Chapter 2

Effects of silymarin or melatonin on behavioural, biochemical, molecular and immunohistochemical indices of maneb + paraquat-induced PD phenotype in mouse
Chapter 2  
**Effect of Silymarin or melatonin on behavioural.....**

2.1. **INTRODUCTION**

PD is typified by the selective degeneration of dopaminergic neurons of the nigrostriatal pathway leading to movement anarchy. Various toxins-induced and genetic models have been developed to understand the etiology of PD and to assess the efficacy of anti-PD drugs. MPTP, MB, PQ and rotenone are the most common chemicals used to induce PD phenotype in the experimental animal (Burns et al., 1983; Beal, 2001; Brooks et al., 1999; Thiruchelvam et al., 2000 a). MB, a fungicide and PQ an herbicide, together induce PD phenotype in the mice and is considered as one of the best models owing to slow and progressive degeneration of dopaminergic neurons, one of the cardinal features of sporadic PD (McCormack, 2002; Thiruchelvam et al., 2000 a; Patel et al., 2008). The diagnosis of PD at an early stage is still enigmatic and symptoms appear only after the degeneration of 60-70% of dopaminergic neurons of the nigrostriatal pathway (Yadav et al., 2012). The mechanisms of pathogenesis and permanent cure of PD are still elusive (Marsden, 1990; de Lau and Breteler, 2006).

Oxidative stress is the main culprit of the nigrostriatal dopaminergic neuronal degeneration. Dopamine metabolism and dopamine auto-oxidation generate high levels of free radicals in dopaminergic neurons (Dabbeni-Sala et al., 2001). Various antioxidants of plant or animal origin, have been tested for assessing their efficacy against PD. Silymarin, an antioxidant derived from plant *Silybum marianum* (milk thistle), offers protection against selected toxin-induced neuroinflammations and toxicities (Upadhyay et al., 2007; Upadhyay et al., 2010; Wang et al., 2008; Jacob et al., 2002). Silymarin is used globally for naturopathy against many neuronal and non-neuronal diseases owing to lack of toxicity even at higher doses (Jacobs et al., 2002; Saller et al., 2001). Silymarin enters the central nervous system by crossing the BBB (Nencini et al., 2007). Silymarin was preferred over other plant derived antioxidants and anti-inflammatory agents, as detailed
knowledge about its efficacy against several diseases is well known and it lacks toxicity even at higher dose. Its antioxidative nature, ability to cross the BBB and lack of toxicity have prompted to investigate its effect against biochemical and molecular indices of MB + PQ-induced PD phenotype in the mice.

Melatonin is present in all the organism starting from lower plants to higher animals (Reiter et al., 2004, Mayo et al., 2005; Paredes et al., 2009). Melatonin and its metabolites act as free radical scavengers and are also used as antioxidants (Tan et al., 1993; Tan et al., 2000; Hardeland et al., 2009; Reiter et al., 2009). The neuroprotective efficacy of melatonin has been tested in MPTP and 6-OHDA rodent models (Mayo et al., 2005). Although the effect of melatonin on biochemical, immunohistochemical and molecular indexes of the nigrostriatal dopaminergic neuroprotection have been reported against a few toxins-induced rodent models of PD (Acuna-Castroviejo et al., 1997; Borah and Mohanakumar, 2009; Chetsawang et al., 2009), its efficacy against MB + PQ-induced PD phenotype has not yet been deciphered. Melatonin was selected as it is naturally present in all the organisms and can be easily supplemented. Despite melatonin is a potent antioxidant, its effect on PD, a disease characterised by an increased oxidative stress is not yet clearly known.

Oxidative stress generates the antioxidant defence mechanism and activates different enzymes to overcome oxidative stress. CYPs and GST are the key players of phase I and phase II xenobiotic metabolism. The involvement of CYP2E1, glutathione-S-transferase A4-4 (GSTA4-4) in MB + PQ-induced PD phenotype have been well known (Patel et al., 2006; Kumar et al., 2010; Gupta et al., 2010; Habig et al., 1974). CYP2E1 participates in the generation of free radicals, which in turn bind to macromolecules and enhance lipid peroxidation. Free radicals oxidize biological macromolecules, leading to DNA damage and cell death (Marchetti et al., 1997; Korsmeyer et al., 2000; Simon et al., 2000).
Oxidation pushes the dopaminergic neurons towards autophagy or ubiquitin proteasome system (UPS)-mediated apoptosis (Tatton et al., 2003; Lev et al., 2003). The roles VMAT 2 and lipid peroxidation have been well known, respectively, for the storage of dopamine and an increased oxidative stress.

The present study was performed to investigate the effects of silymarin and melatonin on the locomotor activities, contents of dopamine and its metabolite, number of dopaminergic neurons, GST and CYP2E1 activities, lipid peroxidation and nitrite content in the nigrostriatal system of MB + PQ-induced PD phenotype in the mice. Furthermore, the expressions of VMAT 2, GSTA4-4 and apoptosis-related proteins were also looked into for elucidation of the molecular and biochemical bases of neuroprotective potentials of silymarin and melatonin.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): Acrylamide, bovine serum albumin (BSA), bromophenol blue, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution, chloroform, 3, 4-dihydroxyphenylacetic acid (DOPAC), disodium hydrogen orthophosphate (Na₂HPO₄), dithiothreitol (DTT), ethidium bromide (EtBr), ethylene-diamine-tetra-acetic acid (EDTA), ethylene-glycol-tetra-acetic acid (EGTA), dimethyl sulphoxide (DMSO), dihydroxybenzylamine hydrobromide (DHBA), glutathione (oxidized), glutathione (reduced), 3, 3’ diaminobenzidine (DAB), heptane sulphonic acid, 3-hydroxytyramine hydrochloride, homovalinic acid (HVA), isopropanol, magnesium chloride, MB, melatonin, nicotinamide adenine dinucleotide phosphate, PQ, paraformaldehyde, phenylmethylsulphonyl fluoride (PMSF), potassium dichromate, serotonin, sodium
chloride (NaCl), sodium dihydrogen phosphate (NaH₂PO₄), sodium dodecyl sulfate (SDS), sucrose, thio-barbituric acid (TBA), tris-base, triton X-100, tween-20, glutamic acid decarboxylase (GAD) (mouse monoclonal) antibody and β-actin. Reverse transcriptase-polymerase chain reaction (RT-PCR) kit, taq DNA polymerase, dNTPs, and 100-bp ladder were purchased from MBI Fermentas, Canada. Agarose, forward and reverse primers for GSTA4-4, VMAT2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Bangalore Genei, India. We procured 4’, 6-Diamidino-2-phenylindole (DAPI) from Vector Laboratory (Burlingame, CA, USA). Polyvinylidene difluoride (PVDF) membrane and Fluoro Jade-B were procured from Millipore (Billerica, MA, USA). Primary antibodies for Serine 20- Pp53 (goat polyclonal), p53 (mouse monoclonal), TH (mouse monoclonal), B-cell lymphoma 2-associated X protein (Bax) (rabbit polyclonal), caspase 9 (casp9) (rabbit polyclonal) and VMAT 2 (rabbit polyclonal) and compatible secondary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Silymarin was purchased from ICN Biomedical (Irvine, CA, USA) and other chemicals, such as acetone, acetic acid, 1-chloro-2, 4-dinitrobenzene (CDNB), hydrogen peroxide, perchloric acid, potassium permanganate, dibutyl phthalate xylene (DPX), potassium phosphate, potassium chloride (KCl), sodium hydroxide, etc., were of analytical grade and purchased locally either from Sisco Research Laboratories (Mumbai, India) or from Bangalore Genei (Bangaluru, India).

2.2.2. Animal treatment and collection of brain tissue

Male Swiss albino mice (20–25 gm) used in this study was obtained from the animal house of the CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow. The animals were kept under standard conditions (temperature: 22 ± 2°C, humidity: 45–55%, light and dark cycle are of 12 h each, light intensity: 300–400 lx) throughout the experiments. The animals were fed standard pellet diet and water ad libitum (Singh et al.,
2008). The institutional ethics committee for the use of laboratory animals approved the study. The mice were treated intraperitoneally daily with silymarin (40 mg/kg) or melatonin (30 mg/kg) for 9 weeks. Subsets of these animals were also treated with MB (30 mg/kg) and PQ (10 mg/kg), twice a week, for 9 weeks. The MB and PQ treatment was given 2 h after the melatonin or silymarin treatment (pre-treatment). The control animals were also treated with saline/0.1% DMSO in saline or in which the respective chemical was dissolved. After the final treatment, animals were sacrificed via cervical dislocation within 24 h, the brain was dissected out the nigrostriatal tissues (striatum and substantia nigra) were isolated and frozen immediately in liquid nitrogen. Expression studies and the measurement of dopamine contents were completed on the day of animal sacrifice and other experiments were performed within a week.

In this study, MB- and PQ-induced PD mice model were used, which is chronic model of disease and take nine weeks to develops the PD symptoms, therefore it does not matter, whether silymarin or melatonin is given pre- or post-treatment of maneb and paraquat. Several studies have been performed in PD model adapted the pre-treatment of antioxidants to assess their neuroprotective potentials (Barthwal et al., 2001; Singh et al., 2012; Zhang et al., 2012), therefore study was performed with pre-treatment of silymarin or melatonin against MB- and PQ-induced PD phenotype in mouse.

2.2.3. Behavioural studies

Since PD is related to movement disorder, therefore, we assessed the locomotor behavioral test: rotarod and optovarimex tests (SLA; spontaneous locomotor activity) (Singh et al., 2008). In rotarod test, the mice were trained for 3 consecutive days before the day of final treatment at a fixed speed (5 rpm) for 5 min. The time after which the animals fell down was determined and the maximal observation time was 5 min. The experimental readings were taken 24 h after the last treatment in all experimental groups.
The SLA was measured in an infrared beam-activated movement monitoring chamber (Opto-varimax-MiniA, Columbus Instruments, Columbus, OH). The animals were placed in the chamber for 1 min before recording the distance travelled and the SLA was recorded further for 5 min. A minimum of four experimental readings were recorded for each animal, and the results were averaged to obtain a single value for each animal. The experiments were performed with five animals (five observations) for each treatment group and the average reading for each group was obtained. Three sets of similar experiments (five observations for each) were performed and final values were calculated.

### 2.2.4. Dopamine, DOPAC, HVA and serotonin measurement

Dopamine, DOPAC, HVA and serotonin content was measured employing standard procedure, as described previously (Singh et al., 2012). The striatum was dissected out from the brain and prepare 10% homogenate in 0.45N perchloric acid having 100 ng/ml DHBA as an internal standard. The samples were sonicated subsequently in dark condition. The homogenate was centrifuged at 15,000 x g for 15 min at 4 °C, the supernatant was filtered through 0.2 µm syringe filter. The n-heptane sulphonic acid in 10% methanol was used as mobile phase. The standard of dopamine, DOPAC, HVA and serotonin of different concentration (10 ng/ml to 100 ng/ml) were run using reverse phase C-18 column attached with high-performance liquid chromatography (HPLC) coupled with electrochemical detector (Waters, Milford, MA, USA). The sample filtrate was run on the HPLC and the area of peaks were taken correspond to their standard peak. The concentration of dopamine, DOPAC, HVA and serotonin was calculated as ng/mg tissue.

### 2.2.5. Cryosectioning

The animals were anesthetized with the help of diethyl ether and intracardiac perfusion was performed with saline (0.9% NaCl), followed by paraformaldehyde (4% w/v in
phosphate buffered saline) at a flow rate of 5 ml/min for the 5 min. The brain was dissected coronally through median eminence, caudal block was postfixed in paraformaldehyde (10%) and serially cryoprotected in sucrose (10%, 20% and 30% w/v in phosphate buffered saline). Cryostat was used to cut 20 µm thick sections for TH and GAD immunoreactivity and 10 µm thick sections for Fluoro-jade B/DAPI staining. The brain was fixed on the block with freezing media and freezed for 2 h at -22 °C. the sections were cut down from the specific area and collect serially in the 24-well plate containing phosphate buffered saline and start the processing of sections immediately.

### 2.2.6. TH immunoreactivity

TH-immunoreactivity was performed, as described previously (Singh et al., 2008). In brief, incubate the brain sections in 0.5% v/v hydrogen peroxide in methanol to reduce endogenous peroxidase activity for 20 min and followed by washing in PBS buffer three times. After this, incubate the sections in blocking buffer (1.5% v/v normal goat serum, 0.1% v/v triton X-100 in phosphate-buffered saline) for 2 h to block nonspecific binding of antibody. The sections were incubated in monoclonal anti-TH antibody and further incubated with secondary antibody, followed by streptavidin–peroxidase complex (Singh et al., 2012). The colour was developed with DAB and the sections were dehydrated in graded ethanol and permanently mounted with DPX. The mounted sections were visualized under Nikon ECLIPSE TE2000-S and the TH-positive cells were counted as described previously (Singh et al., 2012) with slight modifications. Slides were coded by another fellow, I performed the unbiased counting. The anatomic landmarks were used to identify the area and the first section for counting was selected from each animal from a fixed distance. Every third section was then selected and counting was performed bilaterally in three sections per animal (n = 4). Images were captured with CCD camera and the number of TH-positive neurons in the substantia nigra was counted at 20X.
magnification (Leica Microscope DM6000 B, Wetzlar, Germany) using a computerized image analysis system (QWin Pro, Leica, Germany). The same criterion was used for counting as reported elsewhere (Singh et al., 2012). In brief, counting was done using specific dimensions (100 X 100 µm) and one sampling site for each tracing. The average survival was then expressed as the percentage of TH-positive neurons in control.

2.2.7. Fluoro-jade B/DAPI-double staining

For Fluoro-jade B/DAPI staining, the sections were dehydrated with ascending grades of ethanol (25%, 50%, 75% and 100% of ethanol in phosphate buffered saline), hydrated with descending grades of ethanol (100%, 75%, 50% and 25% ethanol in phosphate buffered saline) and rinsed with deionised water three times of 10 min each. The sections were dipped in potassium permanganate solution (0.06% w/v) for 20 min and again washed with deionised water for three times. After this, the sections were incubated with Fluoro Jade-B (0.0004% v/v) and 0.1% (v/v) glacial acetic acid for 20-30 min (Singh et al., 2012) and again washed with deionised water for three times. The processed sections were dried in air and after this dipped in xylene to clear the sections. After this sections were mounted with DPX containing DAPI (0.0002% v/v) and visualized immediately under the fluorescent light microscope at 40X magnification (Leica DM6000 B).

2.2.8. GAD immunoreactivity

The GAD immunostaining was performed as described elsewhere (Hebb and Robertson, 2000) with slight modification. In brief, endoperoxidase activity was minimized and nonspecific labelling was blocked as described above for TH-immunoreactivity. The sections were incubated with monoclonal anti-glutamic acid decarboxylase antibody (1:500) in blocking buffer at 4°C for 48 h, followed by 3 washings with phosphate-buffered saline (15 min each). Further incubation with secondary antibody was performed.
according to standard procedure. The sections were visualised and images were acquired at 20X magnification as described elsewhere (Singh et al., 2012). The integrated densities of sections were measured by ImageJ software freely available at National Institutes of Health.

2.2.9. RNA isolation and cDNA preparation

Total RNA was isolated from the striatum of mice brain following manufacturer’s instructions. In brief, 10% homogenate was prepared in Trizol and put at RT for 15 min and add 0.2 ml of chloroform and kept for 10 min at RT after vigorous mixing. The homogenate was centrifuged at 12,000 x g for 15 min at 4 °C. The upper aqueous layer was collected in fresh tube and isopropanol (0.5 ml) was added, mixed gently and kept at room temperature for 10 min. The samples were centrifuged again at 12,000 x g for 15 min at 4 °C. The RNA pellet was washed with 75% ethanol and centrifuged at 7,500 x g for 10 min. The RNA pellet was semi-dried under electric lamp and dissolved in RNase free water. Quantity and integrity assessment of RNA were assessed by measuring OD at 260 nm spectrophotometrically, ratio of RNA at 260/280 nm and ratio of band density of 28S/18S in denaturing agarose gel electrophoresis. Revert aid TM minus MuLV reverse transcriptase was used to synthesize cDNA from total RNA. RNA was converted into cDNA through reverse transcription using reverse transcriptase enzyme.

2.2.10. PCR amplification of GSTA4-4 and VMAT-2

Primers for VMAT2, GSTA4-4 and GAPDH were synthesized, as reported elsewhere (Singh et al, 2008; Patel et al, 2008; Zimniak et al, 1994) (table 2.1.) and PCR amplifications were performed using standard procedure described in literature. PCR products were visualized on 1.5-3% agarose gel using EtBr (10 mg/ml) under UV light. Image capturing and band density ratio was calculated by a computerized densitometry
system (Alpha Imager System, Alpha Innotech Corporation, South Africa). Band densities for VMAT2 and GSTA4-4 were normalized to β-actin.

**Table 2.1. List of primer sequences and PCR condition**

<table>
<thead>
<tr>
<th>Genes/ Base pair</th>
<th>Primer Sequence</th>
<th>PCR conditions (Denaturing, annealing and extension temperature)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTA4-4 (75bp)</td>
<td>Forward 5’ ATTGCCGTGGCTCCAATTTAA 3’ Reverse 5’ GGTGTAGCTCTTGTACAG 3’</td>
<td>94ºC (30sec), 53.1ºC (30sec) and 72 ºC (2min) for 27 cycle</td>
<td>Zimniak et al., (1994)</td>
</tr>
<tr>
<td>VMAT-2 (507bp)</td>
<td>Forward 5’ACCACCAGCAAGGGACGATAGC3’ Reverse 5’ CCCGAGTCAGCCGAAAGTCAG 3’</td>
<td>94ºC (30sec), 66ºC (30sec) and 72ºC (30sec) for 27 cycle</td>
<td>Singh et al., (2008)</td>
</tr>
<tr>
<td>GAPDH (201bp)</td>
<td>Forward 5’CTCATGACCACAGTCATGC 3’ Reverse 5’CACATTGGGGGTAGGAACAC 3’</td>
<td>Amplified concurrently with the above mentioned primers</td>
<td>Patel et al., (2008)</td>
</tr>
</tbody>
</table>

**2.2.11. Measurements of CYP2E1 activity**

Brain was perfused with normal saline, microsomes were prepared and CYP2E1 enzyme activity was determined (Upadhyay et al., 2007; Koop, 1986). For microsomes preparation tissue was homogenised in suspension buffer (20 mM HEPES buffer, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail) and centrifuge at 1000 x g for 15 min at 4 °C. Took supernatant and centrifuge at 12000 x g for 20 min at 4 °C and again supernatant was centrifuged at 1,00,000 x g for 80 min at 4 °C. The pellet was dissolve in suspension buffer having 20% glycerol. In brief, the reaction mixture containing 4-nitrophenol (0.2 mM) was mixed with Tris-HCl (50 mM, pH 7.4) and MgCl2 (25 mM) and add microsomal fraction (200-250 μg protein) to the mixture and incubated at 37°C for 5 min. The reaction was initiated by the addition of 20 μl NADPH (50 mM) and was further incubated for 10 min. 500 μl of Perchloric acid (0.6 N) was added to stop the reaction. The supernatant was obtained.
following centrifugation at 825 x g for 20 min and sodium hydroxide (100 μl of 10 N) was added to the reaction mixture. 4-nitrocatechol was used for standard curve. The absorbance was recorded at 510 nm and activity was calculated in terms of nM/min/mg protein.

2.2.12. GST activity

GST activity was determined according to method described elsewhere (Upadhyay et al., 2008; Habig et al., 1974). The 10% homogenate was prepared in 0.1 M phosphate buffer containing 0.15 M KCl. The reaction mixture was centrifuged at 10,000 x g for 15 min at 4 °C. The supernatant was added to the mixture, containing 2.8 ml phosphate buffer (0.2M), 100 μl glutathione reduced (9 mM) and 20 μl CDNB (150 mM) and makeup the volume 3 ml with the help of distilled water. The enzymatic activity was determined by monitoring an increase in the absorbance at 340 nm and activity was calculated in terms of nM/min/mg protein.

2.2.13. Lipid peroxidation

Lipid peroxidation was determined as described previously (Ohkawa et al., 1979; Gupta et al., 2010). Tissue homogenate (10% w/v) was prepared in phosphate buffer (0.1M) and incubated for 5 min at room temperature. The 100 μl SDS (10%) was added and further mix with 600 μl glacial acetic acid (20%) and incubated for 2 min. Finally 600 μl TBA (0.8%) was added in the reaction mixture and further incubated for 1 h in a boiling water bath. The reaction mixture was cooled at 4 °C and centrifuged at 10,000 x g for 5 min at 4 °C and the absorbance of the supernatant was recorded at 532 nm against the control blank. The values are expressed in TBARS.
2.2.14. Nitrite estimation

Tissue (10% w/v) was homogenised in NH₄Cl (0.7 M) and the homogenate was centrifuged at 10,000 x g at 4 °C for 10 min. The supernatant of the homogenate was incubated with ammonium chloride (0.7 mM) and mixed with Griess reagent (0.1% N-naphthyl ethylenediamine and 1% sulfanilamide in 2.5% phosphoric acid). The reaction mixture was incubated at 37 °C for 30 min, centrifuged and the absorbance of the supernatant was recorded at 550 nm. The amount of nitrite was calculated in μM using the standard curve of sodium nitrite (Granger et al., 1996; Gupta et al., 2010).

2.2.15. Western blotting

Tissue (100 mg) was homogenized in the homogenizing buffer (50 mM Tris-HCl, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM SDS, protease inhibitor cocktail, PMSF) and sonicated. The homogenate was differentially centrifuged to separate the cytoplasmic and membrane-bound proteins. Protein content was measured and proteins were subjected to polyacrylamide gel electrophoresis. Separated proteins were transferred on to a PVDF membrane and nonspecific sites were blocked by incubating the membrane in 5% w/v nonfat dry milk in tris-buffered saline (TBS) (135 mm NaCl, 2.5 mm KCl, 50 mm tris and 0.1% v/v Tween 20, pH 7.4). Membrane was incubated with primary antibodies in TBS. VMAT2-, Bax-, casp9-, p53- and Pp53-specific bindings were detected using anti-mouse or anti-rabbit alkaline phosphatise-conjugated secondary antibody with BCIP/NBT as substrate. Images were captured and the band density was calculated by computerized densitometry system (Alpha Imager System; Alpha Innotech Corporation, San Leandro, CA, USA). Band densities were normalized with β-actin.
2.2.16. Statistical analysis

The data were analyzed by one-way ANOVA followed by Newman–Keuls test and are expressed as means ± S.E.M. Differences were considered statistically significant when ‘P’ values were <0.05.

2.3. RESULTS

2.3.1. Behavioural Test

Rotarod test find out the grip strength and co-ordination of animal’s limb on the accelerating rod and expressed in terms of time of stay on the rod. MB + PQ treatment decreased the time of stay of animals on rod when compared with controls. However, silymarin or melatonin treatment significantly restored the time of stay on rod in MB + PQ-treated animals toward control values (Figure 2.1a). Similarly, SLA, calculated in terms of distance travelled, was significantly reduced in MB + PQ-treated animals. Silymarin or melatonin led to significant increase in the distance travelled by the mice when compared with MB + PQ-treated animals (Figure 2.1b).

Figure 2.1.
Figure 2.1. Effects of MB + PQ, silymarin and melatonin on locomotor activities. The time of stay on rotarod, measured in sec, is expressed in (a) and the distance travelled by animals, recorded in cm with an automated tracking device, is expressed in (b). The values are calculated as means ± SEM (n = 3 separate experiments and 3–5 mice per experimental group). Significant changes are expressed as ** (P < 0.01) and *** (P < 0.001) in comparison with control and # (P < 0.05), ## (P < 0.01) in comparison with MB + PQ-treated mice.

2.4.2. Levels of dopamine, its metabolites and serotonin in treated animals

MB + PQ- treatment decreased dopamine content in the striatum of treated animals when compared with controls. Silymarin or melatonin significantly restored the dopamine content in MB + PQ-treated animals (Figure 2.2a). Similarly, there was significant decreased in the DOPAC and HVA level in MB + PQ-treated animals, which were restored by silymarin or melatonin (Figure 2.2b, c). No significant change was observed in serotonin level in any of the treated groups as compared with controls (Figure 2.2d).
Figure 2.2.

(a) 

(b)
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Figure 2.2. Effects of MB + PQ, silymarin and melatonin on dopamine, its metabolites and serotonin levels in the striatum. HPLC analyses of (a) dopamine, (b) DOPAC, (c) HVA and (d) serotonin were performed in the striatum. The values are calculated as means ± SEM (n = 3 separate experiments and 3–5 mice per experimental group). Significant changes are expressed as *** (P < 0.001) in comparison with control and # (P <0.05) and ## (P < 0.01) in comparison with MB + PQ-treated animals.

2.4.3. TH-immunoreactivity

The number of TH-positive neurons in the substantia nigra was reduced in the animals treated with MB + PQ when compared with controls (Figure 2.3a, b). Silymarin or melatonin significantly reduced the MB + PQ-induced decrease in number of dopaminergic neurons.

Figure 2.3.
Figure 2.3. TH immunoreactivity in the substantia nigra region of the brain, as observed by bright-field microscopy at 10X magnification. The representative TH immunoreactivity in control, silymarin, melatonin, MB + PQ-treated animals with and without silymarin or melatonin treatment is shown in (a) and the counting of TH-positive neurons in the aforementioned groups in (b). The values are calculated as means ± SEM (n = 3). The data are expressed in terms of percentage of a control and control value expressed as 100%. Significant changes are expressed as *** (P < 0.001) in comparison with control and ## (P < 0.01) and ### (P < 0.001) in comparison with MB + PQ-treated mice.

2.4.4. Fluoro-Jade B and DAPI staining

Fluoro-Jade B/DAPI staining was performed to evaluate the number of degenerating neurons and total live neurons. DAPI stains the nuclei of all the cells, however, Fluoro-Jade B stains the fragmented DNA of the degenerating neurons. MB + PQ-treated animals showed an increased number of degenerating neurons, as compared with controls (Figure...
2.4a, b). Silymarin- or melatonin-treated animals showed lesser number of degenerating neurons when compared with MB + PQ-treated animals. Silymarin or melatonin reduced the statistically significant number of degenerating neurons in MB + PQ-treated animals (Figure 2.4a, b).

Figure 2.4.

(a)
Figure 2.4. The representative images of Fluoro-Jade B/DAPI-stained cells in control, silymarin, melatonin, MB + PQ-treated animals with and without silymarin or melatonin treatment (a) and counting of Fluoro-Jade B-positive cells (b) in the substantia nigra region of control, silymarin, melatonin MB + PQ-treated animals with and without silymarin or melatonin treatment (n = 3). The values are calculated as means ± SEM. The images were captured at 40X magnification under the upright fluorescent microscope. Significant changes are expressed as *** (P < 0.001) in comparison with control and ## (P < 0.01) and ### (P < 0.001) in comparison with MB + PQ-treated animals.

2.4.5. GAD-immunoreactivity

MB + PQ treatment did not induce any change in GAD-immunoreactivity in the substantia nigra region of midbrain. The integrated density of immunoreactivity of GAD-positive neurons in treated groups did not show any significant deviation from the normal value (Figure 2.5).
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Figure 2.5.

(a)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image</th>
</tr>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>MB+PQ</td>
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<tr>
<td>Silymarin+MB+PQ</td>
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</tr>
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</tr>
<tr>
<td>Melatonin+MB+PQ</td>
<td><img src="image6" alt="Melatonin+MB+PQ Image" /></td>
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</table>

(b)

![Bar Diagram](image7)

Figure 2.5. The representative images of GAD immunoreactivity in the substantia nigra of MB + PQ, silymarin and melatonin treated mice at 20X (a). Bar diagrams show the integrated density of neurons (b). (n=3-5).
2.4.6. VMAT 2 expression

VMAT2 mRNA and protein expressions were significantly decreased in the animals treated with MB + PQ as compared with controls. Although the changes were increased toward control values in silymarin- or melatonin-treated animals, significant increase was observed only in the case of protein expression (Figure 2.6a–d).

Figure 2.6.
Figure 2.6. Effects of MB + PQ, silymarin and melatonin on VMAT 2 mRNA (a, b) and protein (c, d) expressions. Lanes 1, 2, 3, 4, 5 and 6 represent control, MB + PQ, silymarin, silymarin + MB + PQ, melatonin and melatonin + MB + PQ-treated groups, respectively. The values are calculated as means ± SEM. (n=3). Significant changes are expressed as *(P < 0.05) in comparison with control and # (P < 0.05), in comparison with MB + PQ-treated mice.

2.4.7. CYP2E1 catalytic activity

A significant increase in CYP2E1 catalytic activity was noted in MB + PQ-treated animals when compared with controls. The catalytic activity of CYP2E1 was decreased towards normalcy significantly in silymarin or melatonin treated animals (Figure 2.7.)
Figure 2.7. Effects of MB + PQ, silymarin and melatonin on CYP2E1 catalytic activity in the striatum (n = 3 separate experiments and 3–5 mice per experimental group). The values are calculated in mean ± SEM. Significant changes are expressed as *** (P < 0.001) in comparison with control and ## (P < 0.01) in comparison with MB + PQ-treated mice.

2.4.8. GST catalytic activity and mRNA expression of GSTA4-4

MB + PQ increased GST catalytic activity significantly when compared with respective controls. Administration of silymarin or melatonin as well in MB + PQ-treated animals led to statistically significant decrease in GST activity (Figure 2.8a).

A significant increase in GSTA4-4 mRNA expressions was noted in MB + PQ-treated animals when compared with control. The expressions were decreased toward basal values in the animals treated with silymarin or melatonin, which was statistically significant in the case of melatonin (Figure 2.8b, c).
Figure 2.8.

(a) 

(b) 

(c)
Figure 2.8. Effects of MB + PQ, silymarin and melatonin on GST catalytic activity (2.8a) and GSTA4-4 mRNA expression (2.8b, c) in the striatum. The values are calculated as mean ± SEM (n= 3 separate experiments, 3–5 mice per experimental group). Lanes 1, 2, 3, 4, 5 and 6 represent control, MB + PQ, silymarin, silymarin + MB + PQ, melatonin and melatonin + MB + PQ-treated groups, respectively. Significant changes are expressed as * (P < 0.05) and ***(P < 0.001) in comparison with control and # (P < 0.05) and ## (P < 0.01) in comparison with MB + PQ-treated mice.

2.4.9. Lipid peroxidation

MB + PQ increased lipid peroxidation significantly as compared with respective controls. Administration of silymarin or melatonin in MB + PQ-treated animals decreased in lipid peroxidation level towards normalcy (Figure 2.9.)

Figure 2.9. Effects of MB + PQ, silymarin and melatonin on lipid peroxidation in the striatum. (n = 3 separate experiments and 3–5 mice per experimental group). Significant
changes are expressed as *** (P < 0.001) in comparison with control # (P < 0.05) and ## (P < 0.01) in comparison with MB + PQ-treated animals.

2.4.10. Nitrite estimation

MB + PQ increased nitrite content significantly as compared with respective controls. Administration of silymarin or melatonin as well in MB + PQ-treated animals led to statistically significant decrease in nitrite level (Figure 2.10)

**Figure 2.10**

![Graph showing nitrite levels](image)

**Figure 2.10** Effects of MB + PQ, silymarin and melatonin on nitrite level in the striatum. The value are expressed as mean ± SEM (n = 3 separate experiments, 3–5 mice per experimental group). Significant changes are expressed as *** (P < 0.001) in comparison with control and # (P < 0.05) and ## (P < 0.01) in comparison with MB + PQ-treated animals.
2.4.11. Western blot analyses of p53, bax and casp9

Combined MB and PQ exposure led to an increase in Pp53, Bax and casp9 expressions in the cytosolic fraction of the treated striatum. However, a decrease in the level of p53 was observed in MB + PQ-treated animals. Silymarin or melatonin treatments restored the protein expressions toward basal levels (Figure 2.11a-h).

Figure 2.11.

(a) 

![Western blot image](image)

(b) 

![Band density ratio image](image)
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(c) Pp53 (53 KDa) 
β-actin (43 KDa)

(d) Band density ratio (Bax/β-actin)

(e) Bax (23 KDa) 
β-actin (43 KDa)

(f) Band density ratio (P-p53/β-actin)
Figure 2.11. Effects of MB and PQ, silymarin and melatonin on p53 (a, b), Pp53 (c, d), Bax (e, f) and casp9 (g, h) protein expressions. The values are calculated as mean ± SEM (n= 3 separate experiments, 3–5 mice per experimental group). Lanes 1, 2, 3, 4, 5 and 6 represent control, MB + PQ, silymarin, silymarin + MB + PQ, melatonin and melatonin + MB + PQ-treated groups, respectively. Significant changes are expressed as ** (P < 0.01), *** (P < 0.001) in comparison with control and ## (P < 0.01) and ### (P < 0.001) in comparison with MB + PQ-treated mice.

2.5. Discussion

The study was performed in male Swiss albino mice as this strain has been used in several studies to examine the molecular mechanisms of MB + PQ-induced PD phenotype (Patel et al., 2006; Patel et al., 2007; Patel et al., 2008). The role of oxidative stress in MB + PQ-
induced PD phenotype is well known. MB inhibits complex-III and PQ inhibits complex I of the respiratory chain, thereby leading to oxidative stress (Castello et al., 2007; Domico et al, 2007). PQ generates the superoxide anion radical through oxidation/reduction cycle (Castello et al., 2007). MB and PQ together induce Bax pathway leading to apoptosis in the nigrostriatal tissues (Cicchetti et al., 2009). MB + PQ-induced animal model is widely used to understand the molecular mechanism of progressive and slow neurodegeneration (Thiruchelvam et al., 2000; Patel et al., 2006) and this model system has been used to explore the neuroprotective potential of antioxidants (Thiruchelvam et al., 2005; Metodiewa and Koska, 2000; Li et al., 2005). Silymarin is a potent antioxidant of plant origin and can cross the BBB. It is nontoxic even at moderate to high doses and possesses free radical scavenging properties (Upadhyay et al., 2007; Upadhyay et al., 2010; Nencini et al., 2007; Kittur et al., 2002; Wang et al., 2002). Melatonin also crosses the BBB and is as a powerful antioxidant especially in the brain (Esteban et al., 2010; Jou et al., 2010) and it has been extensively studied agent for its neuroprotective potential against several models of PD (Reiter et al., 2004; Mayo et al., 2005; Dabbeni-Sala et al., 2001). Its role in a slowly developing progressive model of PD has also been examined (Antolin et al., 2002).

PD is related with the degeneration of dopaminergic neurodegeneration of nigrostriatal pathway, which led to deficiency in dopamine content and locomotor activity. The reduced level of striatal dopamine and its metabolite and the decrease in time of stay on the rotarod and spontaneous locomotor activity after MB + PQ treatment were ameliorated by silymarin or melatonin. The findings suggest that MB + PQ-induced neurobehavioral anomalies were reduced significantly by these two antioxidants. Reductions in neurobehavioral indices and dopamine level after MB + PQ treatment are supported by the previous investigations showing reduced locomotor activities and striatal
dopamine in the chemically induced PD (Acuna-Castroviejo et al., 1997; Thiruchelvam et al., 2000b; Cicchetti et al., 2009; Domico et al., 2007). Serotonin level and GAD immunoreactivity in the substantia nigra region did not show any significant change, suggesting that MB + PQ-induced toxicity did not affected the serotonergic and GABAergic neurons. Significant preservation of these variables by the silymarin and melatonin indicate their efficacy against MB + PQ-induced PD phenotype in experimental animals. TH-positive cells are the markers of dopamine producing cells in the substantia nigra region of the brain. Selective degeneration of the nigrostriatal dopaminergic neurons has been recognized as one of the major characteristics of PD. After MB + PQ-treatment the number of TH-positive cells in the substantia nigra as measured by TH immunoreactivity were reduced as compared with control and silymarin and melatonin ameliorate the TH-positive cell number, which is further supported the neuroprotective role of both the antioxidants.

In PD, the dopaminergic neurons undergo the process of degeneration, which was confirmed by more specific Fluoro-Jade B/DAPI staining. DAPI is known to stain the nuclei, while, Fluoro-Jade B stains the fragmented DNA of the degenerating neurons. MB + PQ-induced dopaminergic neurodegeneration showed an increased number of Fluoro-Jade B-positive cells in the substantia nigra of MB + PQ-treated mice brain when compared with controls. (Singh et al., 2010). The number of Fluoro-Jade B-positive cells was significantly reduced in the antioxidant-treated animals when compared with MB + PQ-treated animals, showing that antioxidants offered the nigrostriatal dopaminergic neuroprotection in MB + PQ models of PD. Melatonin and silymarin could not prevent the neurodegeneration, measured by Fluoro-Jade B/DAPI staining, to the similar extent as they prevented many other studied parameters. It could be attributed to the biological variations, which arose during the counting of Fluoro-Jade B-positive cells, as it was
performed in a fixed and small area of the substantia nigra or because of some other reasons that need to be elucidated further because the exact molecular explanation of its staining is not known.

VMAT2 expression was significantly preserved when the animals were treated with silymarin or melatonin when compared with MB + PQ-treated animals. VMAT2 sequesters dopamine inside the vesicles and protects the neurons from oxidative stress. The reduced expression of VMAT2 in MB + PQ-treated animals showed abnormal dopamine transport, sequestering and storage and could be associated with the degeneration of dopaminergic neurons, as dopamine uptake from the cytoplasm into secretory granules is mediated by VMAT2 along with an ATPase generated proton gradient (Erickson and Varoqui, 2000; Travis et al., 2000). RT-PCR is a semi-quantitative tool and mRNA under some conditions does not translate into proteins, therefore, differences in mRNA and protein expressions were observed in a few cases in the study.

CYP2E1 induces free radical generation by mixed-function oxidase activity, however, increased expression of GSTA4-4 could be an adaptive mechanism to counteract oxidative stress induced by MB + PQ (Patel et al., 2006; Kumar et al., 2010). The increased level of GST and CYP2E1 enzyme activities in MB + PQ-treated mice is consistent with the above-mentioned observations at biochemical and functional levels, as these were observed previously as well (Patel et al., 2006). Silymarin or melatonin reduced the expression of GSTA4-4 and catalytic activities of CYP2E1 and GST, which show that these antioxidants possibly reduce the burden on the nigrostriatal tissues produced by MB and PQ. MB + PQ-exposure increased nitrite level, as observed previously (Gupta et al., 2010). Both antioxidants independently reduced the MB + PQ-induced increases in nitrite content. MB + PQ led to an elevated lipid peroxidation that was significantly restored toward normal level by silymarin and melatonin as seen
previously (Patel et al., 2006; Kumar et al., 2010). Silymarin- and melatonin-mediated reduction in lipid peroxidation in MB + PQ-treated animals is very likely due to their anti-lipid peroxidation properties (Hardeland et al., 2009; Galhardi et al., 2009).

Oxidative stress induces the apoptotic pathway, which may be intrinsic or extrinsic pathway. Intrinsic pathway is very much related with mitochondrial mediated and extrinsic pathway is related with receptor mediated apoptosis. It is well reported that coexposure of MB and PQ activate Bax pathway, which leads to mitochondrial outer membrane permeabilization, which in turn allows cytochrome c release from mitochondria (Fei and Ethell 2008). Cytoplasmic cytochrome c form complexes with Apaf-1 and procaspase-9 to form an apoptosome that can activate caspases, such as caspase-3, leading to apoptosis (Fei and Ethell 2008). Therefore, the levels of Bax, p53, Pp53 and casp9 were measured in this study to assess the impacts of silymarin or melatonin in this PD model. Upregulation of the activated (phosphorylated) form of p53, i.e., Pp53 in mane-b- and paraquat-treated animals supports the notion that toxic insults trigger the rapid induction of post-translational mechanism that phosphorylates p53 (Thompson et al., 2004; Schon et al., 2002). Decrease in the level of p53 in MB + PQ-exposed animals is not an unusual phenomenon, owing to the fact that following genotoxic stress, cytoplasmic pool of p53 is depleted through its conversion into stable Pp53, which ultimately translocates either into nucleus or into mitochondria to exert its transcriptional/nontranscriptional activities, respectively (Lee and Chang, 2010; Mihara et al., 2003). Likewise, upregulation of Bax expression is in accordance with an observation that Bax gene promoter contains a p53-binding site and is responsive to p53 (Benchimol, 2001). Bax plays a critical role in apoptotic cell death in MB + PQ-induced PD phenotype (Cicchetti et al., 2009). MB potentiates the mitochondrial complex I inhibitory property of PQ and both of them together sensitize neurons to mitochondria-dependent apoptosis. It
has been reported that Bax-mediated cell death accompanies the activation of casp9 and paraquat also augments the expression of casp9 (Yang and Tiffany-castiglion, 2008). An activation of casp9 in dopaminergic cells after a toxic insult is well documented in the animal models of PD. In this study, silymarin and melatonin downregulate the enzymatic activities and expression of toxicant responsive genes, such as CYP2E1 and GST and pro-apoptotic genes, like Pp53 and Bax and casp9 and offer neuroprotection against MB + PQ-induced PD phenotype. Silymarin- and melatonin-mediated changes at the mitochondrial levels likely contributed to their protective actions in the current study (Jou et al., 2010; Paradies et al., 2010).

Epidemiological investigations have reported that MB and PQ could induce incidences of PD in humans (Pezzoli and Cereda, 2013; Tsuboi, 2012; Freire and Koifman, 2012; Wang et al., 2011), which prompted investigators to develop an animal model based on the combined exposure to MB and PQ. Combined MB and PQ model is the only well-established model of PD, which is considered to be environmentally relevant (Kachroo et al., 2010). Other chemicals used to develop PD in animals, such as MPTP, 6-OHDA, etc., are neither environmentally relevant nor humans are now exposed to them in their day-to-day life. Therefore, the results obtained from the current model could be of direct human relevance and may be appropriately correlated with human since extrapolation of the findings to humans employing this model are expected to be more accurate. On the other hand, silymarin is naturally present in many plants and reported to be non-toxic even at very high doses. Similarly, melatonin is present in almost all the organisms starting from unicellular organisms to complex multi-cellular organisms, like human and is reported to be non-toxic up to moderated doses (Singhal et al., 2011; Yadav et al., 2012). Owing to non-toxic and naturally occurring nature, the data obtained from the animal could be extrapolated to humans.