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ORIGINAL RESEARCH

Neuroprotective Effects of Bikaverin on H_2O_2-Induced Oxidative Stress Mediated Neuronal Damage in SH-SY5Y Cell Line

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Abstract The generation of free radicals and oxidative stress has been linked to several neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease, and Amyotrophic lateral sclerosis. The use of free radical scavenging molecules for the reduction of intracellular reactive oxygen species is one of the strategies used in the clinical management of neurodegeneration. Fungal secondary metabolism is a rich source of novel molecules with potential bioactivity. In the current study, bikaverin was extracted from *Fusarium oxysporum* f. sp. *lycopersici* and its structural characterization was carried out. Further, we explored the protective effects of bikaverin on oxidative stress and its anti-apoptotic mechanism to attenuate H_2O_2-induced neurotoxicity using human neuroblastoma SH-SY5Y cells. Our results elucidate that pretreatment of neurons with bikaverin attenuates the mitochondrial and plasma membrane damage induced by 100 μM H_2O_2 to 82 and 26 % as evidenced by MTT and LDH assays. H_2O_2 induced depletion of antioxidant enzyme status was also replenished by bikaverin which was confirmed by Realtime Quantitative PCR analysis of SOD and CAT genes. Bikaverin pretreatment efficiently potentiated the H_2O_2-induced neuronal markers, such as BDNF, TH, and AADC expression, which orchestrate the neuronal damage of the cell. The H_2O_2-induced damage to cells, nuclear, and mitochondrial integrity was also restored by bikaverin. Bikaverin could be developed as a preventive agent against neurodegeneration and as an alternative to some of the toxic synthetic antioxidants.

Keywords H_2O_2 · Oxidative stress · Bikaverin · Neuroprotection · Realtime Q-PCR

Introduction

Living organisms obtain the energy for biological processes through oxidation. Reactive oxygen species (ROS) are unwanted metabolic byproducts of normal aerobic metabolism, which are usually highly reactive and short-lived (Uttara et al. 2009). When ROS levels exceed the antioxidant capacity of a cell, a detrimental condition known as oxidative stress occurs (Klein and Susan 2003). Oxidative stress induced by reactive oxygen species is associated with cell apoptosis and plays an important role in the pathological process of neurodegenerative disorders. The nervous system is highly vulnerable to oxidative stress because of its increased oxygen consumption rate, presence of high concentrations of polyunsaturated fatty acids that are susceptible to lipid peroxidation, and is relatively deficient in antioxidant systems (Friedman 2011; Muller 1996). Neurodegeneration resulting from oxidative stress is believed to be implicated in the onset and progression of many neuronal disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic lateral sclerosis (Andersen 2004; Uttara et al. 2009).

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Hydrogen peroxide (H_2O_2) is one of the most important ROS generated through oxidative stress. It is a byproduct of enzymatic activity and dopamine oxidation and it produces highly reactive hydroxyl radicals via the Fenton reaction (Cho et al. 2009). Hydrogen peroxide is of physiological and pathological significance as it is highly diffusible and can cross the plasma membrane (Forman 2007; Li et al. 2003). ROS evoke tissue and cellular injury by various mechanisms like altering the functions of biomolecules leading to inactive enzymes, oxidation of proteins, DNA breakage, peroxidation of lipids of cellular membranes, and alter lipid–protein interactions. They cause cell injury, necrosis, and apoptosis ultimately resulting in pulmonary, cardiovascular, neurodegenerative diseases, aging, cancer, and other disorders (Freidovich 1999; MacNee and Rahman 2001; Valko et al. 2007). Enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and thioredoxin, and the peroxiredoxin family of proteins are active scavengers of superoxide and hydrogen peroxide.

Mitochondrial dysfunction, the induction of oxidative damage and the propagation of cell death pathways; excitotoxicity and neuroinflammation- all these interconnected aspects of a cellular dysfunction are involved in the pathogenesis of numerous neurological disorders (Zádori et al. 2012). The relative preservation of neuronal structure and function is called Neuroprotection, which aims to prevent or slow disease progression and damage by preventing or slowing the loss of neurons (Seidl and Potashkin 2011; Casson et al. 2012). Limiting oxidative stress and excitotoxicity are the important aspects of neuroprotective treatments (Zádori et al. 2012).

The mechanism of neuroprotection involves: antioxidant activity, mainly inhibition of the NADPH oxidase and subsequent reduction of reduced NADPH oxidase-mediated generation of reactive oxygen species (Zhang et al. 2010); scavenging activity and the ability to activate key antioxidant enzymes in the brain, and thus limiting oxidative stress and tissue damage (Lau et al. 2005); activation of endothelial nitric oxide release and inhibitory action on both neuronal and inducible nitric oxide synthase activity and subsequent NO production (Ritz et al. 2008; Pechanova et al. 2004); reduction of neuroinflammation via the inhibition of the release of inflammatory mediators like cytokines, interleukin-1beta, and tumor necrosis factor-alpha and the downregulation of the pro-inflammatory transcription factors such as nuclear factor-kappaB (NF-κB); and modulation of signaling pathways leading to the improvement of memory and cognitive performance (Kovacsova et al. 2010). Selective interactions with protein kinase and lipid kinase signaling cascades (i.e., phosphoinositide-3 kinase/Akt and mitogen-activated protein kinase pathways), which regulate transcription factors and gene expression; activation of the extracellular signal-regulated kinase (ERK1/2) and the protein kinase B/Akt signaling pathways, leading to the activation of the cAMP response element-binding protein (CREB), represent the key pathways leading to neuroprotection (Spencer 2010). Potential mechanisms of neuroprotection are also by lowering cholesterol, reducing oxidative damage and ROS, impairing β-amyloid production and serum apolipoprotein E levels, enhancing the levels of endothelial nitric oxide synthase and cerebral blood flow, and modulating cognitive related receptors and matrix metalloproteases (Wang et al. 2011).

Natural products are rich sources of diverse bioactive compounds with possible health benefits. Many plant species in diverse regions of the world have been screened for their neuroprotective activity. Extracts of plants such as Green tea (Cho et al. 2008), *Tripterygium regelii* (Choi et al. 2010), *Rhus verniciflua* (Sapkota et al. 2011), *Eucommia ulmoides* (Kwon et al. 2012), *Hyptis suaveolens* (Ghaffari et al. 2014), *Bacopa monnieri* (Thomas et al. 2013), and *Cyperus rotundus* (Kumar et al. 2013) were reported to possess antioxidant properties and neuroprotective effects. However, there are very few reports on the neuroprotective compounds of fungal origin. Chrysogenamide A from *Penicillium chrysogenum* exhibited a neurocyte protection effect against oxidative stress-induced cell death (Lin et al. 2008) and coprismycins A and B isolated from a culture of *Streptomyces* sp. displayed neuroprotective effects (Kim et al. 2011). The current situation demands the immediate need for the investigation of new, safe, and effective antioxidants of microbial origin.

Fungal secondary metabolism is a rich source of novel bioactive compounds. Strains of *Fusarium oxysporum* are known to be prolific producers of metabolites with potential application. Polyketides constitute the largest and diverse group among secondary metabolites. Bikaverin is a polyketide compound, frequently biosynthesized by various *Fusarium* species (Bell et al. 2003). It exhibited a broad spectrum of bioactivity such as an antibiotic effective against *Leishmania braziliensis* (Balan et al. 1970), nematicidal activity against the pine wood nematode *Bursaphelenchus xylophilus* (Kwon et al. 2007), and antioomycete activity in the control of tomato late blight disease (Son et al. 2008). Bikaverin actively uncoupled oxidative phosphorylation of tumor cells and of rat liver mitochondria (Kovac et al. 1978). Bikaverin proven activity against metastatic cancer cell lines (Zhan et al. 2007) and an active antitumoral agent against different tumor cells as well. However, there is no negative incidence on human health of bikaverin-contaminated products, which was reported (Carmen et al. 2010).

The antioxidant effects of bikaverin have not been assessed until now. The principal objective of this study

was to study the effect of bikaverin on H_2O_2-induced oxidative stress and to explore its neuroprotective mechanism of action in human dopaminergic cell line. The study is an attempt to elucidate the antioxidant and anti-apoptotic defense mediated neuroprotective potential of bikaverin.

Materials and Methods

Chemicals

Bovine serum albumin (BSA), minimum essential medium eagle (MEM), trypsin (0.1 %), MTT [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide], fetal calf serum (FCS), 2′, 7′-DCFH$_2$DA, Rhodamine 123 were purchased from Sigma Chemical Co. (Saint Louis, Missouri, USA). Trichloroacetic acid (TCA), Agarose, trypan blue, hydrogen peroxide (H_2O_2), DMSO, RNA isolation kit, single-strand cDNA synthesis kit, and Quiagen fast cyber green master mix were procured from Quiagen-Gamb (Hilden, Germany). All other chemicals and solvents were purchased from Sisco Research Laboratories (Mumbai, India) and Merck (Bangalore, India).

Fungal Isolation, Extraction, and Characterization of Bikaverin

F. oxysporum f. sp. *lycopersici* was isolated from rhizosphere soil of tomato plant. Sterilized Erlenmeyer flasks (500 ml) containing 200 ml of Potato dextrose broth were inoculated with mycelium plugs from a 5-day-old culture of *F. oxysporum* f. sp. *lycopersici* grown on Potato dextrose agar and then incubated for 5 days at 150 rev min^{-1} and 28 °C. Cultures were filtered through Whatman No 1 filter paper and the mycelium was macerated thoroughly. A modified extraction protocol was developed. Extraction was carried out repeatedly with acetone +20 gL^{-1} aqueous ascorbic acid (90:10 v/v) until most of the red pigment was removed. The filtered extract was reduced under vacuum to leave only the aqueous phase, which was then extracted repeatedly with chloroform. The chloroform extract was reduced in volume and treated with 5 % aqueous Sodium bicarbonate (3:1 v/v). The insoluble sodium salt of the pigment recovered from the interface was suspended in 0.1 N hydrochloric acid, re-extracted with chloroform, and dried (anhydrous sodium sulfate). The extract was concentrated by rotary evaporation; the purple colored compound was re-crystallized with chloroform yielding the pure pigment (Bell et al. 2003; Brewer et al. 1973). The pigment was TLC purified. LC- MS was carried out to determine the molecular weight and for the structural elucidation, ^{1}H NMR spectrum was recorded in $CDCl_3$ using a Bruker Spectrometer at 400 MHz with reference to TMS.

Neuroprotective Studies of Bikaverin Against H_2O_2-Induced Stress

Cell Culture and Treatments

The SH-SY5Y human neuroblastoma cell line used in the current study was procured from the National Center for Cell Sciences, Pune, India. The cells were equally seeded into plates, flasks, or dishes in 1:1, DMEM/F-12 mixture supplemented with 10 % FBS, 2 mM L-glutamine, antibiotic and antimycotic solution (Sigma, St. Louis, MO, USA) in a humid atmosphere of 5 % CO_2 and 95 % air at 37 °C. Before experiments, 0.5 % serum was provided to differentiate the cells, and freshly prepared H_2O_2 was added for 24 h to the cells with or without pretreatment with bikaverin for 2 h before any experiment. To examine possible toxic effects, the cells were treated with bikaverin at concentrations ranging from 0.1 to 1 mg/mL for 24 h. Similarly, to induce oxidative stress, cells were treated with freshly prepared H_2O_2 (from 30 % stock) at a concentration of 100 μM for 24 h prior to each experiment.

Analysis of Cell Viability Using MTT Assay

MTT assay was performed as described previously (Mosmann 1983). Based on the preliminary observations, cells in the exponential phase were seeded onto 96-well plates (10×10^4 cells/well), allowed to adhere (for 24 h), and treated with various concentrations of bikaverin, vehicle, and H_2O_2. The medium following treatments was removed, cells were washed with PBS and 100 μL of the MTT stock (5 mg/mL) was added to each well. After 4 h of incubation, the solution was removed, and 100 μL of DMSO was added to each well. Following 10 min, the wells were read at 540 nm on an ELISA reader (Tecan, Austria). The data were recorded using the software package Magellan 6.3. The viability (%) was calculated as follows:

$$\text{Viability} = \frac{\text{Average O.D of treated wells} - \text{Average O.D of blank wells}}{\text{Average O.D of control wells} - \text{Average O.D of blank wells.}} \times 100,$$

where O.D. = Optical density.

Lactate Dehydrogenase (LDH) Release Assay

LDH is a marker for cell degeneration. Therefore, we measured the amount of LDH using LDH-estimation kit (Agappe-11407002) according to the manufacturers' instructions. In brief, the SH-SY5Y cells were plated at a density of 5×10^4 cells/well in 24-well plates; after 24 h, the cells were treated with various concentrations of bikaverin for 24 h. Following pretreatment with bikaverin, cells were treated with 100 μM H_2O_2 for 2 h. The cells were precipitated by centrifugation at 2,500×*g* for 5 min at 4 °C. The supernatant (100 μL) was mixed with 900 μL of kit reaction mixture. The total LDH activity was measured by lysis (2 % Triton X-100) of untreated cells.

Observations of Morphological Changes

The cells were seeded in Petri dishes (1×10^6 cells) and then treated with different concentrations of bikaverin for 2 h and with H_2O_2 at a concentration of 100 μM for 24 h. The cellular morphology was observed and photographed using a bright-field microscope.

Estimation of Intracellular ROS

The cells were seeded in 24-well plates at a concentration of 4.0×10^5 cells/mL and treated as mentioned earlier. After treatments, the oxidation-sensitive dye DCFH-DA (5 mg/mL) was added to the cells and incubated for 30 min. The cells were then collected after washing twice with PBS and the intracellular ROS formation was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Hidex plate chameleon™ V (Finland). For imaging, the cells were grown on cover slips which were precoated with poly L-lysine. After experimental treatments, the cells were treated with DCFH-DA as mentioned above and excess dye was removed by washing twice with PBS. The fluorescent intensity percentage was measured, and cells were imaged using fluorescence microscope (ZEISS).

Measurement of Mitochondrial Membrane Potential (MMP)

The protective effect of bikaverin on mitochondrial damage induced by H_2O_2 was determined by measuring the MMP using the fluorescent dye rhodamine 123. The cells were cultured in 24-well plates for fluorimetric analysis. After the treatments, rhodamine 123 (10 μg/mL) was added to the cells and incubated for 1 h at 37 °C. After washing twice with PBS, the cells were collected and the fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Hidex plate chameleon™ V (Finland).

Single Cell Gel Electrophoresis (Comet Assay)

Alkaline comet assay (Singh et al. 1988) was performed to measure the DNA damage and evaluate the protective effect of bikaverin against apoptosis induced by H_2O_2. Exponentially growing cells were treated with different concentrations of bikaverin for 2 h, washed, and then exposed to H_2O_2. After treatments, the comet slides were prepared. 1 mL aliquots containing 1×10^5 harvested cells were centrifuged and the pellets were re-suspended in 200 μL of 0.75 % low melting agarose layered to the frosted slides precoated with 1.0 % (w/v) normal melting agarose. Finally, a third layer of 0.75 % low melting agarose without cells was coated. Subsequently slides were exposed to lysing solution for 1 h at 4 °C, rinsed with water, and placed in electrophoresis buffer for 20 min and electrophoresed at 20 V for 20 min. The slides were dipped in neutralization buffer and treated with ethanol for 5 min before staining with 40 μL of ethidium bromide. The photographs were taken with fluorescence microscope (ZEISS), and measurements were made by Image Pro® plus software to determine the tail length. Olive tail moment (OTM) was used as the parameter to reflect DNA damage using the formula: % OTM = (head mean) × tail % DNA/100.

Relative Quantification of Target Gene Expression by RT-PCR

SH-SY5Y cells were cultured (1×10^7) in 75 cm^2 flasks and treated with 500 μg of bikaverin for 2 h. After exposure to bikaverin, cells were treated with 100 μM of H_2O_2 for 24 h. Total cellular RNA was isolated with a commercial kit according to the manufacturer's instructions (Sigma, St Louis, MO, USA). Equal amounts (2 μg) of RNA were primed with oligo (dT) primers and reverse-transcribed using a HS-RT PCR kit (Sigma, St Louis, MO, USA). Amplification of cDNA was performed in a total volume of 20 μL of SYBR Green I Mastermix (Roche Diagnostics, Germany) containing appropriate primers (Table 1), using a Roche LightCycler 480. After initial denaturation (95 °C for 10 min), 40 PCR cycles were performed using the following conditions: 95 °C, 15 s; 60 °C, 15 s; and 72 °C, 20 s, and at the end of PCR reaction, samples were subjected to a temperature ramp (from 70 to 95 °C, 2 °C/s) with continuous fluorescence monitoring. For each PCR product, a single narrow peak was obtained by melting curve analysis at the specific temperature. Each sample for targeted gene, SOD; (CAT); Brain-derived neurotrophic factor (BDNF); Tyrosine hydroxylase (TH); and Amino acid decarboxylase (AADC)

Table 1 Sequences of primers used in the study

Primer name	Sequence (5′-3′)	Gene target	Reference
CAT F	-CCTTTCTGTTGAAGATGCGGCG-	Catalase	(Hyejin et al. 2012)
CAT R	-GGCGGTGAGTGTCAGGATAG-		
SOD F	-AGGCCGTGTGCGTGCTGAAG-	Superoxide dismutase	(Hyejin et al. 2012)
SOD R	-CACCTTTGCCCAAGTCATCTGC-		
BDNF F	-ATGACCATCCTTTTCCTTACT-	BDNF	(Venkataramana et al. 2014)
BDNF R	-GCCACCTTGTCCTCGGAT-		
TH F	-GAGGAGAAGGAGGGGAAG-	TH	(Venkataramana et al. 2014)
TH R	-ACTCAAACACCTTCACAGCT-		
AADC F	-AACAAAGTGAATGAAGCTCTTC-	AADC	(Venkataramana et al. 2014)
AADC R	-GCTCTTTGATGTGTTCCCAG-		

expression, was assayed in duplicate, and the ΔCT method was used to quantify expression levels based on normalization to housekeeping, β-2 myoglobulin gene. The analysis was performed with Light Cycler and relative quantification software. Primers used in the study are shown in Table 1.

Statistical Analysis

All the experiments were performed in triplicates and the mean values ± standard deviations (SD) are represented. The means were analyzed statistically with the SPSS program version 20. Statistical differences between control and target groups for all experiments were determined using the analyses of variance (ANOVA) determined through Duncan's Multiple Range Test (DMRT) at ($p < 0.05$).

Results

Identification and Structural Characterization of the Compound

At the end of extraction, the pigment was in the form of a deep purple powder. Bikaverin from *F. oxysporum* f. sp. *lycopersici* had the following spectrum: ^{1}H NMR ($CDCl_3$) δ: 2.219 (3H, C-1); 3.861 (3H, C-3); 3.884 (3H, C-8); 5.27 (1H, C-6); 6.489 (1H, C-9); 7.44 (1H, C-2); 7.64 (1H, C-4) and *m/z* 383.17 $[M + H]^+$ as determined by LC/MS. These spectral data were consistent with those reported in the literature for bikaverin.

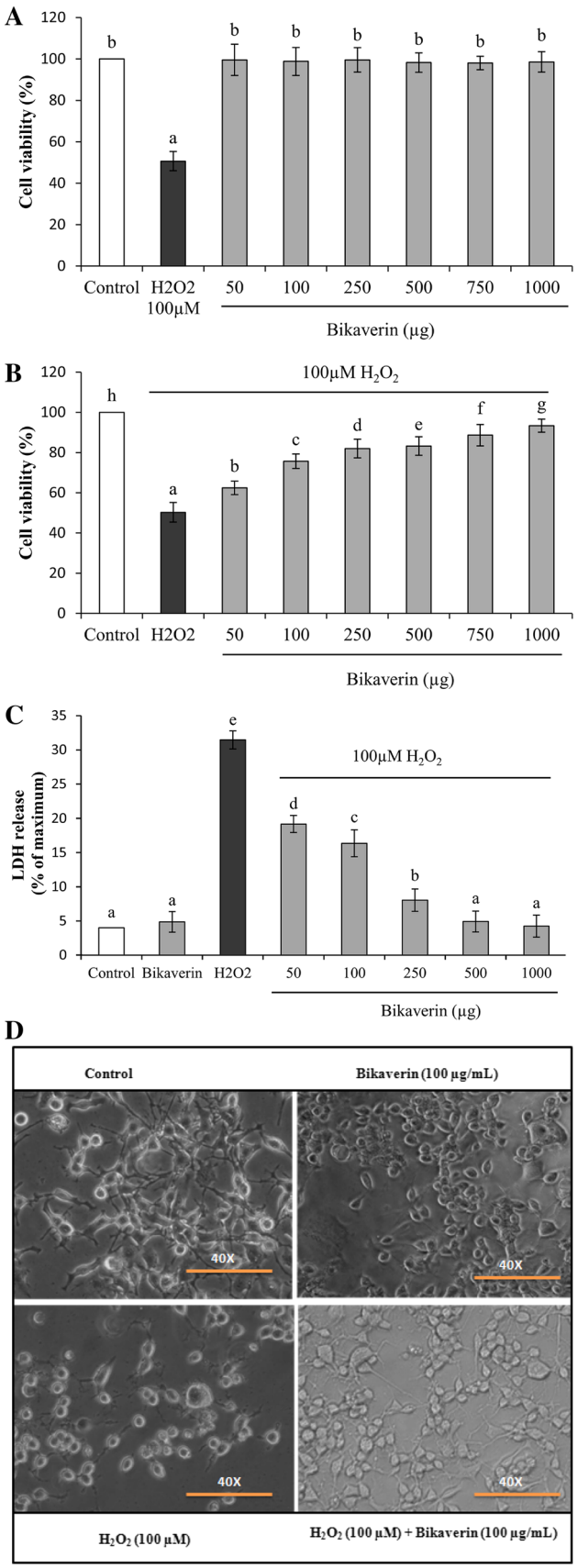
The Protective Effect of Bikaverin Against H_2O_2-Induced Cell Death

Bikaverin prevents H_2O_2-induced oxidative stress and LDH leakage in SH-SY5Y cells. Exposure of SH-SY5Y cells to various concentrations of bikaverin (50–1,000 μg) alone for 24 h did not alter the viability. However, exposure of cells to 100 μM H_2O_2 induced significant oxidative stress and cell death, and cell viability was almost half of control after 24 h exposure (50.4 ± 6.4, Fig. 1a). Pretreatment of cells with various concentrations of bikaverin followed by exposure to H_2O_2 did not drastically affect cell viability. As demonstrated in Fig. 1b, bikaverin pretreatment dose dependently prevents cell death due to H_2O_2 treatment. Bikaverin at 500 μg almost neutralized H_2O_2-induced oxidative stress (95.3 ± 3.6, $p < 0.05$). Moreover, at 1,000 μg, H_2O_2-induced stress was completely neutralized (99.6 ± 2.6, $p < 0.05$) demonstrating the effectiveness of bikaverin in preventing oxidative stress to neuronal cells.

LDH release is an indirect measure of dead cells. To further investigate the protective effect of bikaverin, the release of LDH was measured in the presence and absence of H_2O_2 (Fig. 1c). When SH-SY5Y cells were treated with 500 μg bikaverin, the levels of LDH remained identical to control indicating non-toxic nature of bikaverin. However, upon exposure to 100 μM H_2O_2, the cell supernatant contained sevenfold increased LDH compared to control (4.0 ± 0.4 vs. 31.46 ± 1.4, $p < 0.05$) indicating the cytotoxicity of H_2O_2. On the contrary, cells pretreated with 50–500 μg of bikaverin demonstrated decreased amounts of LDH leakage signifying dose-dependent protective effect of bikaverin against H_2O_2-induced cytotoxicity (Fig. 1c). Extract at 1,000 μg completely prevented H_2O_2-induced LDH outflow.

Morphological Observations

The protective effect of bikaverin was confirmed by morphological observation using bright-field microscope. The H_2O_2-challenged SH-SY5Y cells exhibited disappearance of the neurites and shrinkage of cells which were attenuated with bikaverin pretreatment (Fig. 1d).



◀**Fig. 1** Effect of bikaverin and H_2O_2 on SH-SY5Y cell viability (**a**), Dose-dependent protective effect of bikaverin on H_2O_2-induced cytotoxicity in SH-SY5Y cells. (**b**), Effect of bikaverin on LDH leakage (**c**) and The effects of bikaverin in H_2O_2-induced morphological alterations in SH-SY5Y cells by light microscopic observation of cell morphology (**d**). A: SH-SY5Y cells were exposed to various concentrations of Bikaverin and 100 μM H_2O_2. B: SH-SY5Y cells treated with various concentrations of Bikaverin were exposed to 100 μM H_2O_2 for 2 h. Cell proliferation was determined by MTT assay. C: SH-SY5Y cells treated with various concentrations of Bikaverin were exposed to 100 μM H_2O_2 for 2 h. LDH levels in the cells supernatant was determined using ELISA. D: Morphological changes in SH-SY5Y cells in presence of bikaverin, toxicant and cells pretreated with bikaverin and exposed to toxicant, observations were made under bright filed microscopy at 40× magnification. Results represent mean ± SD ($n = 3$) for each concentration. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to the DMRT

Bikaverin Inhibits H_2O_2-Induced ROS Generation

The exposure of SH-SY5Y cells to 100 μM H_2O_2 elicited a 2.5 fold increase in ROS production as compared to control group. ROS generation was attenuated significantly when cells were pretreated with bikaverin followed by 100 μM H_2O_2 treatment for indicated time periods. The fluorescence intensity decreased to 145 % indicating the potent antioxidant activity of bikaverin which was further confirmed by fluorescence imaging (Fig. 2a, b).

Effects of Bikaverin on H_2O_2-Induced Reduction of the MMP

To examine whether H_2O_2-induced apoptosis and its protection by bikaverin involve MMP pathway, its measurement was carried out using Rhodamine 123 and results are presented in Fig. 3. Here, we observed 35 % decrease in MMP with 100 μM H_2O_2 challenge which indicates the depolarization of mitochondrial membrane. However, the cells pretreated with bikaverin prior to the addition of H_2O_2 showed a significant recovery in the fluorescence intensity to an extent of control.

Bikaverin Decreased H_2O_2-Induced Genotoxicity in SH-SY5Y Cells

Comet assay is performed to determine the genotoxicity of a substance due to single strand breaks of DNA. In order to determine the protective potential of bikaverin on the H_2O_2-induced DNA damage, cells were treated with various concentrations of bikaverin for 2 h, followed by exposure to H_2O_2 (100 μM) for 24 h (Fig. 4a). When SH-SY5Y cells were treated with 100 μg bikaverin, the Olive tail moment (OTM) used as the parameter to reflect DNA damage remained same as control cells treated with

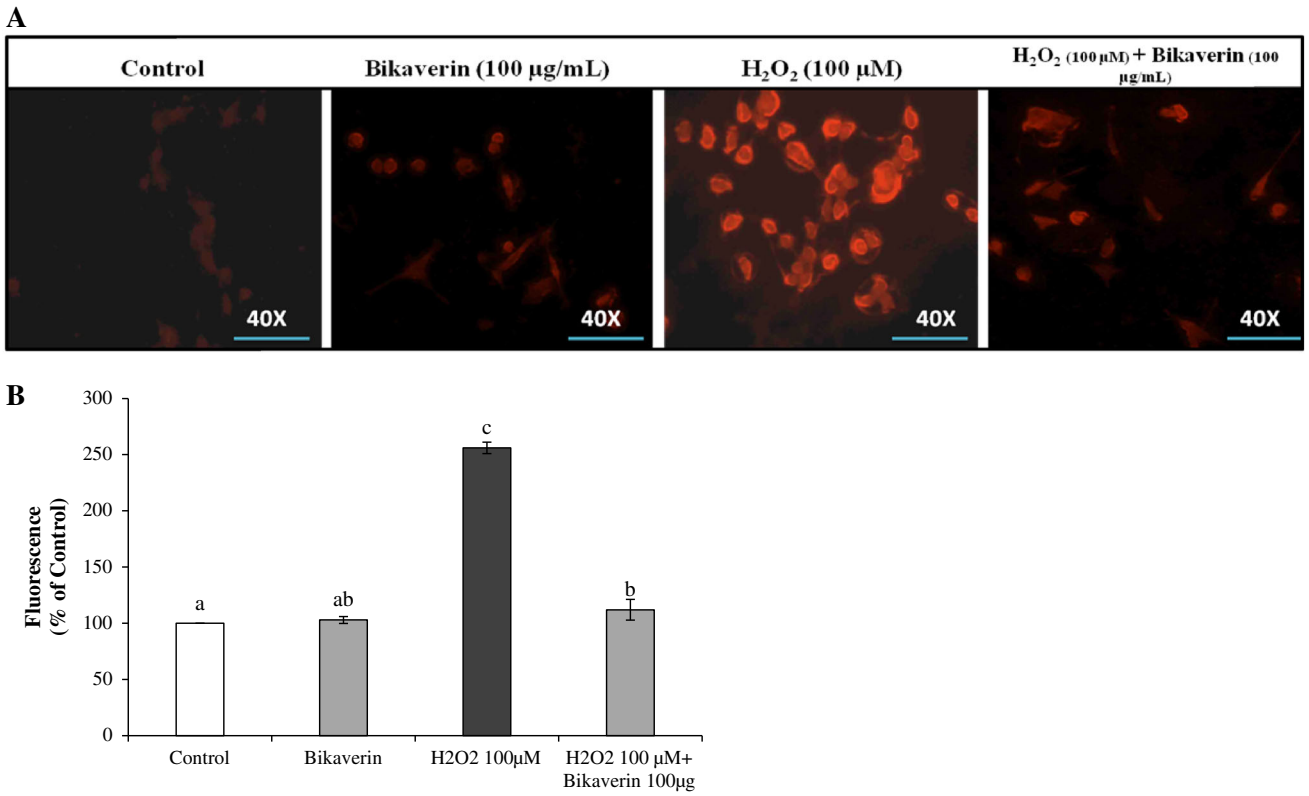


Fig. 2 Effect of bikaverin on the intracellular ROS formation in SH-SY5Y cells. **a** The ROS production in SH-SY5Y cells was monitored by fluorescence microscopy. Control cells without any treatment, 100 µg/mL bikaverin, 100 µM H_2O_2, and cells pretreated with 100 µg/mL bikaverin then treated with 100 µM H_2O_2, fluorescent images were taken using ZEISS fluorescent microscope under 40× magnification. **b** Estimation of ROS production by 2′,7′,DCFH-DA using spectrofluorimeter. The fluorescence intensity was expressed as relative value of control (% of control). Results represent mean ± SD ($n = 3$) for each concentration. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to the DMRT

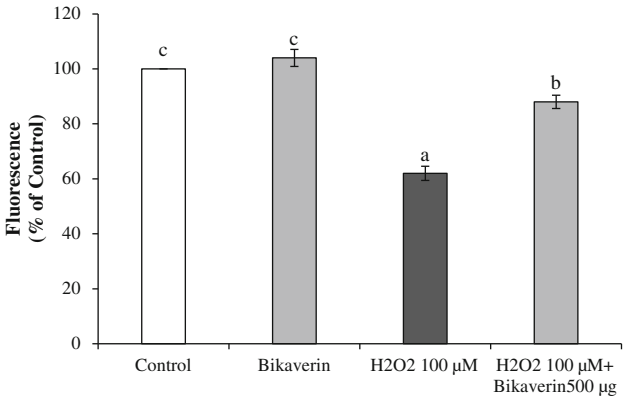


Fig. 3 Attenuation effect of bikaverin on H_2O_2 induced decrease of mitochondrial membrane potential. Results represent mean ± SD ($n = 3$) for each concentration. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to the DMRT

vehicle. Whereas, exposure to 100 µM H_2O_2 for 24 h, the OTM enhanced by 4 fold (22.94 ± 2.34 vs. 6.03 ± 0.46, $p < 0.05$) compared to control demonstrating the extent of DNA damage (Fig. 4b). On the converse, reduced OTM was observed after treatment of SH-SY5Y cells with 100–500 µg bikaverin followed by H_2O_2. As shown in Fig. 4b, after 500 µg bikaverin treatment, the OTM remained significantly less than H_2O_2 group (10.82 ± 0.9) but not significantly different from control.

Bikaverin Stimulates Enhanced Expression of Antioxidant Enzymes in SH-SY5Y Cells

The gene response for the SOD and CAT was monitored by quantitative real time RT-PCR (Fig. 5a). Cells treated with 500 µg of bikaverin alone displayed 2.7 and 2.5 fold increase in expression of CAT and SOD genes respectively ($p < 0.05$). However, treatment with 100 µM H_2O_2 alone resulted in decreased expression of the same genes. Surprisingly, SH-SY5Y cells pretreated with 500 µg of bikaverin following exposure to 100 µM H_2O_2 demonstrated very significant increase in 4.6 and 6.3 fold in CAT and SOD genes, respectively ($p < 0.05$) (Fig. 5a).

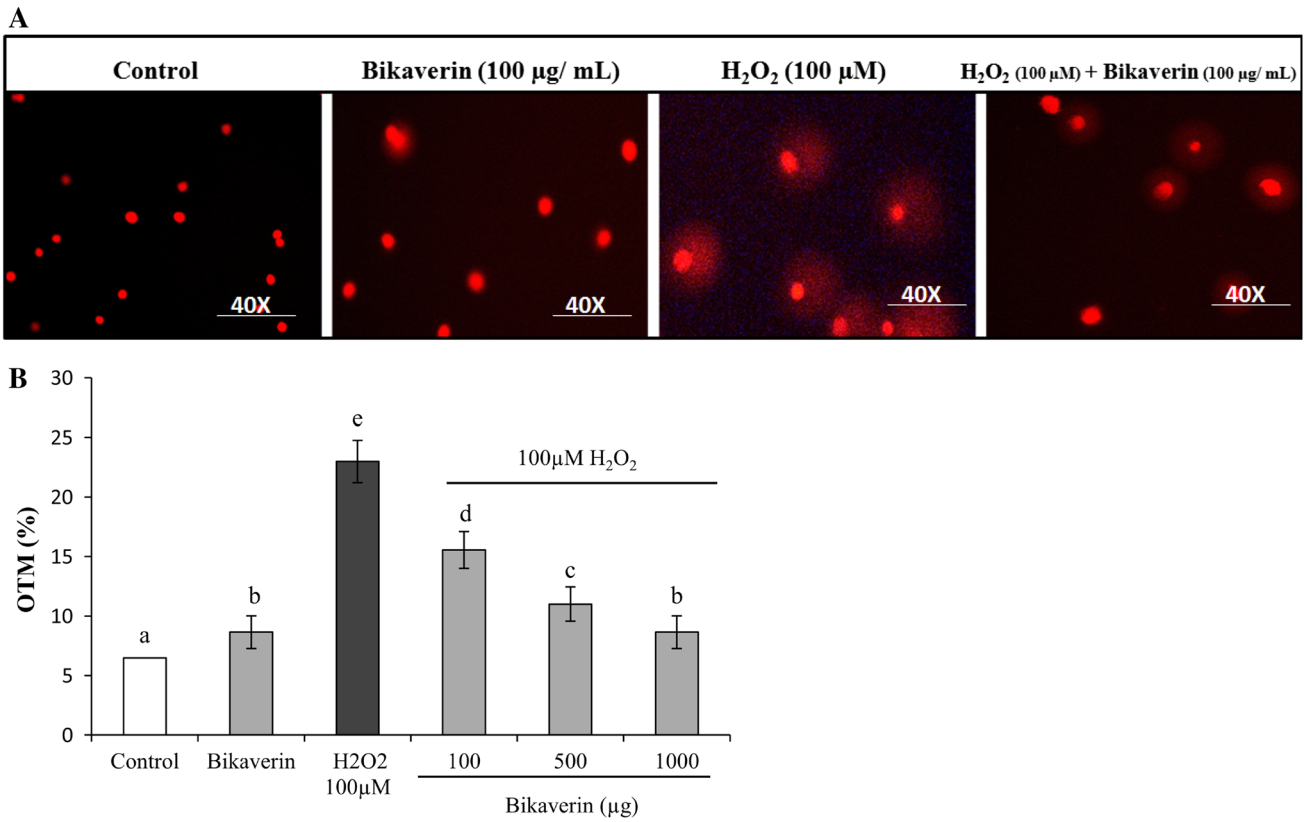


Fig. 4 Effect of bikaverin on DNA damage induced by H_2O_2 in SH-SY5Y cells determined using comet assay. The photomicrographs represent the levels of DNA damage in SH-SY5Y cells following treatment with bikaverin and 100 µM H_2O_2, fluorescent images were taken using ZEISS fluorescent microscope under 40× magnification. **b** The tail lengths of the comet was measured in each cell using Image pro® plus software and % OTM is represented as mean ± SD for each concentration ($n = 3$). In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to the DMRT

Bikaverin Stimulates Enhanced Expression of Neuronal Stress Biomarkers in SH-SY5Y Cells

The gene response for the neuronal biomarkers BDNF, TH, and AADC was monitored by quantitative real time RT-PCR (Fig. 5b). Cells treated with 500 µg of bikaverin alone displayed 2.7, 2.5, and 1.8 fold increase in expression of BDNF, TH, and AADC genes, respectively ($p < 0.05$). Treatment with 100 µM H_2O_2 alone resulted in decreased expression of the same genes. However, SH-SY5Y cells pretreated with 500 µg of bikaverin following exposure to 100 µM H_2O_2 demonstrated significant 2.6, 2.69, and 2. 63 fold increase in BDNF, TH, and AADC genes, respectively ($p < 0.05$) (Fig. 5b).

Discussion

Neurological disorders have been linked to elevated levels of oxidative stress and apoptosis. Oxidative stress and free radical generation have been shown to play pivotal role in regulating redox reactions in vivo contributing ROS, the main culprits in neurodegeneration. Several evidences indicate that oxidative stress plays a major role in modulating the biochemical changes resulting in aging and neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple Sclerosis (MS), and amyolotrophic lateral sclerosis (ALS) (Uttara et al. 2009). Antioxidants are exogenous or endogenous molecules which act against oxidative stress by neutralizing ROS and other kinds of free radicals, thus exhibiting their therapeutic potential. Hydrogen peroxide-induced cytotoxicity is the general method employed for the measurement of potential neuroprotective antioxidants (Ghaffari et al. 2014; Hemanth Kumar and Khanum 2013). The principal objective of this study was to explore the protective effects of bikaverin, a secondary metabolite produced by *F. oxysporum* f. sp. *lycopersici*, against oxidative stress-induced injury in SH-SY5Y cells and to elucidate some potential protection mechanism.

Apoptosis is known to be one of the most sensitive biological markers for evaluating oxidative stress caused by the imbalance between ROS generation and efficiencies of the antioxidant system (Gutteridge 1995; Kassie et al.

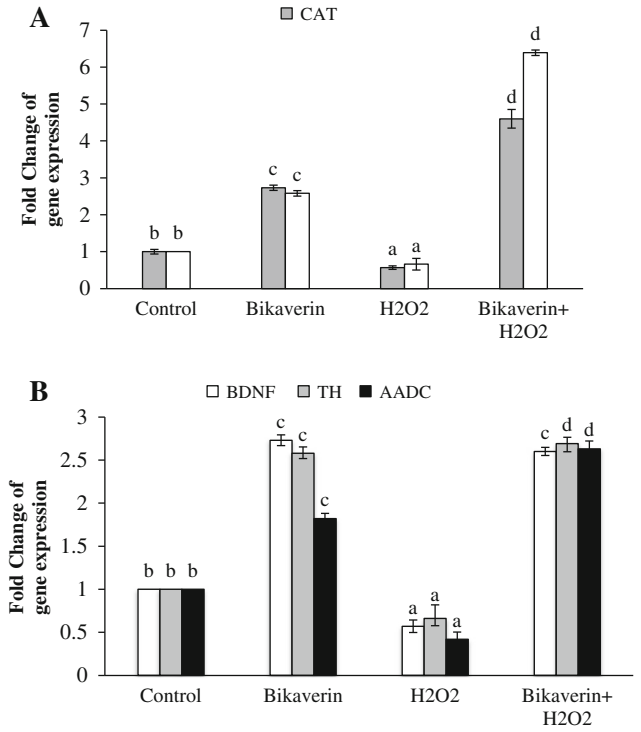


Fig. 5 **a** Real-time PCR for quantification of antioxidant enzymes expression levels following exposure to bikaverin, bikaverin ± H_2O_2, **b** Real-time PCR for quantification of neuronal marker expression levels following exposure to bikaverin, bikaverin ± H_2O_2. The fold change was calculated based on normalization with β-2 myoglobulin gene expression. The analysis was performed with Light Cycler and relative quantification software. Each experiment was performed in triplicates. Results represent mean ± SD ($n = 3$) for each treatment. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to the DMRT. *CAT* catalase, *SOD* superoxide dismutase, *BDNF* brain-derived neurotrophic factor, *TH* tyrosin hydroxylase, *AADC* amino acid decarboxylase

2000). Apoptotic cell death is characterized by morphological changes, including cell shrinkage, condensation of nuclei (Fernandez et al. 1999), and biochemical such as activation of caspase, nuclease, and the inactivation of nuclear repair polymerases, thereby resulting in the degradation of chromosomal DNA and cell death (Enari et al. 1998).

In the present investigation, we observed the cytotoxic effects of H_2O_2 as well as the protective effect of bikaverin by MTT and LDH assays which were further supported by the morphological observation. The cell viability as determined by the MTT assay revealed that the viability of SH-SY5Y cells exposed to various concentrations of bikaverin was similar to the untreated control cells. In the evaluation of neuronal cytotoxicity induced by H_2O_2 in SH-SY5Y cells, cell death was observed in a dose-dependent manner. In order to determine the protective effects of bikaverin against H_2O_2-induced cytotoxicity, the SH-SY5Y cells pretreated with various concentrations of bikaverin were exposed to 100 μM of H_2O_2. The H_2O_2-induced cell death was significantly attenuated by bikaverin pretreatment. The protective effect of bikaverin was further confirmed by the LDH assay. The assay is based on the principle that there is an increase in the leakage of cytosolic LDH with increasing number of dead cells. Pretreatment of SH-SY5Y cells with bikaverin showed lower LDH leakage compared to the H_2O_2-exposed cell group demonstrating its protective effect against H_2O_2-induced oxidative stress. The protective effect exerted by bikaverin on H_2O_2-induced cytotoxicity determined by the MTT assay correlated to the LDH assay and was further supported by the morphological observation. Thus, the evidence indicates SH-SY5Y cells die as the result of apoptosis after H_2O_2 insult, and that pretreatment with bikaverin attenuates the H_2O_2-induced neuronal cell damage. Zhan et al. (2007) reported the toxicity of bikaverin toward non-small-cell-lung, pancreatic, breast, and CNS glioma cancer cell lines. Interestingly, 6-deoxybikaverin was devoid of activity, signifying the requirement of the C-6 hydroxy group of bikaverin for its cytotoxic activity. Thus, indicating a high specificity of the bikaverin chemical structure required for its bioactivity. In the previous study, Lin et al. (2008) reported the ability of Chrysogenamide A, a macfortine group of alkaloids identified from *P. chrysogenum*, to inhibit cell death induced by hydrogen peroxide by improving cell viability by 59.6 %. In the present study, the cell viability and morphology of cells treated with bikaverin were very similar to those of the untreated control cells. In their study, Hiort et al. (2004) reported, aspernigrin B extracted from culture of *Aspergillus niger* isolated from the Mediterranean sponge *Axinella damicornis* displayed a strong neuroprotective effect by significantly reducing the increase of intracellular calcium concentration in rat cortical neurons stimulated with glutamic acid or quisqualic acid.

H_2O_2, the major ROS associated with oxidative stress, readily penetrates into cells and generates highly reactive hydroxyl radicals that successively attack cellular components, including lipids, protein, and DNA-inducing oxidative damages (Andersen 2004; Denisova et al. 2001; Halliwell and Aruoma 1991). The generation of ROS was quantified spectrofluorimetrically using the oxidant-sensing fluorescent probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA). 2′, 7′-dichlorodihydrofluorescein (DCFH) is very sensitive to several ROS and can be oxidized to a highly fluorescent 2′, 7′-dichlorofluorescein (DCF). The DCF fluorescence indicates the resultant oxidative stress due to overproduction of ROS or the depletion of antioxidants without any identification of specific ROS (Marchesi et al. 1999).

The nonpolar and non-ionic dye (DCFH-DA) gets converted into the polar derivative DCFH by cellular esterases that are non-fluorescent but switched to highly fluorescent

DCF when oxidized by intracellular ROS and other peroxides (Rastogi et al. 2010). The fluorescence is directly proportional to the oxidative stress induced by H_2O_2. The fluorescence intensity in SH-SY5Y cells treated with 100 μM H_2O_2 was 1.5 times higher compared to the control group. However, in the cells pretreated with bikaverin followed by H_2O_2 treatment, we observed a significant decrease in ROS indicating the ROS inhibitory effect of bikaverin. Our results are in accordance with earlier findings of Kumar et al. (2013) who reported *C. rotundus* rhizome extracts, exhibited inhibitory effects on ROS generation. Flavonoids are known to have powerful antioxidant properties, which are generally attributed to the presence of phenolic hydroxyl groups. The presence of one or more aromatic hydroxyl moiety is responsible for their antioxidant activity (van Acker et al. 2000). Chemically, bikaverin is a polyketide with a tetracyclic benzoxanthone structure. The presence of aromatic hydroxyl groups in the chemical structure of this compound might be responsible for its antioxidant potential.

Mitochondrial dysfunction and oxidative stress have been implicated in the pathophysiology of many diseases; therefore, the ability to determine mitochondrial membrane potential (MMP) and ROS can provide important clues about the physiological status of the cell and the function of the mitochondria (Joshi and Bakowska 2011). Rhodamine 123 (RH-123), a mitochondrial selective, cationic, and lipophilic dye, was used to monitor the membrane potential of mitochondria. Mitochondrial energization induces quenching of RH-123 fluorescence and the rate of fluorescence decay is proportional to the mitochondrial membrane potential (Baraccaa et al. 2003). Thus, it is imperative to measure the fluorescence intensity of the probe after the application of a specific stimulus. This allows one to determine the percentage of change in fluorescence intensity between the baseline level and a stimulus. This change in fluorescence intensity reflects the change in relative levels of MMP or ROS (Joshi and Bakowska 2011). A decrease in MMP could be correlated with apoptosis and its estimation helps to evaluate stress-induced apoptotic cell damage (Lee et al. 2010). In the present study, cells treated with 100 μM H_2O_2 exhibited 35 % decrease of MMP compared to that of control indicating the depolarization of MMP. However, we observed the restoration of MMP by bikaverin pretreatment, which was dissipated with H_2O_2 treatment. In their study, Sapkota et al. (2011) showed the extract of *R. verniciflua* markedly inhibited MMP disruption, offering protection against rotenone-induced oxidative stress. Kwon et al. (2012) reported *E. ulmoides* extract increased cell viability, inhibited cytotoxicity, and DNA condensation. It also attenuated the increase in ROS production and MMP reduction against H_2O_2-induced neuronal cell death in SH-SY5Y cells.

Apoptosis is morphologically characterized by nuclear disintegration due to the fragmentation of DNA. The H_2O_2-induced cell death through the induction of apoptosis and nuclear damage was measured by agarose gel electrophoresis. The comet assay (SCGE-Single Cell Gel Electrophoresis) was performed to assess the protective effect of bikaverin on H_2O_2-induced DNA damage in SH-SY5Y cells. The comet assay is a tool for the evaluation of local genotoxicity. The assay detects a broad spectrum of primary DNA lesions, including single strand breaks and oxidative base damage (Susanne et al. 2005). Undamaged DNA retains a highly organized association with the protein matrix in the nucleus which gets disrupted due to DNA damage. During electrophoresis, undamaged DNA remains compact but damaged DNA fragments move faster and are visualized in the form of a tail, like a comet. In the present study, a protective effect of bikaverin against H_2O_2-induced DNA damage was observed in the comet assay, which was based on the tail length and tail intensity. The tail dispersion showed a dose-dependent decrease with increasing concentration of bikaverin. Bikaverin showed inhibitory effects against DNA damage as measured in terms of tail length at a concentration of 500 μg clearly demonstrating that the H_2O_2-induced DNA damage was successfully overcome by bikaverin pretreatment. Cho et al. (2008) reported the ability of L-theanine, a component of green tea, to attenuate both rotenone- and dieldrin-induced DNA fragmentation and apoptotic death in SH-SY5Y cells. Kumar and Khanum (2013) have also reported the ability of *C. rotundus* extract to ameliorate DNA damage caused by H_2O_2 in a similar model.

Oxidative stress results to an imbalance of cellular oxidants and antioxidants. Enzymes, such as SOD, CAT, glutathione peroxidase (GPx), are active scavengers of superoxide and hydrogen peroxide. However, increased production of ROS or a poor antioxidant defense mechanism leads to physiological dysfunction, progressive cell damage, and disease emergence (Gilgun-Sherki et al. 2001; Mario et al. 1994). In the present study, the effect of bikaverin pretreatment on the expression of SOD and CAT genes was quantified by RT-PCR. The SH-SY5Y cells when treated with 100 μM of H_2O_2 resulted in a lower expression of the SOD and CAT genes. However, pretreatment with bikaverin attenuated the change in the expression of these genes that was induced by H_2O_2, demonstrating an increased expression of the SOD and CAT genes. The decrement of ROS may be associated with increased expression and activities of SOD, CAT quantified through RT-Q-PCR. Similarly, in earlier reports, Martin et al. (2011) and Sunil et al. (2011) showed the neuroprotective effects of isolated polyphenols of spanish red wine, and total oligomeric flavonoid extract of *C. rotundus* rhizomes was through decreased reactive oxygen

species generation and enhancement of enzyme activities and the protein expression of the antioxidant enzymes catalase, superoxide dismutase.

Brain-derived neurotrophic factor (BDNF), Tyrosine hydroxylase (TH), and Amino acid decarboxylase (AADC) play a major role in brain functioning as well as neurotransmitter synthesis (Chen et al. 2003). The effect of H_2O_2-induced oxidative stress mediated neuronal damage is well known. The expressions of TH, BDNF, and AADC play pivotal role in the survival as well as differentiation of dopaminergic neurons. Schapira (1999) has reported decreased expression of TH and AADC with simultaneous depletions of catecholamines in Parkinson's disease. Park et al. (2010) reported that rosmarinus extract significantly attenuated the H_2O_2-induced downregulation of TH, and AADC gene in SH-SY5Y cells. Choi et al. (2010) reported an increased expression of TH and BDNF genes on pretreatment with *T. regelii* extract accounting for its neuroprotective actions. Sapkota et al. (2011) reported detoxified extract of *R. verniciflua* prevented the downregulation of BDNF and TH against rotenone-induced toxicity. Ghaffari et al. (2014) observed *H. suaveolens* methanol extract treatment demonstrated enhanced expression of neuronal biomarker genes BDNF and TH. In present study, we observed similar results when the cells were pretreated bikaverin. Bikaverin prevented the decrease in BDNF, TH, and AADC expression levels in SH-SY5Y cells induced by H_2O_2. Thus, bikaverin conferred protection to human dopaminergic cells against H_2O_2-induced oxidative stress by multiple functions contributing to neuroprotection. The potent neuroprotective capacity of bikaverin, shown in these experiments, suggests that bikaverin might be developed as a preventive agent against oxidative stress.

The present study revealed that bikaverin from *F. oxysporum* f. sp. *lycopersici* can attenuate the H_2O_2-induced oxidative stress by improving the antioxidant status, cell viability, mitochondrial membrane integrity, and regulation of gene expression. Our studies have paved the way for future efforts toward the production of semi-synthetic derivatives of the compound or its expression in suitable hosts for antibiotic and anticancer applications. The results demonstrate that bikaverin can be an alternative to some of the toxic synthetic antioxidants, which are used in food and cosmetics. However, further in vivo investigation is required to study the use of this natural compound for pharmaceutical applications to prevent various ROS-mediated neuronal disorders.

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Conflict of interest Authors declare that there is no conflict of interest.

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