INTERNATIONAL JOURNAL
OF
PHARMACEUTICAL SCIENCES
AND
RESEARCH

NEURO-PROTECTIVE PROPERTIES OF *ORTHOSIPHON STAMINUS (BENTH)* LEAF METHANOLIC FRACTION THROUGH ANTIOXIDANT MECHANISMS ON SH-SY5Y CELLS: AN *IN-VITRO* EVALUATION

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ABSTRACT: In present study to evaluate the mechanisms behind the antioxidant and neuro-protective potential of *Orthosiphon staminus* methanol bio active guided fraction (OMF) using SH-SY5Y human neuroblastoma cell culture model was carried out. In *in-vitro* studies, mechanisms of neutralization of H_2O_2 induced toxicity by OMF using MTT, LDH, ROS, MMP, MDA, Comet assay was performed. Regulation of anti-oxidant enzyme levels by western blot analysis and neuronal biomarker genes (BDNF, TH and AADC genes) at genetic level by quantitative RTPCR was performed. Cells pretreated with OMF effectively prevents the H_2O_2 induced anti cell proliferation (cell viability), oxidative stress, genotoxicity and significantly enhance (~ 6 fold, p < 0.01) the expression of antioxidant enzymes and increase in (~ 4 fold, p < 0.01) neuronal biomarker gene expression. Present study demonstrated that, significant neuro-protective activity of *O. staminus* methanol bioactive guided fraction against H_2O_2 induced oxidative stress by antioxidant mechanisms and enhancement of neuronal biomarker gene expression. This study validate its use by folk medicinal practitioners in India. Therefore, OMF of *O. staminus* can serve as a strong candidate for development of herbal neuro-protective compositions.

INTRODUCTION: Even in 21st century after tremendous advancements in modern medicine, protective drugs from neuronal damage stimulate brain function or help to regenerate neuro-glial cells are scanty [1]. Medicinal herbs play a major role in the management of health practice throughout the World [2]. Studies on medicinal herbs and their bioactive compounds has become an important source of drugs due to increasing recognition of herbal medicinal systems [3, 4]. Herbal-derived remedies need a powerful and deep assessment of their pharmacological qualities [5].

Neurological disorders have been linked to elevated levels of oxidative stress and apoptosis. Free radical generation and oxidative stress have been shown to play major role in regulating redox reactions in neurodegeneration. Several reports indicate that oxidative stress plays a key role in modulating the biochemical changes resulting in many metabolic disorders [6]. Oxidative stress leads to many degenerative diseases such as cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, variety of neuronal disorders [7].

Orthosiphon staminus Benth (Lamiaceae) commonly known as "Poonai meesai" is well distributed ornamental plant in South East Asian countries. Several previous studies have demonstrated the effectiveness of the plant related

to phytochemical properties [8] and pharmacological importance [9]. The bioactive compounds including highly oxygenated isopimarane-type diterpenes, orthosiphols A-E were reported in addition to monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene and myoinositol [10]. The polyphenols are the predominant constituents in the plant, which have been reported to be effective in reducing oxidative stress by inhibiting lipid peroxidation in biological systems [11]. Recently, the diuretic activity of methoxy flavonoids isolated from *O.staminus* was reported [8].

The important pharmalogical properties of the plant include diuretic, hypouricemic, renal protective, antioxidant, anti-inflammatory, hepatoprotective, gastroprotective, antihypertensive, antidiabetic, antihyperlipidemic, antimicrobial, and anorexic activities [12]. However cytoprotective and neuro-protective effects of *O. staminus* extracts are not yet investigated. Moreover, the reports related to neuroprotective properties of medicinal plants are scanty. Therefore, the present study was conducted to investigate the cytoprotective and neuroprotective potency of *O. stamineus* against oxidative stress and neuronal damage induced by H_2O_2 in SH-SY5Y cells respectively.

Further, present study reveals the principle behind the beneficial activity of *O. staminus* as an antioxidant and neuro-protective plant, and validates its use by the folk medicinal practitioners in Western Ghats of India.

MATERIALS AND METHODS:
Chemicals:
Glutathione reductase (GR), 1-chloro-2,4-dinitrobenzene (CDNB), oxidised glutathione (GSSG), glutathione (GSH), pyrogallol, thiobarbituric acid (TBA), bovine serum albumin (BSA), 2,4- dinitrophenyl hydrazine (DNPH), tetraethoxypropane, Reduced nicotinamide adenine dinucleotide phosphate (NADPH), Eagle's minimum essential medium (MEM), fetal calf serum (FCS), trypsin (0.1%), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], 2',7'- CFH2DA, rhodamine 123 were obtained from Sigma Chemical Co. (Saint Louis, Missouri, USA). Agarose, trypan blue, dimethyl

sulphoxide (DMSO), Trichloroacetic acid (TCA), 5, 5 dithiobis (2-nitrobenzoic acid) (DTNB), hydrogen peroxide (H_2O_2), RNA isolation kit, Avian reverse transcriptase-single strand cDNA synthesis kit and 2 X fast cyber green master mix were purchased from Quiagen-Gamb (Hilden, Germany). All other chemicals were purchased from Sisco Research Laboratories (Mumbai, India) and Merck (Bangalore, India).

Plant Materials and extraction:
Fresh *O. staminus* leafs were collected from the Western Ghats of India, during August- September 2012 and were authenticated at Acharya Nagarjuna University, Guntur, India. The voucher specimen was deposited in the herbarium at the Department of Pharmacognosy, Chalapathi Institute of Pharmaceutical Sciences. The shade dried leaf material was extracted with methanol using Soxhlet apparatus. The resulted extract was flash evaporated under reduced pressure. The lyophilized powder of the extract was used for further studies.

HPLC analysis of methanolic extract and their antioxidant properties:
Eluted methanolic fraction containing 3 mg/ml extract was used for HPLC analysis. Jasco HPLC system equipped with gradient control with fluorescent detector inbuilt heat devise. The chromatographic separation was carried out using a mobile phase methanol-acetic acid-water (10:2:88, v/v) as solvent A and methanol-acetic acid-water (90:2:8, v/v) as solvent B programmed in gradient.

In-vitro evaluation of neuro-protective property of OMF
Cell culture and treatments:
Human neuroblastoma cell line SH-SY5Y was obtained from National Centre for Cell Science (Pune, India). Cells were grown in 25-cm2 flasks (Falcon, Dickinson, USA) with loosened caps, containing Ham's F12 supplemented with 2 mM L-glutamine and 10% fetal bovine serum at $37^{o}C$ (NuAire, Plymouth, MN, USA) in an atmosphere of humidified 5% CO_2. To examine possible toxic effects, the cells were treated with OMF at concentrations ranging from 0.01 to 1 mg/ml for 24 hr.

Analysis of cell viability using MTT assay:

MTT assay was performed as described previously [13] with minor modifications. Based on the preliminary observations, SH-SY5Y 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 OMF, vehicle and H_2O_2 (100μM) and incubated for 24 hrs. After the incubation time, cells were washed with PBS, followed by the addition of MTT (100 μl;5 mg/ml) to each well. After 4 h of incubation, the MTT solution was removed and 100 μl of DMSO was added. After 10 min, the absorbance was recorded at 540 nm with the help of an ELISA reader (Tecan, Austria).

The data was recorded using the software package Magellan 6.3. The viability (%) was calculated as follows:

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

(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 plate. After a period of 24hrs, the cells were treated with various concentrations of OMF for 2 hr, followed by the treatment of H_2O_2 treatment(100μM) for a period of 24 hrs. The cells were precipitated by centrifugation at 2500 rpm for 5 min at 4°C. The supernatant (100μl) was mixed with 900μl of kit reaction mixture. The total LDH activity was measured by lysis (2% Triton X-100) of untreated cells.

Attenuating effects of OMF on H_2O_2 induced oxidative stress in SH-SY5Ycells:
Effects on ROS generation:
The cells were seeded in 24-well plates at a concentration of 4.0 x 105 cells/ml and treated as mentioned earlier. After treatments, the oxidation-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (5 mg/ml) was added to the cells and incubated for 30 min. Intra cellular production of ROS was measured by fluorometric detection of DCF oxidation on a fluorimeter with an excitation wavelength of 485 nm and emission wavelength of 522 nm. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. Results are expressed as the ratio of DCF-induced H_2O_2 fluorescence/DCF-Induced control fluorescence. Fluorescent intensity percentage was measured using florescent microscope (ZEES).

Effects on Lipid peroxidation (MDA assay):
MDA, the lipid peroxidation product, was measured following the method of Ohkawa et al.[14] with slight modifications. The SH-SY5Y cells were seeded in 75 cm^2 flasks at a concentration of 1.0 x 10^7cells/ml and incubated at 37°C. After confluence is reached, the cells were treated as described earlier. The collected cells were lysed in ice-cold PBS by sonication followed by centrifugation at 12,000 rpm for 5 min at 4°C. The resulting supernatants were used for measuring the levels of malondialdehyde (MDA) by spectro-photometer at 532 nm.

Effects on Mitochondrial Membrane Potential (MMP):
The electrical potential across the inner mitochondrial membrane was measured using the fluorescent dye rhodamine 123 to estimate the mitochondrial health according to the method described by Russell et al.[15] The protective effect of OMF on mitochondrial damage induced by H_2O_2 was determined by measuring the MMP. 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 1h at 37°C. The plates were allowed to wash with PBS and then were collected for fluoroscence detection at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Tecan Plate Reader (Austria).

Single cell gel electrophoresis (Comet assay):
Alkaline comet assay [16] was performed to measure the DNA damage and evaluate the protective effect of OMF against apoptosis induced by H_2O_2. Exponentially growing SH-SY5Y cells were treated with different concentrations of OMF for 2h, washed, and then exposed to H_2O_2 for 24hr.

After treatment, the comet slides were prepared with agarose gel and run electrophoresis. The photographs were taken with fluorescence microscope (Zesis, Germany) equipped with Cool SNAP® Pro color digital camera, 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) x tail % DNA / 100.

Western blot analysis of oxidative biomarkers (SOD, CAT and GPX):

Western blot analysis was followed by previously described method[17] with minor modifications. The cells (1×10^7 cells) were cultured in 75 cm^2 flasks and treated with H_2O_2 with or without pre-treatment of OMF. After treatments, the cells were washed twice with PBS and lysed in ice-cold RIPA buffer with protease and protease inhibitor cocktail. The supernatants were collected from cell lysates and the protein contents were determined by FC method. The proteins (25μg) were resolved on 8–12% SDS-PAGE and electro-blotted onto nitrocellulose membranes.

The membranes were blocked overnight at 4°C with 5% (v/v) non-fat dry milk in phosphate-buffered saline (PBS) and incubated with primary antibodies namely monoclonal anti-SOD, anti-CAT and anti- Gpx at 1:1000 dilution for 2h separately. The membranes were then washed in PBS-T followed by incubation for 1h at room temperature in dark with horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibodies (Sigma, USA) at 1:2000 dilutions. Further washed with PBS-T, the immunoreactivity of the membranes was detected by using the enhanced chemiluminescence peroxidase substrate kit (CPS-160, Sigma, St. Louis, MO, USA). The band intensity was calculated using 'Image-J' software.

Relative quantification of target gene expression by RT-PCR:

SH-SY5Y cells were cultured (1×10^7) in 75cm^2 flasks and treated with 1000 μg of OMF for 12 h. After exposure to OMF, cells were treated with 100 μM of H_2O_2 for 24 h. Total cellular RNA was isolated with a commercial RNA extraction 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 (Quiagen, Germany) containing appropriate primers (**Table 1**) of target neuronal biomarker genes (BDNF, TH and AADC), using a Roche Light Cycler 480.

After initial denaturation (95°C for 10 min), 35 PCR cycles were performed using the following conditions: 95°C, 15s; 60°C, 15s; and 72°C, 20s, at the end of PCR reaction, samples were subjected to a temperature ramp (from 70°C 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 was assayed in replicates and the ΔCT method was used to quantify expression levels based on normalization with β-2 myoglobulin as housekeeping gene. The analysis was performed with Light Cycler and relative quantification software.

TABLE 1: PRIMERS USED FOR REALTIME Q-PCR ANALYSIS OF NEURONAL MARKER GENE EXPRESSION.

Name	Primer sequence (5'-3')	Gene target	Tm (^{o}C)
BDN-F	ATGACCATCCTTTTCCTTACT	Brain derived Neurotrophic factor	56
BDN-R	GCCACCTTGTCCTCGGAT		
Th-F	GAGGAGAAGGAGGGGAAG	Tyrosine Hydroxylase	58
Th-R	ACTCAAACACCTTCACAGCT		
AAD-F	AACAAAGTGAATGAAGCTCTTC	Amino Acid Decarboxylase	58
AAD-R	GCTCTTTGATGTGTTCCCAG		
B2m-F	ACAGGTTGCTCCACAGGTA	B-2 Myoglobulin	57
B2m-R	GAGTGCAAGAGATTGAAGAG		

Statistical analysis:

Data was expressed as mean ± standard deviation (std) of minimum of six independent experiments. Statistical differences between control and target groups for all experiments were determined using Student's t-test. The comparison between the groups were considered significant if $p \leq 0.05$.

RESULTS:

Orthosiphon staminus **OMF prevents H_2O_2 induced oxidative stress and LDH leakage:**

Exposure of SH-SY5Y cells to various concentrations of OMF (50-2000 µg) alone for 24h did not alter the cell viability. However, exposure of cells to 100 µM H_2O_2 induced significant oxidative stress and cell death. The cell viability was almost half of control after 24h exposure (50.4 ± 8.4, **Fig. 1A**). Following pretreatment of cells with various concentrations of OMF, exposure to H_2O_2 did not drastically effect cell viability. As demonstrated in figure.1B, OMF pretreatment dose dependently prevents cell death due to H_2O_2 treatment (**Fig.1B**).

OMF at 1000 µg almost neutralized H_2O_2 induced oxidative stress (95.3 ± 3.6, p< 0.01). Moreover, at 2000 µg H_2O_2 induced stress was completely neutralized (99.6 ± 2.6, p< 0.01) demonstrating the effectiveness of OMF in preventing oxidative stress to SH-SY5Ycells (**Fig.1B**).

LDH release is an indirect measure of dead cells. To further investigate the protective effect of OMF, the release of LDH was measured in presence and absence of H_2O_2 (**Fig.1C**). When SH-SY5Y cells were treated with 1mg OMF, the levels of LDH remained identical to control indicating non-toxic nature of OMF. However, upon exposure to 100 µM H_2O_2 for 2h, the cell supernatant contained 9 fold increased LDH compared to control (4.0 ± 0.4 vs. 36.4 ± 1.4, p< 0.01) indicating the cytotoxicity of H_2O_2. On the contrary, cells pretreated with 50-1000µg of *O. staminus* OMF demonstrated decreased amounts of LDH leakage signifying dose dependent protective effect of OMF against H_2O_2 induced cytotoxicity (**Fig.1C**). Extract at 500µg completely prevented H_2O_2 induced LDH outflow (4.8 ± 0.4, p< 0.01).
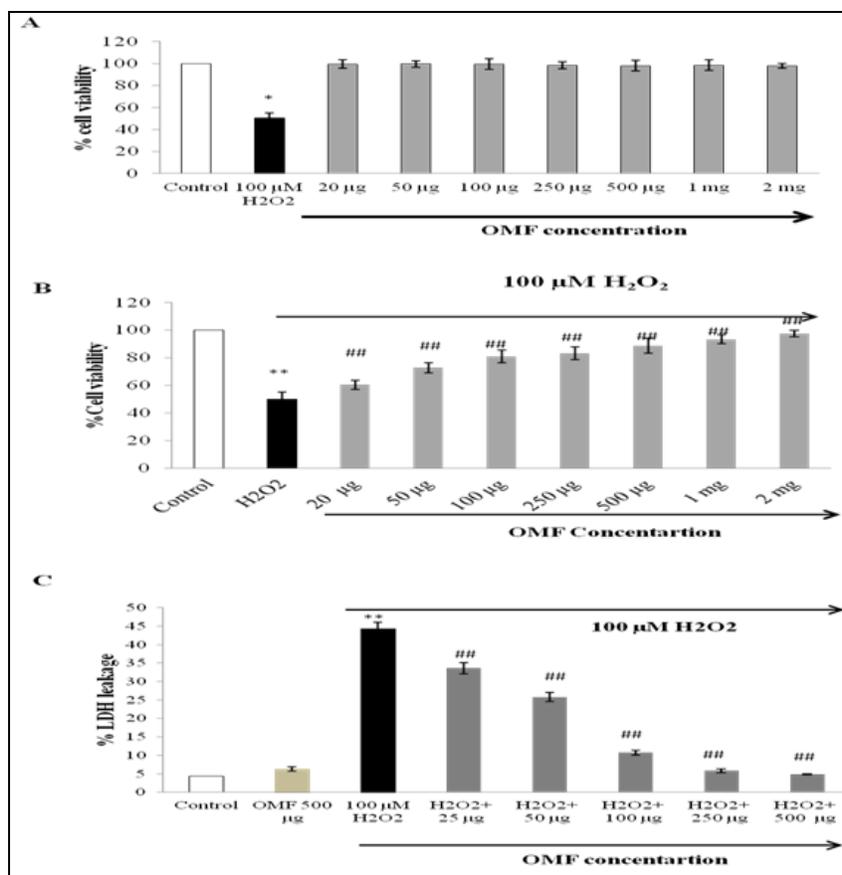
FIG.1: EFFECTS OF OMF ON CELL PROLIFERATION AND H_2O_2 INDUCED ANTI CELL PROLIFERATION AND LDH LEAKAGE IN SH-SY5Y CELLS

OMF of *O. staminus* inhibits H_2O_2 induced ROS generation:

The exposure of SH-SY5Y cells to 100μM H_2O_2 elicited 2.2 fold increase in ROS production as compared to control group. ROS generation was attenuated significantly when cells were pre-treated with OMF followed by 100μM H_2O_2 treatment for indicated time periods. The fluorescence intensity decreased to 1.3 folds indicating the potent antioxidant activity of OMF which was further confirmed by fluorescence imaging (**Figure. 2A, Figure. 2B**).
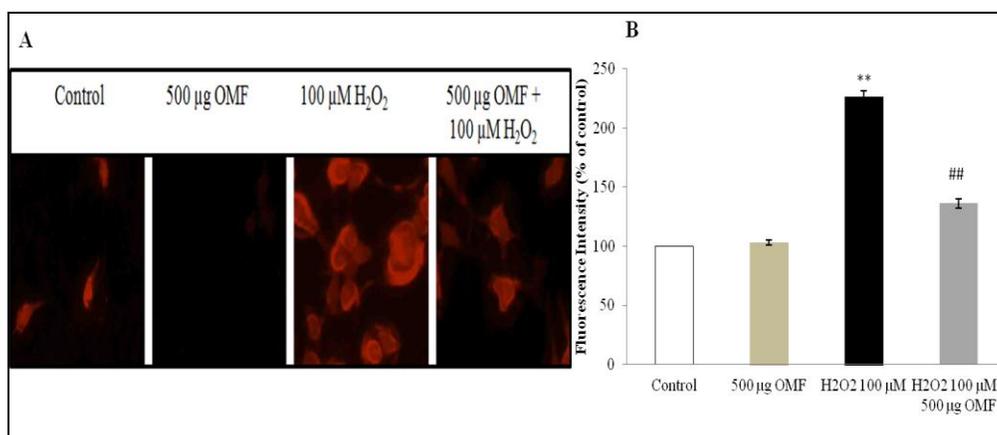
FIG: 2. EFFECTS OF OMF ON H_2O_2 INDUCED ROS GENERATION IN SH-SY5Y CELLS.

Effects of OMF on H_2O_2 induced reduction of the MMP:

To examine whether H_2O_2 induced apoptosis and its protection by OMF involve MMP pathway, its measurement was carried out using rhodamine 123 and results are presented in **Fig.3A, Fig.3B**.

Here, we observed 27 % decrease in MMP with 100μM H_2O_2 challenge which indicates the depolarization of mitochondrial membrane. However, the cells pre-treated with OMF prior to the addition of H_2O_2, showed a significant recovery in the florescence intensity to an extent of control.
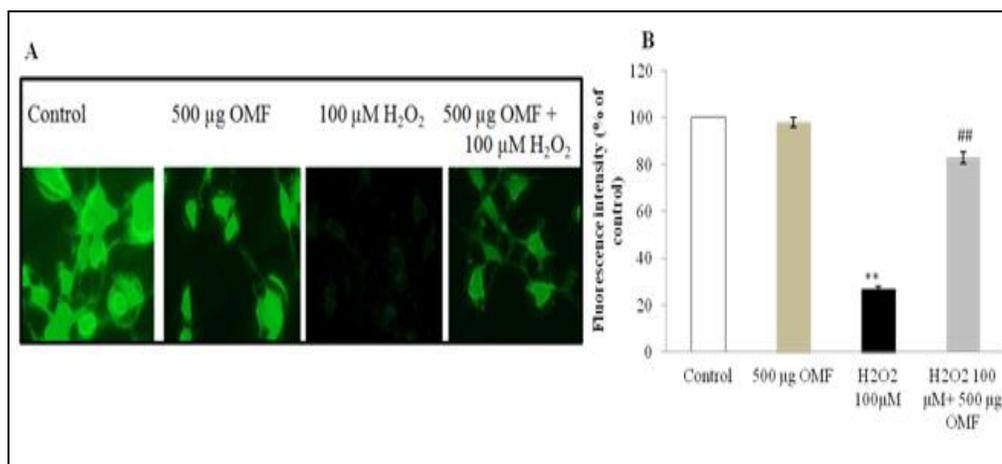
FIG. 3: EFFECTS OF OMF ON H_2O_2 INDUCED MITOCHONDRIAL MEMBRANE INTEGRITY (MMP) IN SH-SY5Y CELLS.

Affects of OMF on H_2O_2 induced genotoxicity in SH-SY5Y cells (Comet assay):

Comet assay was performed to determine the genotoxicity of a substance due to single strand breaks of DNA. In order to determine the protective potential of OMF on the H_2O_2-induced DNA damage, cells were treated to various concentrations of OMF for 2h, followed by exposure to H_2O_2 (100μM) for 24h (**Fig.4A**). When SH-SY5Y cells were treated with 1mg OMF, the Olive tail moment (OTM) used as the parameter to reflect DNA damage remained same as control cells treated with vehicle. Whereas, exposure to 100μM H_2O_2 for 24h, the OTM enhanced by 4 fold (25.4 ± 2.34 vs. 6.23 ± 0.46, $p< 0.01$) compared to control demonstrating the extent of DNA damage

(**Fig.4B**). On the converse, reduced OTM was observed after treatment of cells with 100-1000µg OMF of *O. staminus* followed by H_2O_2. As shown in **Fig.4**, after 1000µg MAF treatment, the OTM remained significantly less than H_2O_2 group (9.6 ± 0.9) but not significantly different from control.
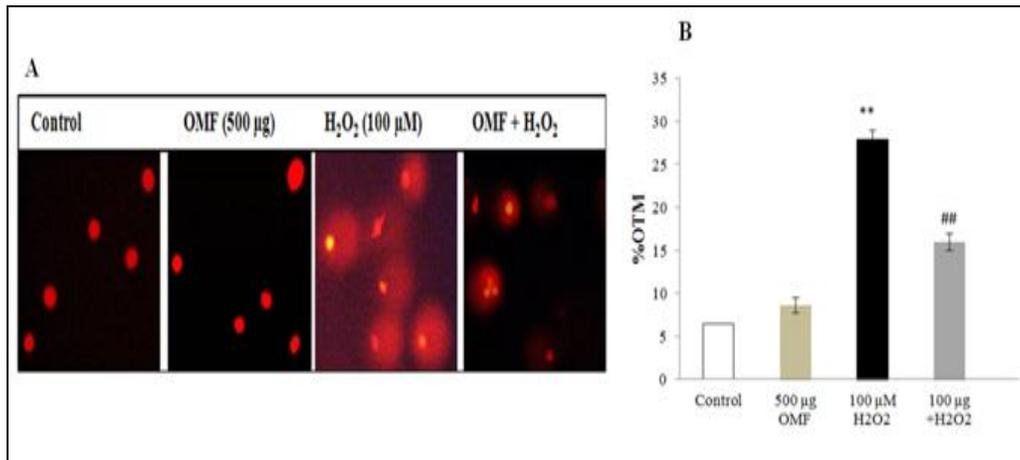
FIG. 4: DNA DAMAGE IN SH-SY5Y CELLS TREATED WITH VARIOUS CONCENTRATIONS OF OMF AND H_2O_2 USING ALKALINE COMET ASSAY.

OMF of *O. staminus* stimulates enhanced expression of genes for antioxidant enzymes in SH-SY5Y cells:

The gene response of the CAT, SOD and GPx were monitored by western blot analysis (**Fig.5**). Cells treated with 1000µg of OMF alone was not observed significant change in expression of CAT, SOD and GPx genes respectively (p< 0.05). However, treatment with 100µM H_2O_2 alone resulted in decreased expression of the same genes. However, cells pretreated with 100µg of OMF following exposure to 100µM H_2O_2 demonstrated very significant increase in CAT, SOD and GPx genes respectively (p< 0.01) (**Fig.5**).
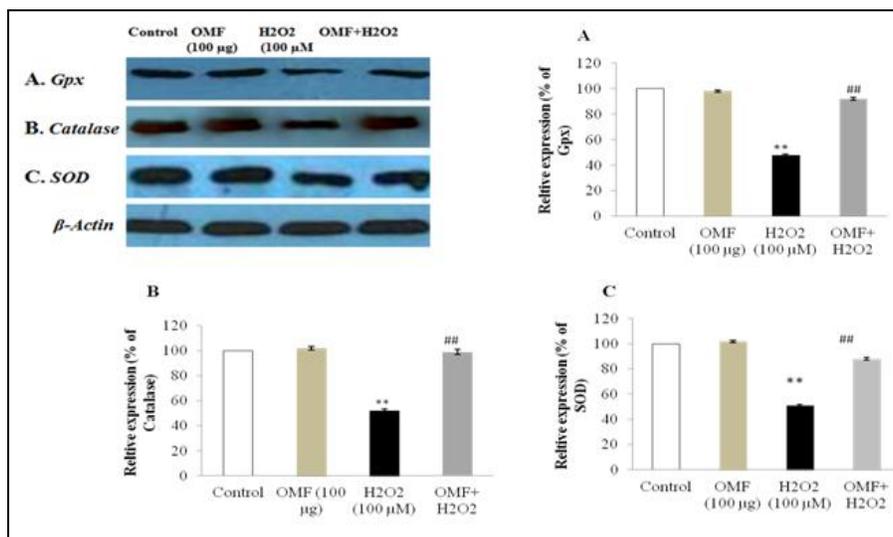
FIG. 5: EFFECTS OF OMF ON H_2O_2 INDUCED ANTIOXIDANT ENZYME EXPRESSION (WESTERN BLOT ANALYSIS).

Affects of OMF on neuronal biomarker gene expression in SH-SY5Y cells:

The gene response of the BDNF, TH and AADC were monitored by quantitative real time RT-PCR (**Fig.6**). Cells treated with 1000 µg of OMF alone displayed 3.7, 2.5 and 1.2- fold increase in expression of BDNF, TH and AADC genes respectively (p< 0.05). However, treatment with 100µM H_2O_2 alone resulted in decreased expression of the same genes. Surprisingly, SH-SY5Y cells pretreated with 100 µg of OMF

following exposure to 100 µM H_2O_2 demonstrated very significant increase in 5.6, 6.3 and 4.3-fold in BDNF, TH and AADC genes respectively (p< 0.01) (**Fig.6**).
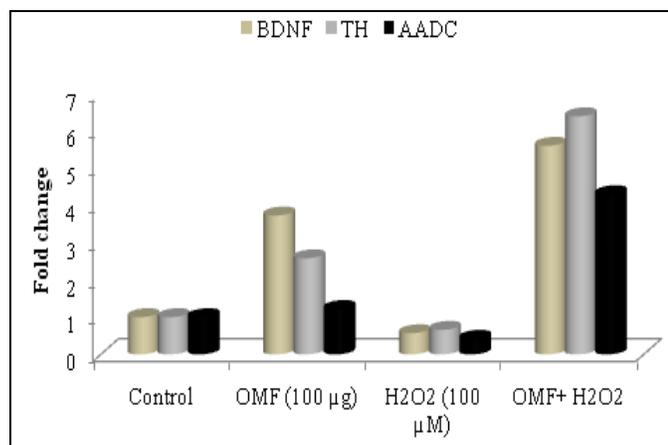
FIG.6: REAL-TIME PCR FOR QUANTIFICATION OF NEURONAL MARKER EXPRESSION LEVELS FOLLOWING EXPOSURE TO OMF, OMF ± H_2O_2.

DISCUSSION: Since ancient times, several diseases are being treated by using herbal extracts. Scientific investigations of these traditionally used herbs are valuable as a source of potential chemotherapeutic drugs as well as a measure of safety for their continuous use. 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 [18]. Scientific reports on *O. staminus* depicts that it is a popular herb among the various ethnic groups, Ayurvedic and folk medicinal practitioners for treatment of various disorders [4, 8, 9, 19]. Thus, the principal objective of this study was to explore the protective effects of OMF of *O. staminus*, against oxidative stress-induced injury in SH-SY5Y cells and to elucidate some potential protection mechanism.

In the present investigation, we observed that the cytotoxic effects of H_2O_2 as well as protective effect OMF of *O. staminus* by MTT and LDH assays which were further supported by the morphological observation. The cytoprotective effect of *O. staminus* against H_2O_2 was confirmed by MTT and LDH assays. The previous works of Abdelwahab et al [20] showed the results of H_2O_2 induced SH-SY5Y cytotoxicity which were in line with Kumar and Khannum [17] who has reported inhibitory activity of *Cyperus rotendus* against

H_2O_2 induced cell injury. In the evaluation of neuronal cytotoxicity induced by H_2O_2 in SH-SY5Y cells, cell death was observed in dose dependent manner. Protective effect of OMF 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 OMF showed lower LDH leakage when compared to the H_2O_2 exposed cell group, demonstrates its protective effect against H_2O_2 induced oxidative stress. The protective effect exerted by OMF on H_2O_2 induced cyototoxicity determined by the MTT assay correlated with the LDH assay. Thus, the evidence indicates SH-SY5Y cells die as the result of apoptosis after H_2O_2 insult, and pretreatment with OMF attenuates the H_2O_2 induced neuronal cell damage.

H_2O_2 is 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[21]. The generation of reactive oxygen species (ROS) was quantified spectrofluorimetrically using the oxidant-sensing fluorescent probe DCFHDA. 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 [22]. However, we observed in the cells pretreated with OMF ROS inhibitory effect even after H_2O_2 treatment.

Flavonoids are known to have powerful antioxidant properties, which are generally attributed to the presence of phenolic hydroxyl groups. Previous reports of Akowuah et al[19] indicated that the extracts of *O. staminus* are known to be free radical inhibitors and primary antioxidants. This is because of the presence of several bioactive phytocompounds in the extracts.

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 [23]. 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 [24].

In present study, we observed that the cells treated with H_2O_2 decreased the integrity of mitochondrial membrane, however cells pretreated with OMF prevented the loss of mitochondrial membrane integrity (**Fig 3**).

Apoptotic cell death is characterized by morphological changes, including cell shrinkage, condensation of nuclei [25] and biochemicals such as activation of caspase, nuclease and the inactivation of nuclear repair polymerases, thereby resulting in the degradation of chromosomal DNA and cell death[26]. The comet assay (SCGE- Single Cell Gel Electrophoresis) was performed to assess the protective effect of OMF on H_2O_2 induced DNA damage in SH-SY5Y cells. The assay detects a broad spectrum of primary DNA lesions, including single strand breaks and oxidative base damage [27].

Our Comet assay results exhibits that OMF decreased H_2O_2 induced genotoxicity in SH-SY5Y cells (**Fig 4**). Using this assay we demonstrated that Olive tail moment (OTM) used as the parameter to reflect DNA damage remained same as control cells treated with vehicle following treatment with OMF indicating the magnitude of protection that is offered following treatment with OMF. The observations of kumar et al.[28] on H_2O_2 induced effect on PC12 cells and protective effect of herbal extracts like *Terminalia arjuna* was supporting present study.

Oxidative stress results due to an imbalance of cellular oxidants and antioxidants. Enzymes, such as superoxide dismutase (SOD), catalase (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 [29].

In the present study, the effect of OMF of *O. staminus* pretreatment on the levels of SOD, CAT and Gpx were analyzed by western blotting analysis. The SH-SY5Y cells when treated with 100µM of H_2O_2 resulted in lower levels of the SOD, CAT and GPx enzymes. However, SH-SY5Ycells pretreated with OMF stimulates enhanced expression of genes for antioxidant enzymes such as SOD, CAT and GPx (**Fig 5**). The decrement of ROS may be associated with increased expression and activities of Glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT).

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 [30, 31]. 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 dopamenergic neurons. Schapira [32] has reported decreased expression of TH and AADC with simultaneous depletions of catecholamines in Parkinson's disease.

In the present study, observed that, the treatment of SH-SY5Y cells with OMF enhanced the expression neuronal biomarker genes viz, BDNF, TH and AADC, which are, monitored by real time-Q-PCR analysis (**Fig 6**). Our findings are in line with the previous studies of Park et al [33] who reported that *rosmarinus* extract is a potent inducer of TH and AADC against H_2O_2 stress in SH-SY5Y cells and Cho et al [34], who reported the expression of TH and BDNF gens with treatment of *Tripterygium* extract which has been lowered with H_2O_2 radical stress. Therefore, OMF can help in protect SH-SY5Y cells through different mechanisms and useful in preparation of herbal based medicines with further investigation.

CONCLUSION: The present study revealed OMF of *O. staminus* can attenuate the H_2O_2 induced oxidative stress by improving the antioxidant status, cell viability, ROS formation, mitochondrial

membrane integrity and regulation of gene expression. Our studies have paved the way for future efforts towards the production of semi-synthetic derivatives of the compound or its expression in suitable hosts for antibiotic and anticancer applications. The results demonstrate that OMF of *O. staminus* 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 plant extract for pharmaceutical applications to prevent various ROS mediated neuronal disorders.

REFERENCES:

1. Chattopadhyay RR: Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: Part II. Journal of ethnopharmacology 2003; 89: 217-219.
2. Stickel F and Schuppan D: Herbal medicine in the treatment of liver diseases. Digestive and liver disease 2007; 39: 293-304.
3. Cooper EL: Drug discovery, CAM and natural products. Evidence-Based Complementary and Alternative Medicine 2005; 13: 215–217.
4. Firenzuoli F and Gori L: Herbal medicine today: clinical and research issues. Evidence-based Complementary and Alternative Medicine 2007; 4: 37–40.
5. Rabe T and Van Staden J: Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 1997; 56: 81–87.
6. Uttara B, Singh AV, Zamboni P and Mahajan RT: Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology 2009; 7: 65-74.
7. Hensley K, Robinson KA, Gabbita, SP, Salsman S and Floyd RA: Reactive oxygen species, cell signaling, and cell injury. Free radical biology & medicine 2000; 28: 1456-1462.
8. Adam Y, Somchit MN and Sulaiman MR: Diuretic properties of *Orthosiphon stamineus Benth*. Journal of Ethnopharmacology 2009; 124: 154–158.
9. Akowuah GA and Zhari I: Determination of betulinic acid from *Orthosiphon stamineus* leaf extract in rat plasma. Pharmazie 2008; 63: 788–790.
10. Olah NK, Hanganu D, Oprean R, Mogosan C, Dubei N and Gocan S: Selective extraction of caffeic acid derivatives from *Orthosiphon stamineus Benth*. (Lamiaceae) leaves. Journal of Planar Chromatography-Modern TLC 2004; 17: 18–21.
11. Hollman PCH and Katan MB: Dietary flavonoids: intake, health effects and bioavailability. Food and Chemical Toxicology 1999; 37: 937–942.
12. Ameer OZ, Salman IM, Asmawi MZ, Ibraheem ZO and Yam MF: *Orthosiphon stamineus*: traditional uses, phytochemistry, pharmacology, and toxicology. Journal of Medicinal Food 2012; 15: 678-90.
13. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods 1983; 65: 55-63.
14. Ohkawa H, Ohishi N and Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituricacid reaction. Analytical Biochemistry 1979; 95: 351–8.
15. Scaduto RC and Grotyohann LW: Measurement of Mitochondrial Membrane Potential Using Fluorescent Rhodamine Derivatives. Biophysical Journal 1999; 76: 469–477.
16. Singh NP, McCoy MT, Tice RR and Schneider EL: A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 1988; 175:184–191.
17. Kumar KH and Khanum F: Hydroalcoholic extract of *Cyperus rotundus* ameliorates H2O2 induced human neuronal cell damage via its anti-oxidative and anti-apoptotic machinery. Cellular and Molecular Neurobiology 2013; 33: 5-17.
18. Kelly GSND: Clinical Applications of N-acetylcysteine. Alternative Medicine Review 1998; 3: 114-127.
19. Akowuah GA, Zhari I, Norhayati I and Sadikun A: Radical scavenging activity of methanol leaf extracts of *Orthosiphon stamineus*. Pharmaceutical Biology 2004; 42: 629 - 635.
20. Abdelwahab SIA, Syam M, Manal ME, Nabil AM, Abdelbasit AM, Ahmad BA, Mahmood AA and Khalid MA: Antiapoptotic and Antioxidant Properties of *Orthosiphon stamineus* Benth (Cat'sWhiskers): Intervention in the Bcl-2-Mediated Apoptotic Pathway. Evidence-Based Complementary and Alternative Medicine 2012; 156765: 1-11.
21. Denisova NA, Cantuti-Castelvetri I, Haasan WN, Paulson KE and Joseph JA: Role of membrane lipids in regulation of vulnerability to oxidative stress in PC12 cells: Implication for aging. Free Radical Biology and Medicine 2001; 30: 671–678.
22. Marchesi E, Rota C, Fann YC, Chignell CF and Mason RP: Photoreduction of the fluorescent dye 2',7'-dichlorofluorescein: a spin trapping and direct electron spin resonance study with implications for oxidative stress measurements. Free Radical Biology and Medicine 1999; 26: 148–161.
23. Joshi DC and Bakowska JC: Determination of Mitochondrial Membrane Potential and Reactive Oxygen Species in Live Rat Cortical Neurons. Journal of Visualized Experiments 2011; 51: 2704.
24. Baraccaa A, Gianluca S, Giancarlo S and Giorgio L: Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F0 during ATP synthesis. Biochimica et Biophysica Acta 2003; 1606: 137– 146.
25. Fernandez SE, Canizares FJ, Cubero MA, Warley A and Campos A: Changes in elemental content during apoptotic cell death studied by electron probe X-ray microanalysis. Experimental Cell Research 1999; 253: 454–462.
26. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S: A caspaseactivated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998; 391: 43–50.
27. Susanne BS, Andreas H, Stefan P and Gunter S: The *in vivo* comet assay: use and status in genotoxicity testing. Mutagenesis 2005; 20: 245-254.
28. Kumar GP, Navya K, Ramya EM, Venkataramana M, Anand T and Anilakumar KR: DNA damage protecting and free radical scavenging properties of *Terminalia arjuna* bark in PC-12 cells and plasmid DNA. Free Radicals and Antioxidants 2013; 3: 35-39.
29. Gilgun-Sherki Y, Melamed E and Offen D: Oxidative stress induced-neurodegenerative diseases: the need for

antioxidants that penetrate the blood brain barrier. Neuropharmacology 2001; 40: 959-975.

30. Chen BY, Wang X, Wang ZY, Wang YZ, Chen LW and Luo ZJ: Brain-derived neurotrophic factor stimulates proliferation and differentiation of neural stem cells, possibly by triggering the Wnt/β-catenin signaling pathway. Journal of Neuroscience Research 2013; 91: 30-41.

31. Karasawa N, Hayashi M, Yamada K, Nagatsu I, Iwasa M, Takeuchi T, Uematsu M, Watanabe K and Onozuka M: Tyrosine Hydroxylase (TH)- and Aromatic-L-Amino Acid Decarboxylase (AADC)-Immunoreactive Neurons of the Common Marmoset (*Callithrix jacchus*) Brain: An Immunohistochemical Analysis. Acta Histochemica et Cytochemica 2007; 40: 83–92.

32. Schapira AHV: Science, medicine, and the future: Parkinson's disease. British Medical Journal 1999; 318: 311–314.

33. Park SE, Kim S, Kumar S and Kim SJ: Neuroprotective Effect of Rosmarinus *officinalis* Extract on Human Dopaminergic Cell line, SH-SY5Y. Cellular and Molecular Neurobiology 2010; 30: 759-767.

34. Cho ES, Jang YJ, Hwang MK, Kang NJ, Lee KW and Lee HJ: Attenuation of oxidative neuronal cell death by coffee phenolic phytochemicals. Mutation Research. 2009; 661: 18–24.