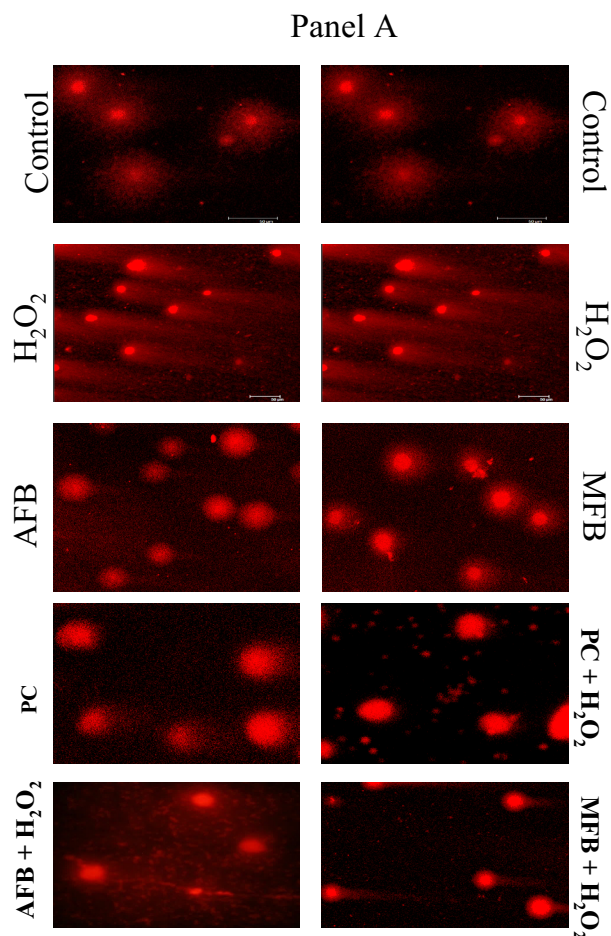
of lipid peroxidation products by scavenging free radicals like superoxide anion, hydroxyl and peroxy-free radicals<sup>6,7,13</sup>. Miranda *et al.*<sup>14</sup> also reported that protection may be afforded by the antioxidant activity of the mate tea's bioactive compounds.

### H<sub>2</sub>O<sub>2</sub> induced DNA damage in neuroblastoma cells and protective effects of FB extracts

As described in our previous studies<sup>10</sup>, 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<sub>2</sub>O<sub>2</sub> (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<sub>2</sub>O<sub>2</sub> 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. The mutagenic effects of ROS, in particular H<sub>2</sub>O<sub>2</sub>, that induces lesions similar to those resulting from ionizing radiation, have been well documented in V79 cells and SKNSH cells<sup>10,15</sup>. H<sub>2</sub>O<sub>2</sub>-induced DNA damage in cells has been thought to occur through the Fenton reaction which produces hydroxyl radicals that attack DNA, resulting in damage.

H<sub>2</sub>O<sub>2</sub>-mediated oxidative DNA damage in SK-N-SH cells was reflected by the magnitude of tail length and the intensity of comets. The neuroprotective activity of FB extracts against DNA damage was assessed by measuring comet tail length in cultured cells treated with H<sub>2</sub>O<sub>2</sub> that were pre-treated with FB extracts (Fig. 3). For this, we selected concentrations of FB (0.2 mg/ml) extract showing optimal activity was added to cells and cultured for 24 h and then exposed to 1.0 mM of H<sub>2</sub>O<sub>2</sub> for 24 h. 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. Aqueous FB extract was found to be more effective than methanol extract in controlling the H<sub>2</sub>O<sub>2</sub> induced oxidative DNA damage and bring the cell growth back to the normal comparable to positive control catechin (flavanoid). Thus, the results showed that FB extracts conferred significant protection from peroxide-mediated DNA damage in neuroblastoma cells.

Indeed, H<sub>2</sub>O<sub>2</sub> treatment was cytotoxic, genotoxic and mutagenic in SK-N-SH cells. Addition of FB extract to the culture medium prior to H<sub>2</sub>O<sub>2</sub> led to an increase in



**Figure 3:** Neuroprotective effect of FB extracts on H<sub>2</sub>O<sub>2</sub> induced DNA damage in neuroblastoma cells.

SK-N-SH cells were pretreated with 200 µg/ml media of FB extract or 20µM catechin (PC - positive control) for 24 h and then with 1.0 mM H<sub>2</sub>O<sub>2</sub> for 24h. Panel A represents comets by confocal microscopy and Panel B shows quantified tail length. Corresponding bar charts depict Mean ± SEM. An average of 50 cells was chosen for each experiment (n=3). DNA damage was measured in terms of comet tail length and quantified using Comet-IV software (Perceptive Instruments, UK). Scale bar = 100µm. Using Student t-test, decrease in tail length was found to be highly significant in AFB and MFB pretreated cells compared to H<sub>2</sub>O<sub>2</sub> treatment alone (\*\*\*) p < 0.001).

the number of survived cells, suggesting that FB extract prevented cell damage mediated by H<sub>2</sub>O<sub>2</sub>. Oxidative DNA damage in these FB loaded cells has shown a significant reduction of comet length and intensity of DNA damage and also brought it to the control level (fig 3). It is plausible that substances in FB extract 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 plant to repress cellular oxidative stress. Antioxidant activity was thought to be one mode of action of *Ficus bengalensis* extract regarding its neuroprotective effect. A well-built association between vegetable or fruit consumption and protection against oxidative DNA-damage was found in recent comet experiments<sup>16,17</sup>. Some authors demonstrated with supplementation of standard antioxidants like quercetin or borneol and observed significant reduction in oxidative DNA damage against H<sub>2</sub>O<sub>2</sub> challenge<sup>18,19</sup>.

The neuroprotective effect of FB extract may be attributed to its antioxidant properties that neutralize the oxidative environment generated by H<sub>2</sub>O<sub>2</sub> treatment. Though the ability of the active component of FB extract in crossing the blood-brain barrier has to be determined, considering the evidence as a whole, FB seems to have alternative therapeutic potential for preventing or delaying progression of oxidative damage mediated neurodegenerative diseases.

## ACKNOWLEDGMENTS

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## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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## Abbreviations Used

- MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide  
 AFB – Aqueous extract of *Ficus bengalensis*,  
 MFB – Methanol extract of *Ficus bengalensis*  
 PC – Positive control  
 ROS – Reactive oxygen species