

Phyllanthus niruri Protects against Fe²⁺ and SNP Induced Oxidative Damage in Mitochondrial Enriched Fractions of Rats Brain

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Abstract—The potential neuroprotective effect of *Phyllanthus niruri* against Fe²⁺ and sodium nitroprusside (SNP) induced oxidative stress in mitochondria of rats brain was evaluated. Cellular viability was assessed by MTT reduction, reactive oxygen species (ROS) generation was measured using the probe 2,7-dichlorofluoresce diacetate (DCFH-DA). Glutathione content was measured using dithionitrobenzoic acid (DTNB). Fe²⁺ (10μM) and SNP (5μM) significantly decreased mitochondrial activity, assessed by MTT reduction assay, in a dose-dependent manner, this occurred in parallel with increased glutathione oxidation, ROS production and lipid peroxidation end-products (thiobarbituric acid reactive substances, TBARS). The co-incubation with methanolic extract of *Phyllanthus niruri* (10-200 μg/ml) reduced the disruption of mitochondrial activity, glutathione oxidation, ROS production as well as the increase in TBARS levels caused by both Fe²⁺ and SNP in a dose dependent manner. HPLC analysis of the extract revealed the presence of gallic acid (20.54±0.01), caffeic acid (7.93±0.02), rutin (25.31±0.05), quercetin (31.28±0.03) and kaempferol (14.36±0.01). This result suggests that these phytochemicals account for the protective actions of *P. niruri* against Fe²⁺ and SNP -induced oxidative stress. Our results show that *P. niruri* consist important bioactive molecules in the search for an improved therapy against the deleterious effects of Fe²⁺, an intrinsic producer of reactive oxygen species (ROS), that leads to neuronal oxidative stress and neurodegeneration.

Keywords—*Phyllanthus niruri*, mitochondria, antioxidant, oxidative stress, synaptosome.

I. INTRODUCTION

REACTIVE oxygen and reactive nitrogen species production is a normal physiological event essential for the functioning of the nervous system in healthy individuals [1]. The mitochondrion has been identified as a major source of ROS, and its dysfunction with time appears to contribute to neural decay and ageing. Oxidative stress results from a misbalance between ROS generation and antioxidant defenses. The generation of reactive oxygen species (ROS) leads to oxidative damage that is involved in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [2].

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Iron participates directly as a donor or acceptor in electron transfer reactions, making it an essential trace element for cell function. This property makes iron the most common cofactor within the oxygen handling biological machinery [3], [4]. Additional roles for iron have been described in neurons, such as its participation in myelin synthesis [5] and neurotransmitter metabolism [6]. However, excessive iron accumulation in vital organs and neurons is associated with an overproduction of ROS which can lead to the loss of function of those organs [7]. Iron deregulation and oxidative stress have been linked to neurodegenerative diseases [8].

Antioxidants are our crucial defense against free radical induced damage, and are critical for maintaining optimum health and wellbeing. Exogenous consumption of antioxidants especially from plant sources has proved beneficial to human health and effective to reduce the incidence of free radical induced diseases [9].

Phyllanthus niruri is an annual shrub that grows mostly on undisturbed environment and is often regarded as weeds. It is distributed throughout the tropical and subtropical areas [10]. It is widely distributed across India and West African countries like Sierra Leone, Ivory Coast, Ghana and Nigeria [11]. In Nigeria, It is locally called "Eyin-Olobe" by the Yorubas (South West). *P. niruri* has attracted the attention of researchers because of its hepato-protective properties especially its efficacy in viral Hepatitis B. It is known for its liver healing properties and is commonly used in Chinese medicine for treatment of liver diseases [12]. The plant is of medicinal importance for numerous ailments like dysentery, influenza, vaginitis, tumors, diabetes, diuretics, jaundice, kidney stone, dyspepsia, anti-hepatotoxic, anti-hepatitis-B, anti-hyperglycemic and also as antiviral and antibacterial [13]. However, there is no report in the literature on its potential effect on mitochondria, hence, this study was conducted to investigate the neuroprotective effect of *P. niruri* on Fe²⁺ and SNP induced oxidative stress in mitochondria enriched fraction of rats brain.

II. MATERIAL AND METHODS

A. Chemicals

Reduced glutathione (GSH), malonaldehydebis-(dimethyl acetal) (MDA), 5,5'-dithiobis (2-nitrobenzoic acid (DTNB), methylthi-azolyldiphenyl-tetrazolium bromide (MTT), thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS) were

purchased from Sigma-Aldrich (USA). All other chemicals used in this study were of the highest analytical grade.

B. Plant Material

The whole plant of *P. niruri* was collected from the premises of Adekunle Ajasin University, Akungba Akoko. They were identified and authenticated at the herbarium of Plant Science and Forestry department, University of Ado Ekiti, Nigeria (voucher specimen number UHAE: 301). The leaves were separated from the whole plant and dried at room temperature (23–26°C) in the laboratory for three weeks and pulverized into fine powder using the Laboratory Hammer-mill at the Department of Agronomy, Faculty of Agriculture, University of Ibadan, Oyo State, Nigeria. The pulverized plant sample was then kept in air-tight and water-proof containers prior to use.

C. Extraction Process

The pulverized leaves of *P. niruri* (200g) were soaked at room temperature in 70 % ethanol for 72 h, after which the ethanolic extract was filtered and the solvent was fully evaporated under reduced pressure.

D. Animals

Male Wistar rats, weighing 270–320 g from our own breeding colony (Animal House-holding, UFSM, Brazil) were kept in cages with free access to food and water in a room with controlled temperature (22±3°C) and in 12 h light/dark cycle. The protocol of this study has been approved by the Brazilian Association for Laboratory animal Science (COBEA) of the Federal University of Santa Maria.

E. Preparation of Mitochondria Enriched Fraction

Rat brain mitochondrial enriched fractions were prepared as described previously [14]. Briefly, adult (8-10 weeks) male Wistar rats were sacrificed by decapitation. The whole brain (minus the cerebellum) was removed and homogenized on ice in 15 volumes of isolation medium (10 mM HEPES buffer, pH 7.0, containing 220 mM mannitol, 68 mM sucrose, 10 mM KCl, and 0.1% serum albumin) and the homogenate was centrifuged at 4°C for 10 min at 1000 g. The supernatant was then centrifuged at 17,500g for 20 min at 4°C, providing a myelin-rich supernatant and a pellet (P2) consisting of synaptosomes and free (extra-synaptosomal) mitochondria. The supernatant was discarded, and the pellet was suspended in the isolation medium without albumin. The samples were kept on ice until the experiments were performed, usually within 10-15 min.

F. Treatment

P2 (2 mg protein) was incubated with or without (control) different concentrations of *P. niruri* (10-200 µg/ml) in the presence or absence of the pro-oxidant (i.e. Fe²⁺ (10µM) and SNP (5µM)) for 30 min at 25 °C in an incubation medium containing in mM: 10 HEPES buffer, 220 mannitol, 68 sucrose, and 10 KCl, pH 7.0 (total incubation volume = 300 µL). After incubation, cell viability, non-protein thiol (NPSH),

total thiol content, lipid peroxidation (TBARS) and ROS production were determined.

G. Assessment of Cell Viability or Mitochondrial Activity

Mitochondrial activity was assessed by the conversion of the MTT dye to formazan insoluble salt described by [15]. This assay is based on the ability of mitochondrial enzymes to metabolize MTT into formazan, a reaction that takes place only in functional mitochondria. Briefly, after incubation with iron or SNP, 300 µL of MTT (0.5mg/ml) was added to the medium and the mixture was incubated for an additional 30 min period (at 25°C). The purple formazan crystals were pelleted by centrifugation at 10,000 rpm for 5 min, and the supernatant was discarded. The pellets were dissolved in DMSO and the absorbance was measured spectrophotometrically using microplate reader (SpectraMax, USA) at 550 nm. Data are reported as percentage of control.

H. Assessment of Non-Protein Thiol (NPSH) and Total Thiol (T-SH) Content

After treatment of P2 with different concentrations of *P. niruri* (10-200 µg/ml) in the presence or absence of Fe²⁺ (10µM) or SNP (5µM), 300 µL of 10% trichloroacetic acid was added to the samples. After centrifugation (4000 g 4°C for 10 min), the protein pellet was used for total thiol determination (T-SH), whereas, the free thiol groups (NPSH) were determined in the clear supernatant (which was neutralized with 0.1 M NaOH) by the method described by [16]. The results were corrected with protein content and expressed in percentage.

I. Assessment of Lipid Peroxidation

The lipid peroxidation end-products were determined by the TBARS assay originally described by [17]. After 30 min of incubation at 25°C as described above, samples were incubated with 0.45 M acetic acid/HCl buffer, pH 3.4, 1.2% SDS, 0.28% thiobarbituric acid, at 95°C for 60 min. The color reaction developed was measured at 532 nm using microplate reader (SpectraMax, USA). Malondialdehyde (0 to 3nmol/mL) was used for the curve and the protein concentration was determined by the method of [18] using bovine serum albumin as standard.

J. Estimation of ROS

ROS production in isolated synaptosomal P2 fraction was measured using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence probe as described by [7]. Briefly, after treating P2 (2 mg protein) with or without *P. niruri* (10-200 µg/ml), in the presence or absence of Fe²⁺ (10µM) or SNP (5µM), the samples were centrifuged at 4000 rpm for 10 min. The supernatant were removed and 10 µL of DCFH-DA (1µM final concentration) was added to the medium and incubated in the dark for additional 10 min. The formation of the oxidized fluorescent derivative (DCF) was measured in the supernatant as the result of reactive oxygen/nitrogen species (ROS/RNS) generation using a spectrofluorimeter (Shimadzu RF-5301) with excitation and emission wavelengths of 488 and 525 nm respectively and with slit widths of 1.5 nm.

K. Statistical Analysis

The results are expressed as mean ± S.E.M (standard error of mean) of 3-4 independent determinations. Statistically significant differences among groups were analyzed by one-way ANOVA followed by the Duncan multiple range test where appropriate. Differences were considered to be statistically significant when $p < 0.05$.

III. RESULTS

A. Protective Effect of P. niruri against Fe²⁺ and SNP - Induced Cell Death

P. niruri at different concentrations tested did not have any effect on cellular viability evaluated by MTT reduction (Fig. 1 A). However, exposure of mitochondria to Fe²⁺ (Fig. 1B) and SNP (Fig. 1 C) resulted in a significant decrease in MTT reduction when compared to their respective controls ($p < 0.05$, Figs. 1 B and C). Co-treatment for 30 min with *P. niruri* (10–200 µg/mL) mitigated the neurotoxicity of Fe²⁺ (Fig. 1 B, $p < 0.05$) and SNP (Fig. 2C, $p < 0.05$) and restored the cellular viability to control values at concentration 100 µg/mL ($p > 0.05$) (Figs. 1 B and C).

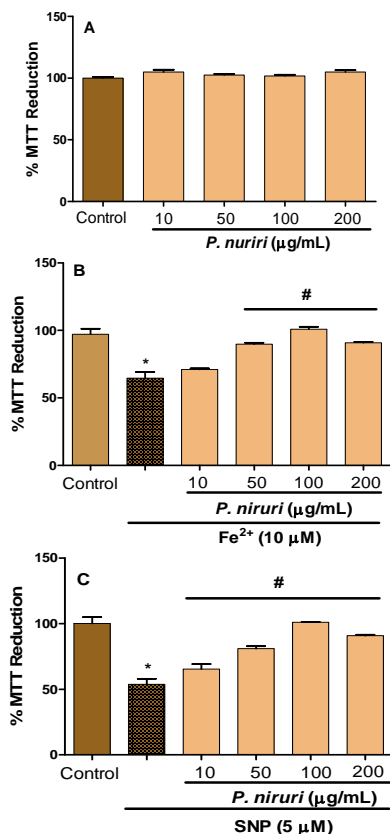


Fig. 1 Effect of *P. niruri* on mitochondrial activity of rats brain (A), co-treatment with Fe²⁺ (B) and SNP (C). Columns represent mean ± S.E.M. of three independent experiments. * $p < 0.05$ compared to control, # $p < 0.05$ compared to Fe²⁺ and SNP treated mitochondria

B. Effects of P. niruri on TBARS Production Induced by Fe²⁺ and SNP

Incubation of brain mitochondria with Fe²⁺ (Fig. 2 B) and SNP (Fig. 2 C) for 30 mins caused marked increase in TBARS production in mitochondria when compared to their respective controls. Co-treatment of mitochondria with *P. niruri* extract (100 and 200 µg/mL) prevented lipid peroxidation induced by Fe²⁺ and SNP (Fig. 2 B and C, $p < 0.05$).

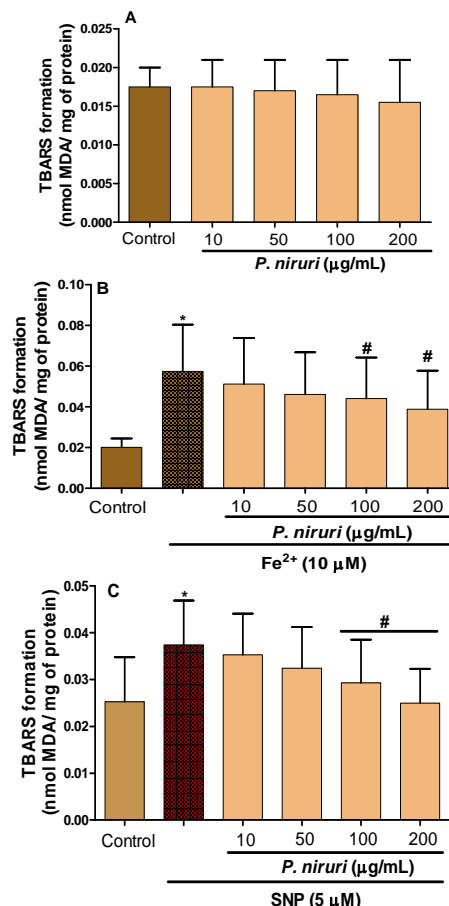


Fig. 2 Effect of *P. niruri* on lipid peroxidation production in mitochondrial of rats brain (A), co-treatment with Fe²⁺ (B) and SNP (C). Columns represent mean ± S.E.M. of three independent experiments. * $p < 0.05$ compared to control, # $p < 0.05$ compared to Fe²⁺ and SNP treated mitochondria

C. Effect of P. niruri Extract on ROS Production in Mitochondria

Under basal conditions, *P. niruri* did not decreased DCFH oxidation indicating that it did not cause significant ROS production when compared to control ($p < 0.05$; Fig. 3 A). Exposure of mitochondria to Fe²⁺ (Fig. 3 B) and SNP (Fig. 3 C) for 30 mins caused a significant increase in DCF fluorescence in the incubation medium when compared to control medium ($p < 0.05$; Figs. 3 B and C). Co-treatment with *P. niruri* (200 µg/ml) for 30 min caused a significant reduction in ROS production in Fe²⁺ treated mitochondria, while *P.*

niruri (10-200 µg/ml) caused a significant reduction in ROS production in SNP treated mitochondria.

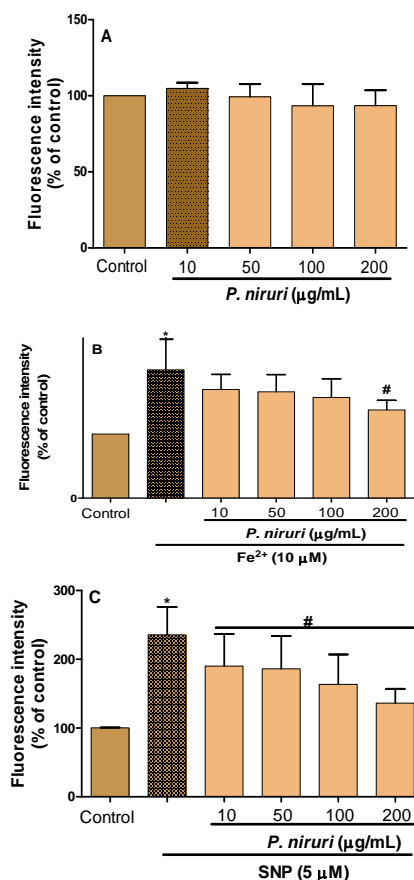


Fig. 3 Effect of *P. niruri* on ROS production in mitochondrial of rats brain (A), co-treatment with Fe²⁺ (B) and SNP (C). Columns represent mean ± S.E.M. of three independent experiments. * p<0.05 compared to control, # p<0.05 compared to Fe²⁺ and SNP treated mitochondria

TABLE I

PHENOLICS AND FLAVONOIDS COMPOSITION OF *PHYLLANTUS NIRURI*

Compounds	Mg/g	Percent (%)
Gallic acid	27.76 ± 0.02	2.77
Caffeic acid	30.15 ± 0.03	3.01
Rutin	7.52 ± 0.04	0.75
Quercetin	8.36 ± 0.01	0.83
Kaempferol	3.91 ± 0.05	0.39

Results are expressed as mean ± standard deviations (SD) of three determinations

D. Effect of *P. niruri* Extract on ROS (GSH) Nonprotein Thiol(NPSH)

P. niruri under basal condition did not decrease NPSH level in mitochondria when compared to control (p < 0.05; Fig. 4 A). Fe²⁺ (Fig. 4 B) and SNP (Fig. 4 C) caused a significant reduction in NPSH level when compared to their respective controls. This effect was attenuated by co-treatment of mitochondria with *P. niruri* (10-200µg/ml) (Figs. 4 B and C). *P. niruri* was able to restore to control level the nonprotein-SH

content that was significantly reduced by Fe²⁺ and SNP (Figs. 4 B and C).

E. Phenolics and Flavonoids Profile of *P. niruri* Barks Extract by HPLC-DAD

The HPLC fingerprinting of *P. niruri* leaves extract revealed the presence of phenolic compounds (gallic and caffeic acids), flavonoids (quercetin, rutin and kaempferol) (Fig. 5, Table I). They were identified by comparing their retention time and UV spectra to authentic standards analyzed under identical analytical conditions. The quantification of these compounds by HPLC-DAD is shown in Table I.

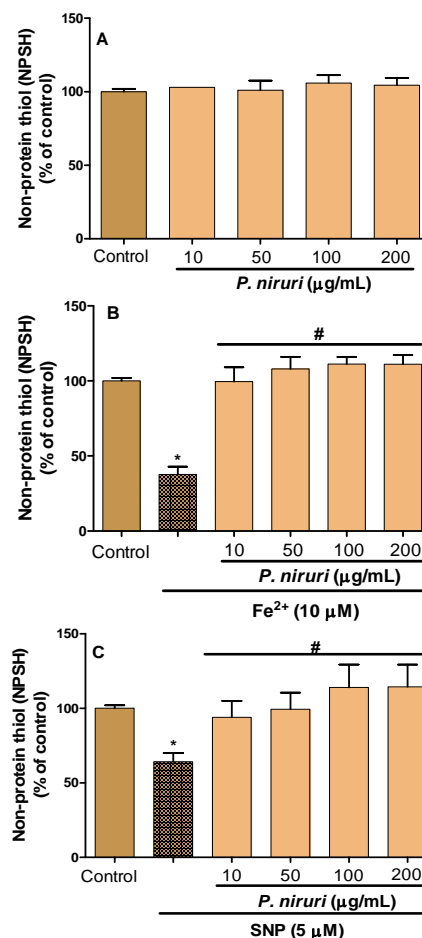


Fig. 4 Effect of *P. niruri* on (GSH) non protein thiol (NPSH) level in mitochondrial of rats brain (A), co-treatment with Fe²⁺ (B) and SNP (C). Columns represent mean ± S.E.M. of three independent experiments. * p<0.05 compared to control, # p<0.05 compared to Fe²⁺ and SNP treated mitochondria

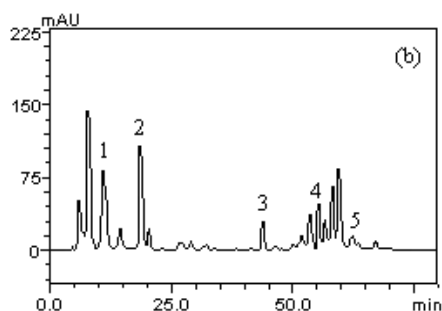


Fig. 5 Representative high performance liquid chromatography profile of *Phyllanthus niruri* leaves

IV. DISCUSSION

Neurodegenerative diseases are disorders characterized by gradually progressive, selective loss of anatomically or physiologically related neuronal systems [19]. Mitochondria contribute to ageing and neurological diseases through the accumulation of mitochondrial DNA (mtDNA) mutations and net production of reactive oxygen species (ROS) [20]. Numerous plants have been used to treat age related brain disorders and some of them have given a new direction for drug discovery [7]. In the present study, we used isolated brain mitochondria as a model to investigate Fe^{2+} and SNP toxicity and the protective effect of *P. niruri* extract on mitochondria.

Under normal physiological conditions, ROS and free radicals are generated and maintained at a relatively high steady-state level in mitochondria of tissue cells. Production of superoxide anions and hydrogen peroxide in mitochondria is increased with age in animal tissues [21], [22]; hence, it is imperative to identify compounds and/or plant extracts that could protect the mitochondria from ROS induced toxicity [23]. It has been reported that SNP can release several toxic compounds such as NO, CN^- , iron, and ROS which are responsible for SNP-induced neurotoxicity [24]. The production of ROS have been reported to inhibit mitochondrial respiratory enzymes in an irreversible manner [7], [25], resulting in disruption of electron transfer in electron transport chain and Krebs cycle. This leads to impairment of mitochondria function and ATP depletion [26]. In this study, the interaction of ROS on mitochondrial redox potential was evaluated by estimation of MTT reduction. Our results showed that exposure of mitochondria to Fe^{2+} and SNP for 30 mins resulted in a significant decrease in MTT reduction hence, mitochondrial dysfunction. Significant decrease in MTT reduction is an indication of cell death and could be due to exposure of mitochondrial to oxidative stress. The co-treatment of mitochondria with *P. niruri* in the presence of Fe^{2+} or SNP significantly maintained cellular viability. The ability of *P. niruri* extract to maintain cellular viability could be due to the antioxidant potential of phytochemicals (gallic acid, caffeic acid, rutin, quercetin and kaempferol) present which are potent free radical scavenger.

Lipid peroxidation (LPO) is a free radical mediated process. Membrane lipids present in subcellular organelles are highly susceptible to free radical damage leading to alteration in

structure and function of cell membrane and cellular metabolism resulting in cytotoxicity [27], [28]. In this study, treatment of mitochondria with Fe^{2+} and SNP resulted in accumulation of MDA (a lipid peroxidation product), an effect that was inhibited by co treatment with *P. niruri*.

The toxicity of Fe^{2+} proceeds via the Fenton reaction, in which iron catalyses a one-electron transfer reaction that generates ROS, such as the OH^\cdot from H_2O_2 [29]. SNP release cyanide, nitric oxide (NO) and free iron which also generate ROS [30], [31]. The current study demonstrated that *P. niruri* showed concentration-dependent and significant inhibition of iron and SNP induced peroxidation in mitochondria.

In the current study, we investigated the effect of *P. niruri* on basal ROS generation as well as Fe^{2+} and SNP-induced ROS generation in mitochondria isolated from rat's brain. Mitochondria in cells are the major producers of ROS that can result in the oxidative stress that has been linked to mitochondrial damage in a wide range of pathologies [32]. *P. niruri* showed a protective effect by attenuating ROS production in the brain mitochondria. This effect could be attributed to its capacity to scavenge ROS.

Reduced glutathione (GSH) is the most abundant thiol containing molecule in mammalian cells and plays a critical role in controlling intracellular burdens of oxidative stresses. It is an important antioxidative molecule in brain cells [33]. GSH non-enzymatically reacts with O_2^\cdot , NO, OH^\cdot , and peroxynitrite (ONOO^-) [24], [34]. GSH reacts directly with radicals and is substrate of the glutathione peroxidase-dependent detoxification of peroxides [35]. In both processes, GSH becomes oxidized to glutathione disulfide (GSSG) which is reduced in cells by glutathione reductase to regenerate GSH [33]. Thus, the cellular GSH content and the cellular ratio of GSH to GSSG are good indicators for ROS-induced alterations in cellular metabolism. It has long been known that mitochondria produce hydrogen peroxide [36], and free Fe^{2+} react with H_2O_2 to produce toxic hydroxyl radical (OH^\cdot). Several possible pathways can be invoked for disposal of hydrogen peroxide [37]. However, GSH dependent reactions are clearly of major importance for reducing hydrogen peroxide in mitochondria [36]. Iron promotes the production of a hydroxyl radical at the expense of O_2 and GSH consumption [38], this could account for the decrease in GSH level observed in this study in the presence of iron. In this study, a significant reduction in NPSH (GSH) by Fe^{2+} and SNP was observed in mitochondria. However, co-treatment with *P. niruri* significantly increased the level of NPSH (GSH) in mitochondria protecting the organelle from Fe^{2+} and SNP-induced neurotoxicity.

Phytochemicals in medicinal plants and dietary plants have been reported to exert potent antioxidative properties [39]. Hence, it can be speculated that the constituent phenolics of *P. niruri* play a crucial role in the observed mitigation of mitochondrial ROS and LPO formation, increase in cellular viability and increase in GSH level as observed in this study. The phenolics found in *P. niruri* have well-established antioxidant properties. Gallic and caffeic acid, which are major phenolics in *P. niruri* were reported to exhibit a

protective effect on the mitochondria via various mechanisms including attenuation of ROS generation [40], [41]. Flavonoid extracts from medicinal plants could also attenuate toxicant induced mitochondrial ROS formation and oxidative damage.

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