Summary and conclusion
Parkinson’s disease (PD) is the second most common progressive neurodegenerative disorder after Alzheimer’s Disease. The main cardinal features of PD are resting tremor, rigidity, bradykinesia and postural instability. The disease is mainly characterised by the degeneration of dopaminergic neurons in the substantia nigra pars compacta, which results in the depleted level of dopamine in the striatum. The deficiency of dopamine, a catecholamine, in the striatum leads to movement deficits in the body of rodents and primates. Several studies have suggested that increasing age, genetic modification and environmental factors are the main contributory factors of PD pathogenesis. PD is known as the disease of aging, as the prevalence rate of the disease is much higher in the elderly people above the age of 60 years as compared with young individuals. Several genes are also reported as the main causative factors of an early onset of PD. Environmental factors, such as pesticides and heavy metals play critical roles in the onset and progression of PD.

Several pesticides-induced rodent models have been developed to elucidate the molecular mechanisms of PD pathogenesis and to assess the therapeutic efficacy of naturally occurring and synthetic chemical agents. Combined maneb (MB) - and paraquat (PQ) - induced mice model is considered as one of the best rodent models since both of them in combination induce slow and progressive degeneration of dopaminergic neurons, which mimics cardinal pathological features of sporadic PD. MB and PQ inhibit the mitochondrial electron transport chain complex III and I, respectively and induce the formation of reactive oxygen species. Oxidative stress could initiate the process of degeneration by damaging the cell membrane and cell organelles. MB + PQ degenerate the dopaminergic neurons by increasing the oxidative stress, which ultimately leads to motor dysfunction. Oxidative stress is produced by pesticides-mediated complex I and III enzymatic inhibition leading to incomplete reduction of oxygen and also during the phase
Summary and conclusion

I xenobiotic metabolism. Free radicals lead to damage of cell membrane and increase the production of lipid peroxides. MB + PQ also increase nitrite level, suggesting the involvement of inducible nitric oxide synthase, which may further induce the expression of pro-inflammatory cytokines. Cytokines in turn activate several signalling pathways, thereby increase the rate of apoptosis and cell cycle arrest and inhibit the cell proliferation and differentiation.

Oxidative stress is one of the main culprits of PD, therefore, the antioxidants are expected to provide neuroprotection against MB + PQ-induced PD phenotype. The neuroprotective efficacy of silymarin and melatonin, two naturally occurring antioxidants, was therefore assessed in the present study. Silymarin, is naturally present in the fruit of Silybum marianum (commonly called as milk thistle) while melatonin is secreted in almost all the organisms. Since silymarin exhibits anti-cancerous, antioxidant and anti-inflammatory properties and readily crosses the blood brain barrier, therefore, it is expected to offer neuroprotection against PD. Melatonin, on the other hand, is ubiquitous in nature, regulates circadian rhythms and reduces oxidative stress, neuroinflammation and apoptosis. As melatonin and its metabolites scavenge free radicals, therefore, it is also expected to render neuroprotection against MB + PQ-induced PD by reducing the burden of oxidative stress, as reported in a few rodent models of PD.

In this study, male Swiss albino mice (20–25 gm) were injected 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. The respective control animals (vehicles) were treated with an equal volume of 0.1% DMSO in saline. After the final treatment, animals were sacrificed via cervical dislocation within 24 h, the brain was dissected out and the nigrostriatal tissues (striatum and substantia
nigra) were isolated and frozen immediately in liquid nitrogen. The neurobehavioral (rotarod and spontaneous locomotor activity performances) study of controls and treated animals was checked using standard procedures. The levels of dopamine, its metabolites and serotonin were measured in the striatum of control and treated mice. The tyrosine hydroxylase (TH) immunoreactivity and glutamic acid decarboxylase (GAD) immunoreactivities were performed in the substantia nigra pars compacta to assess any change in the number of the dopaminergic and glutaminergic neurons in the treated animals. Fluoro Jade-B/DAPI staining was performed in the substantia nigra pars compacta to assess the number of degenerating neurons in the substantia nigra of treated animals. To assess the microglial activation in the substantia nigra of treated animals, integrin-αM immunoreactivity was performed. Lipid peroxidation and nitrite level were measured using standard procedure to check the involvement of oxidative and nitrosative stresses in dopaminergic neurodegeneration. The expression of vesicular monoamine transporter 2 (VMAT2) and the expressions and activities of genes/proteins (CYP2E1, GSTA4-4, p53, Bax, caspase 9) related to the antioxidant defense system and apoptosis were also assessed in the striatum of the brain. The transcriptome profiling was performed using microarray to investigate the neuroprotective mechanism of silymarin or melatonin in MB + PQ-treated animals. Quantitative real time polymerase chain reaction (qRT-PCR) was performed to confirm the expression patterns of a few differentially expressed genes obtained from microarray experimentations.

Silymarin or melatonin treatment restored the time of stay of the animals on rotarod, spontaneous locomotor activity, which shows the improvement in locomotor activities. Silymarin or melatonin also restored the level of dopamine and its metabolites in the striatum and TH-immunoreactivity in the substantia nigra of MB + PQ-induced PD phenotype in mice. Silymarin or melatonin reduced death of dopaminergic neurons,
which was supported by decreased Fluoro-Jade B staining. Restoration of the VMAT2 expression by the treatment of silymarin or melatonin shows the increased sequestration of dopamine. Furthermore the reduced expression of pro-apoptotic proteins, P-p53, Bax and caspase 9 also shows the antiapoptotic properties of silymarin or melatonin. MB + PQ-induced catalytic activities of cytochrome P4502E1 and glutathione-S transferase and lipid peroxidation/nitrite level were attenuated by silymarin or melatonin, which indicates their antioxidative property. MB + PQ-induced microglial activation was also reduced by silymarin or melatonin, which shows their anti-inflammatory property. In microarray experimentations, silymarin or melatonin modulated the expression of genes related to apoptosis, cell signalling, cell cycle, cytoskeleton, inflammation, mitochondrial and synaptic function, which were altered in the striatum of MB + PQ-treated animals. The expression patterns of BCL2-antagonist/killer 1 (Bak1), v-akt murine thymoma viral oncogene homolog 1 (Akt1), interleukin 1, beta (IL-1β), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFκB1), heat shock transcription factor 1 (Hsf1), synaptotagmin 5 (Syt5), caspase 9 (casp9), measured by qRT-PCR, showed the similar expression patterns, as obtained from microarray experimentations.

In brief, the conclusions derived from the present study are listed as follows:

- Silymarin and melatonin offer neuroprotection, as shown by the increase in dopamine level and number of TH positive cells.
- Silymarin and melatonin reduce the activities and expressions of pro-oxidant and pro-apoptotic proteins in MB + PQ-treated animals.
- The results obtains from the present study delineate the possible molecular mechanism of silymarin/ melatonin-mediated neuroprotection against MB + PQ-induced PD phenotype.
Summary and conclusion

- Silymarin and melatonin offer neuroprotection against PD phenotype in mouse, therefore, their clinical trial, as natural therapeutic agents in humans could be useful against sporadic PD.