The present study investigated the neuroprotective effect of withanolides and curcuminoids in the MPTP model of Parkinson’s disease (PD). MPTP model has been suggested to be a gold standard for PD; since, many of the disease pathological hallmarks can be experimentally recapitulated (Dauer and Przedborski, 2003; Shimohama et al. 2003). In-addition, MPTP model serve as an indispensable tool for the screening of therapeutic drug candidate (Muralikrishnan and Mohankumar, 1998; Dauer and Przedborski, 2003). The present research findings suggested marked dopaminergic degeneration in C57Bl/6 mice strain when MPTP was administered at the dose of 20 mg/kg each in a single day by i.p route (20x4 mg/kg and total dose of 80 mg/kg). These findings were in corroboration with other published reports which also observed marked dopaminergic degeneration at the selected dose (Araki et al. 2004; Mohanasundari and Sabesan 2007). Furthermore, the present research findings and previous studies suggested that acute dose regimen of MPTP (20x4 mg/kg; single day) is primarily associated with dopaminergic cell death through necrosis (Przedborski and Vila 2003) and may initiate inflammatory cascade (Furuya et al. 2004). Moreover, the acute MPTP dosage regimen leads to irreversible and specific dopaminergic neuronal loss which makes it further predominantly acceptable for neuroprotective studies globally (Araki et al. 2004; Anderson et al. 2006). Similarly, several studies have demonstrated that C57Bl/6 mice were more vulnerable to MPTP intoxication than any other strain and exhibited marker parkinsonian symptoms (Heikkila et al. 1985; Przedborski and Jackson-Lewis. 1998; Sedelis et al. 2001).

The present findings suggested that acute dose of MPTP (20x4 mg/kg; i.p.) causes a significant depletion of DA with concomitant reduction of its metabolites, 3, 4-dihydroxy phenyl acetic acid (DOPAC) and homovanillic acid (HVA) in the striatum of MPTP-induced mice. The
loss of dopamine and its metabolites further leads to the elevation in DOPAC/DA and HVA/DA ratio (DA-turnover). Collectively, the changes in DA, its metabolites and elevated turn over suggested the extensive utilization of DA which was produced by the remaining DA neurons in the intoxicated mice brain. Moreover, elevated DA turnover noticed in present study further suggested a unique compensatory mechanism of brain which was also reported in other studies (Muralikrishnan and Mohankumar, 1998).

The present data suggest that pre-treatment with withanolides (100 mg/kg/day; p.o.) and curcuminoids (150mg/kg/day; p.o.) significantly protected the dopaminergic degeneration as manifested by the reduced depletion of DA and its metabolites in the striatum of treated groups when compared with the MPTP alone group. The activity of withanolides and curcuminoids can be compared with the standard drug deprenyl (3 mg/kg/day; p.o.) which elevated the DA and its metabolite content in the MPTP+deprenyl treated group. Consistent with present findings, in one study, administration of Withania somnifera (WS) root extract elevated the dopamine and its metabolites content in 6-OHDA-induced PD model; may be due to its D2 receptor agonist activity (Ahmad et al. 2005). It is evident that D2 receptors are highly vulnerable and directly proportional to dopamine loss, since D1 and D2 receptors are predominantly present in the dorsal striatum-the site which govern motor coordination. Therefore, from the present observations, it is hypothesized that the significant activity of withanolides over dopaminergic neurotransmission was may be in-part due to its D2 receptor agonist activity. Similarly, curcuminoids administration has also ameliorated the dopamine and its metabolite content when compared with diseased control group. Furthermore, in a recent study curcumin has been reported to modulate dopaminergic receptor, CREB and phospholipase c gene expression in the cerebral cortex and cerebellum of streptozotocin induced diabetic rats (Kumar et al. 2010). Therefore, based upon the present observations and previous finding, it is hypothesized that the prevention of dopaminergic degeneration on curcuminoids administration was may be due to the modulation of D1 and D2
receptor and dopaminergic signalling. However, the proposed hypothesis warrants further research.

In-addition, the protective effect of withanolides and curcuminoids over dopaminergic neurotransmission which was further supported by tyrosine hydroxylase (TH) protein expression, which is a rate-limiting enzyme in dopamine biosynthesis. Pre-treatment with withanolides and curcuminoids significantly ameliorated the TH protein expression which was severely compromised in the striatum of MPTP-induced mice (Kurkowska-Jastrzebska et al. 1999). Our observations were in accordance with other published report, where standard root extract of Ws down-regulated the TH expression in 6-OHDA-induced PD model (Ahmad et al. 2005). Similarly, in a recent study conducted by Khuwaja and co-workers (2011), significant elevation of TH level on curcumin administration was reported in 6-OHDA-induced mice brain. Therefore, it is assumed that withanolides and curcuminoids protected the dopamine neurotransmission in the striatum of MPTP-induced mice by modulating the TH protein expression. Significant elevation of TH protein expression was also observed in the deprenyl treated mice which was in accordance with the previous reports (Rodriguez-Gomez et al. 1997).

Besides the activity of withanolides and curcuminoids over DA synthesising enzyme tyrosine hydroxylase (TH), their neuroprotective effect over DA metabolising enzyme monoamine oxidase-B (MAO-B) was also evaluated in the present study. Reduced level of MAO-B activity (although not significant) was observed in the MPTP-induced mice brain, which indicated the excessive utilization of MAO-B enzyme during the conversion of MPTP into its neurotoxic metabolite MPP⁺ (Ransom et al. 1987) and further justifies the extensive dopaminergic neurodegeneration on MPTP treatment (Dauer and Przedborski 2003; Muralikrishnan and Mohankumar 1998). Both withanolides and curcuminoids exerted MAO-B inhibitory activity in in-vitro studies, however no significant changes were observed in the in-vivo studies. The present in-vivo findings of curcuminoids over MAO-B activity was in-contrast with previous published
reports, which may be due to the differences in the mice strain, dose regime of MPTP and drug (Rajeswari and Sabesan 2008; Shrinivas et al. 2008). Nevertheless, our result suggested that withanolides and curcuminoids did not interfere with the MPTP metabolism and its toxicity. Moreover, the present study observations revealed that deprenyl treatment reduced the MAO-B activity, which was in accordance with other published report, where deprenyl has exerted neuroprotective effect through modulating MAO-B activity and thus elevating the DA level in the MPTP-induced model (Saravanan et al. 2006).

Furthermore, histo-pathological observations of the current study revealed that, primarily acute dose regimen of MPTP-intoxication damages the brain microvasculature resulting to blood pooling in the substantia nigra zone. This may in-part resulting to agonal contraction of arterioles which could prevent normal blood flow and may lead to hypoxia, astroglial activation and free radical generation; collectively, exacerbating the cellular homeostasis and may leads to necrotic cell death. The observation was consistent with other published report, where acute MPTP induction causes necrotic dopaminergic cell death in mice (Adams et al. 1991). Further the histo-pathological observations was supported by enzyme linked immunosorbent assay, immunoblotting and immunohistochemistry data which suggest that hampered blood flow and/or blood pooling subsequently triggers the inflammatory cascade as noticed by activated pro-inflammatory cytokines (TNF-α and IL-1β) and astroglial activation (GFAP over expression); culminating to necrotic cell death followed by the MPTP intoxication which is consistent with other published report (Adams et al. 1991) and validates that acute MPTP dose regimen causes necrotic cell death. The present findings suggested that withanolides and curcuminoids administration prevented and/or restored the MPTP-induced cytotoxicity. Based on the present findings, it can be speculated that administration of withanolides and curcuminoids prevented cellular alteration and microvasculature integrity may be in-part through reverting activation of inflammatory cascades and membrane depolarization.
The change in the bio-marker of dopaminergic neurotransmission on MPTP intoxication was further reflected in the compromised motor coordination as assessed in rota-rod and hang time tests which was consistent with other published reports (Rajeswari and Sabesan 2008; Shrinivas et al. 2008). Treatment with withanolides and curcuminoids significantly improved the retention time on rota-rod and hang time tests. It is evident that the motor coordination is directly controlled by the dopaminergic system majorly through dorsal striatum, therefore improvement in rota-rod and hang time test performance indicate the restoration of DA content on withanolides and curcuminoids treatment. The present findings were in corroboration with earlier published reports, where treatment with *Withania somnifera* reversed the alterations in locomotor and muscle coordination on 6-hydroxy dopamine-induced rats via an unknown mechanism (Ahmad et al. 2005). Similarly in another study, Khuwaja and co-workers have reported neuroprotective effects of curcumin on 6-hydroxydopamine-induced Parkinsonism in rats; where curcumin administration significantly restored the motor coordination on rota-rod and gross behavioral activity as assessed by open field test (Khuwaja et al. 2011). Furthermore, the improvement in behavioral performance followed by withanolides and curcuminoids administration can be compared with standard deprenyl, which also restored the behavioral performance in 6-OHDA model of PD (Saravanan et al. 2006).

In PD, mood disturbances such as depression and anxiety are quite common. Anxiety and depression have also been associated with an increased risk of later development of PD. (Schuurman et al. 2002; Shiba et al. 2000). The pathophysiological mechanisms involved in mood disturbances in PD remain unclear, but serotonergic dysfunction has been postulated as such systems are involved in mood disorders in non-PD and the raphe nuclei, as well as hippocampus and prefrontal cortex, appear to be the primary sites affected (Drevets et al. 2007; Groenewegen et al. 2000). In-extension to the significant effect of withanolides and curcuminoids over motor coordination, their anti-depressant and total gross behavioural activity was assessed
through tail suspension and open field test respectively. The present findings showed a significant
elevation in the depressive behaviour in MPTP-induced group which was consistent with other
published reports, where MPTP or 6-OHDA induction demonstrated motor, cognitive,
emotional, neurochemical, molecular and sleep-related disruptions in animals (Da Cunha et al.
withanolides (100mg/kg/day; p.o.) and curcuminoids (150mg/kg; p.o.) specifically elevated the
nor-adrenaline and serotonin content in the MPTP-induced mice brain and the results were in
corroborated with other published report (Bhattacharya et al. 2000; Kulkarni et al. 2008). The
restoration in the monoaminergic neurotransmission on withanolides and curcuminoids treatment
was further supported with significant decrease in the depressive behaviour and improvement in
gross behavioral activity as compared with MPTP control group. In agreement with present
observations, study conducted by Gupta and Rana (2007) has demonstrated that root extract of
Ws holds anti-depression activities in dose dependent manner, against protracted social isolation
induced depressive behaviour in rats. Likewise, several studies have demonstrated that curcumin
administration showed antidepressant-like effects in animal models employed for the prediction
of antidepressant activities (Xu et al. 2005a, b; Kulkarni and Mehta 1985). Moreover, reports are
available that chronic administration of curcumin exhibited antidepressant-like activity on
olfactory bulbectomy model of depression in rats (Xu et al. 2005b). Recently, Wang et al. (2008)
have reported that curcumin modulates 5-hydroxytryptamine (5-HT)1 and 5-HT2 receptors to
attribute antidepressant action. Since, it is evident that PD-related neurodegenerative process
aggravates in brainstem and depressive behaviour is the outcome of intricate involvement of
striatal, frontal and limbic dopaminergic, cholinergic, serotonergic, noradrenergic and
GABAergic pathways that are thought to be involved in the mood regulation (Blandini et al.
2000; Schrag, 2004). The aforementioned alterations in dopaminergic and serotonergic systems
suggest that one or more of these neurotransmitter systems play an important role in depressive-
like behaviours in the MPTP-induced model, further supporting the involvement of these
neurotransmitter systems in PD-related depression (Schrag, 2004). Indeed, there is pathophysiological evidence of 5-HT alterations in patients with PD-associated depression and a hypothesis concerning 5-HT has even been proposed for depression in PD (Mayeux, 1990). Collectively, based upon the present findings it was postulated that withanolides exert multi-targeted activity by ameliorating the catecholamine neurotransmitters, whereas curcuminoids modulate DA and serotonin neurotransmitters in the midbrain and striatum of MPTP-induced mice.

In order to find out the molecular mechanism of action of putative drug candidates withanolides and curcuminoids in MPTP induced model of PD, the present study has comprehensively investigated their effect over mitochondrial dysfunction, oxidative stress and inflammatory responses involved in PD progression. Mounting evidences suggested mitochondrial impairment as one of the protuberant cause in PD pathogenesis (Schapira, 2010). In the present study, significant reduction of mitochondrial complex I (NADH-ubiquinone oxidoreductase) activity was observed in the MPTP-induced mice brain which was consistent with the earlier published reports (Przedborski and Ischiropoulos, 2005; Banerjee et al. 2008). Pre-clinical studies demonstrated that MPP⁺ binds at two distinct sites of mitochondrial complex I viz., N2 and ubiquinone which in-turn inhibit the NADH oxidation (Perier 2007, Ramsay et al. 1986); further disrupt the electron transport chain (ETC) thereby, reducing the ATP content in neuronal cells and thus generates energy crisis in the nigrostriatal dopaminergic system. The present findings are in agreement with proposed hypothesis of ATP crisis and data demonstrated the reduced ATP content in mid brain of MPTP-induced mice as compared with normal control.

In addition to Complex I, the results of the present study also demonstrated the reduced activity of complex II (succinate dehydrogenase), II-III (cytochrome c reductase) and IV (cytochrome c oxidase) in the MPTP-induced mice brain. Although extensive research work has been carried out regarding the involvement of complex I in PD progression, the studies about the
other mitochondrial complex were limited and of varied opinion. Few studies have indicated the
inhibition of complex I activity alone without affecting the other complexes (Nicklas et al. 1985;
Vyas et al. 1986). In contrast reports were also available, which indicated reduced activity of
complex II-III in the PD patients (Haas et al. 1997; Shinde and Pasupathy 2006). It is evident that
mitochondrion are more vulnerable to toxic insult and/or oxidative damage, since it acts both as
the target as well as the site for ROS generation. The mechanism behind the mitochondrial
impairment apparently involved the direct inhibition of Complex I by MPP\(^+\), further leads to the
excessive ROS generation and disrupted electron transfer within the other complexes. The
dysfunction of the remaining complexes appears to be the secondary consequence of ETC
disruption through complex I mediated excessive ROS generation (Keane et al. 2011). Complex I
and III inhibition causes an increase electron release from the ETC into the mitochondrial matrix,
which then reacts with oxygen to form free radicals such as superoxide (O\(^2\)^-), hydroxyl ions (OH\(^-\))
and peroxy nitrites (ONOO\(^-\)). Among all, ONOO\(^-\) acts as a powerful oxidant which directly
damage mitochondrial respiratory chain complexes. Selective damage to complex I activity by
ONOO\(^-\) appears to be the result of protein modifications in the form of S-nitrosation (Zhang and
Dryhurst 1994; Spencer et al. 1998), whereas inhibition of Complex IV activity may be due to
irreversible binding of ONOO\(^-\) in competition with molecular oxygen (Brown, 2001).
Subsequently, excessive ONOO\(^-\) reacts predominately with tyrosine and cysteine residues of
proteins, which are the major component of other associated mitochondrial complexes, thus
inhibit their activity (Radi et al. 2002).

The present findings demonstrated that pre-treatment with withanolides and
curcuminoids significantly attenuated the compromised mitochondrial complex activity in the
mid brain of MPTP-intoxicated mice. Withanolides administration specifically protected NADH
dehydrogenase and cytochrome c oxidase activity which may be due to the modulation of NADH
oxidation by scavenging the ROS and ONOO\(^-\). However, no significant changes were observed in
Complex II-III activity. Several preclinical and clinical findings suggested that root extract of *Withania somnifera* possess immense antioxidant properties (Bhattacharyya et al. 2001; Naidu et al. 2003; Sankar et al. 2007) which scavenges reactive oxygen species and quenches the superoxide and hydroxyl radicals, which may be one of the mechanisms to protect mitochondrial complexes dysfunction. Similarly, another study by Kumar and Kumar (2009) has also reported the neuroprotective effect of WS root extract over 3-Nitropropionic acid (3-NP) induced rat model of Huntington's disease (HD), through modulating antioxidant enzymes and mitochondrial complexes in dose dependent manner. However, the present study have reported for the first time the neuroprotective effect of withanolides against mitochondrial complexes impairment in MPTP model of PD. Interestingly, in our correlation studies significant positive correlation was obtained between the improvement in mitochondrial complex-II and grip retention time test in MPTP induced withanolides treated group. From the correlation analysis it can be speculated that mitochondrial complex II (MC II), via an interaction with D2 receptors, directly regulates the levels of DA in striatal neurons. Moreover, it is evident that MC II has an important role at the intersection of the TCA cycle and the respiratory chain, since the reduction of succinate to fumarate results in electron feeding to ubiquinone and complex III (ubiquinone–cytochrome c oxidoreductase). Thus the present findings suggest that MC II plays a key role in oxidative energy metabolism. Therefore it can be suggested that in diseased condition where DA metabolism or levels are increased, as during hypoxia, ischemia and intoxication with mitochondrial poisons, the down-regulation of MC II would further increase the vulnerability of dopaminergic cells to cell death.

Correspondingly, curcuminoids administration significantly protected and restored the complex I activity in treated group as compared to diseased control group. The present findings were in compliance with previous studies which reported that curcumin protect mitochondria from oxidative damage and attenuate apoptosis in cortical neurons (Zhu et al. 2004). In vitro
findings of present study suggested that curcuminoids possess potent NO scavenging activity by inhibiting the nitrite formation and thus reducing the peroxynitrite formation. The findings are consistent with previous published reports where curcumin has attenuated the peroxynitrite formation in various cell lines (Kim et al. 2003; Liu et al. 2011). It has been suggested that chronic exposure of ONOO' modifies the tyrosine residue of Complex I to 3-nitrotyrosine (3-NT) at post translational levels resulting to reduced Complex I activity (Muray et al. 2003). Considering the fact that curcuminoids exert potential ONOO- scavenging activity even better than the standard antioxidant vitamin E (Reddy and Lokesh, 1992) and ONOO- contribute majorly in mitochondrial Complex I impairment, it was suggested that in the present study the prevention of MPTP induced mitochondrial impairment on curcuminoids treatment was attributed to its significant antioxidant activity.

In-addition to mitochondrial Complex I activity, significant prevention of other mitochondrial complex i.e II, II-III and IV activity were also observed in curcuminoids treated MPTP induced mice when compared with diseased control group. This is in accordance with another study, where curcuminoids treatment significantly ameliorated the mitochondrial impairment in diabetic rat brain (Rastogi et al. 2008). Moreover, the same may also be due to the direct inhibition of MPP' neurotoxicity as evident in one study where curcumin can directly inhibit MPP' toxicity in PC12 neuronal cell line (Chan et al. 1998). In-addition, to the attenuation of dopaminergic degeneration, significant improvement was also observed in the depressive behaviour among curcuminoids treated MPTP mice due to the amelioration of mitochondrial functioning as evident in correlation studies where a positive correlation was obtained between complex-II and tail suspension test. Though, the exact pathophysiology of depression is not clearly understood. Several reports are available which suggest impairment in brain metabolism as a mechanism underlying depression. In this context, Gardner and co-workers (2003) have demonstrated a significant decrease of mitochondrial ATP production rates and mitochondrial enzyme ratios in
muscle compared to controls in major depressive disorder patients. Moreover, Madrigal et al (2001) have reported that chronic stress inhibited complexes I-III and II-III of mitochondrial respiratory chain activity in rat brain. Therefore it can be suggested that the oxidative damage induced by stress may be either the cause or the consequence of the mitochondrial dysfunction (Madrigal et al. 2001; Boekema and Braun 2007; Torres et al. 2004).

However, present findings demonstrate that deprenyl treatment did not exerted significant effect on mitochondrial complexes. Although reports are available that deprenyl treatment attenuates 6-OHDA toxicity by modulating the mitochondrial complex activity (Sarvanan et al. 2006). Furthermore, cumulative research findings suggest that hyper activation of nNOS triggers excessive NO production which plays a key role in PD pathogenesis (Shen et al. 2005). Reports are available that NO and its toxic metabolite ONOO− exacerbates mitochondrial respiratory chain by attenuating complex I, II, and IV activity resulting to energy failure and ultimately cell death (Stewart and Heales 2003). In the present study western blot data showed enhanced neuronal nitric oxide synthase (nNOS) expression in the mid brain of MPTP-induced mice as compared with normal control group. The observations are consistent with other published report, where MPTP induction enhances the nNOS expression in striatum and concomitant increment of NO and ONOO− level in the mouse brain (Halasz et al. 2004) henceforth, it is worthy to note that inhibition of nNOS could be a novel neuroprotective strategy for the prevention and treatment of PD. The present findings suggest that pre-treatment with withanolides and curcuminoids, significantly attenuated nNOS expression in the MPTP+treated groups. These observations were in corroboration with other published reports where root extract of Withania somnifera and curcumin significantly reduced the nNOS activity on in-vivo and cell line culture studies (Bhatnagar et al. 2009; Chan et al. 1998; Epstein et al. 2010; Braidy et al. 2010). Moreover, our findings suggested that pre-treatment with withanolides and curcuminoids significantly reduced the total nitrite level, which were in accordance with our previous report and other published
reports where withaferin A, curcumin and curcuminoinds reduces the nitric oxide level in the treated groups (Rastogi et al. 2008; Khan et al. 2010; Song et al. 2011). Furthermore, as it is evident that nNOS attached to the NMDA receptor by PSD-95 protein (Christopherson et al. 1999) is enriched with Ca\(^{2+}\), which binds to calmodulin, and further Ca\(^{2+}\)-calmodulin complex activates the nNOS and thus excessive NO production occurs on toxic insult. The NO formed is ready to diffuse through membranes to neurons nearby, where it can react with superoxides, which further triggers the generation peroxynitrite which diffuses throughout the cells and membranes and exert deleterious effect over several biomolecules (Salvemini et al. 2006). Pertaining to it, it can be assumed that withanolides and curcuminoinds have greater affinity for NMDA receptor and it may modulates Ca\(^{2+}\)-calmodulin complex and thus inhibits NO generation, however it warrants further research to test the hypothesis. The present findings suggest that deprenyl treatment did not exerted significant effect on nNOS expression, which was in contrast with other published report (Czerniczynece et al. 2007).

The significant effect of both the putative drug candidates over mitochondrial functioning in MPTP induced model of PD was further reflected in the overall ATP content which was found to be elevated in the treated groups when compared with the MPTP control. Therefore, based upon the present observations it was suggested that the amelioration of mitochondrial functioning was one of the neuroprotective mechanism involved in the prevention of dopaminergic degeneration in the MPTP-induced PD model.

In addition to the perturbed energy metabolism, mitochondrial impairment leads to dopaminergic cell death through the elevated generation of ROS and RNS thus impairing the redox homeostasis. Despite numerous proposed hypotheses, oxidative stress still remains the leading theory behind the etiology of PD (Miller et al. 2009). The data of the present study has demonstrated elevated oxidative stress as indicated by the compromised activity of endogenous antioxidant enzymes in the ventral mid brain of MPTP-induced mice. The results showed a
significant decrease in glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase activity with concomitant increased levels of TBARS, LHP and protein carbonyl content (PCC) in MPTP-induce mice as compared with normal control. These observations were in accordance with the previous published reports (Mohanasundari et al. 2006; Han and Zhao 2010). Dopaminergic neuronal cells of substantia nigra pars compacta (SNpc) are particularly more vulnerable for oxidative damage due to the presence of high concentrations of iron, low contents of endogenous antioxidants relative to the other regions of the brain (Sofic et al. 1991; Kedar et al. 1999) as well as dual production of ROS from impaired mitochondria and dopamine (DA) metabolism (Chinta and Andersen, 2008).

Oral administration of withanolides, curcuminoids or standard deprenyl significantly enhanced the SOD activity in MPTP induced mice brain when compared with diseased group and the results were in compliance with the previous published reports (Rajasankar et al. 2007; Maheshwari et al. 2010; Rajeshwari et al. 2006; Sarvanan et al. 2006). Moreover, in the present correlation studies a positive correlation was obtained between super oxide dismutase and motor coordination in the withanolides treated group (Rajasankar et al. 2007). Based upon the present correlation studies it was suggested that the significant quenching of superoxide anions on withanolides treatment might have modulated the oxidative stress mediated mitochondrial dysfunction as well reduced autoxidation of dopamine content thereby reduced progressive dopamine depletion as indicated in improved motor performance in MPTP induced mice.

Treatment with curcuminoids significantly protected and restored the catalase and GPx whereas withanolides was found to exert significant effect over catalase level alone as compared to diseased control group. However, deprenyl did not exert significant effect over any of the enzymes. The present results were in accordance with the previous reports (Maheshwari et al. 2010; Rajeshwari et al. 2006).
Among the various endogenous antioxidant marker enzymes, the magnitude of GSH depletion appears parallel to the severity of disease and may also act as earliest known indicator of the nigral dopaminergic degeneration (Bharat et al. 2002). In the present study, withanolides, curcuminoids and deprenyl administration significantly restored the GSH content in treated groups and the results were consistence with other published report (Rajasankar et al. 2009; Rastogi et al. 2008; Sarvanan et al. 2006). Curcumin has been reported to induce GSH synthesis through up-regulation of γ-GCL (Dickinson, 2003) or inhibiting the glutathione reductase enzyme activity. Similarly, a recent study has demonstrated that administration of root extract of *Withania somnifera* restored the GSH content in the striatum of MPTP-induced mice and thus supports its potent antioxidant activity (Rajasankar et al. 2009). Furthermore, deprenyl treatment also reduces the oxidative stress by ameliorating the GSH content in the rat brain (Leret et al. 2002).

GSH depletion has also been demonstrated to be involved in Complex I inhibition via direct thiol oxidation of important complex I residues (Jha et al. 2000, Chinta et al. 2007). In addition decrease in GSH content in the dopaminergic neurons leads to the poor scavenging dopaminergic quinones and superoxide radicals produced during the DA auto-oxidation, which are also capable of inhibiting mitochondrial complex I activity and thus reduced ATP production (Berman et al. 1999; Zhang and Dryhurst 1994; Spencer et al. 1998). Thus, the significant effect of withanolides and curcuminoids over mitochondrial complexes with overall improvement in dopaminergic content may also be attributed to their prominent GSH enhancing activity.

The findings of the present study further demonstrated the reduced level of oxidative stress on withanolides and curcuminoids or deprenyl administration as indicated by reduced thiobarbituric acid reactive species (TBARS), lipid hydro peroxide (LHP) and protein carbonyl contents in MPTP induced mice brain. Previous studies also supported the present findings where leaf extract of *Withania somnifera* and curcumin demonstrated significant peroxy radical scavenging effect in various models (Bhattacharya et al. 2001; RajaSankar et al. 2009, Rajeshwari
Moreover, deprenyl treatment also reduced the TBARS content but did not exerted effect over LHP content. It is evident that lipid peroxidation is initiated by free radicals on the membrane lipids, which are capable of abstracting a hydrogen atom from the methylene group. The radical thus formed is stabilized by molecular rearrangement to produce conjugated diene, which easily reacts with an O₂ molecule to give a peroxy radical. The peroxy radical can further abstract a hydrogen atom from another lipid molecule to form lipid hydro-peroxides, which exert deleterious effect over neurons. Further in correlation analysis, negative correlation trend was observed between LHP and open field activity in withanolides treated group; supports the present findings that withanolides inhibits lipid peroxidation through scavenging the TBARS content and thus alleviate the depressive symptoms through modulating the oxidative stress (Tsuboi et al. 2004). Although, it is apparent that depression may contribute to the production of reactive oxygen species, and thus lead to increased oxidation of lipid molecules. Therefore, based upon the correlation studies it is hypothesized that the improvement in gross behavioural activity on withanolides administration was may be due to its significant anti-lipid peroxidation activity. The interesting correlation between LPO and depression supports the hypothesis that depressive symptoms are closely related to LPO; however the underlying mechanism linking depressive symptoms and lipid peroxidation associated oxidative stress warrants further research.

Similarly, among the curcuminoids treated group a negative correlation was obtained between lipid peroxide/lipid hydro peroxide and rota rod performance. From the results and subsequent correlation analysis, it is apparent that curcuminoids attenuated lipid peroxidation as depicted by enhanced TBARS content, following the MPTP intoxication and ameliorates the DA level and thus improving the motor coordination. Agreement with present observation, several reports are available, which suggest that the by-products of lipid peroxidation particularly malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) could disrupt many important membrane functions such as oxidative phosphorylation, signal transduction, and regulation of
electron, iron, and metabolite transport and thus may be in-part contribute to nigral dopaminergic degeneration in PD (Yoritaka et al. 1996; Dexter et al. 1989). Therefore, based upon correlation studies it is proposed that significant prevention of dopaminergic degeneration on curcuminoids administration was due to its potent anti-lipid peroxidation activity and thus validates its antioxidant activity.

The present findings demonstrated the significant reduction of protein carbonyls content on withanolides and curcuminoids administration in MPTP induced mice brain (Parihar et al. 2004; Lim et al. 2001). Increased protein carbonyl content is a direct indicator of protein oxidation and oxidative damage as evident in various pre clinical and clinical studies (Alam et al. 1997; Floor and Wetzel 1998; Nystrom 2005). Several studies (Oliver et al. 1987; Stadtman et al. 1992) have demonstrated that in PD brain protein carbonyl modification primarily raised arises from iron catalysed oxidation. Since, substantia nigra is rich in neuromelanin content therefore it is highly susceptible for protein carbonyls mediated dopaminergic neuronal damage. The protective effect exerted by the curcuminoids are may be in-part due to their chelating activity (Jiao et al. 2006).

Apart from potential effect of withanolides and curcuminoids over endogenous antioxidant marker enzymes and ROS mediated oxidative damage, the present study also evaluated the effect of putative drug candidates over reactive nitrogen species as detected by the total nitrite level and nNOS protein expression in MPTP induced mice brain. Pre-treatment with withanolides and curcuminoids significantly reduced the total nitrite level, which were in accordance with other published reports where withaferin A and curcumin reduced the nitric oxide level in the treated groups (Khan et al. 2010; Song et al. 2011). In the protein expression studies, both withanolides and curcuminoids significantly down regulated the neuronal nitric oxide synthase (nNOS) expression and NO level in the striatum of MPTP-induced mice as compared with diseased control group (Halasz et al. 2004, Bhatnagar et al. 2009; Braidy et al.
NO mediated neurotoxicity has been implicated in the pathogenesis of PD. It is reported that nNOS attached to the NMDA receptor by PSD-95 protein (Christopherson et al. 1999) is enriched with Ca$^{2+}$ which binds to calmodulin and further activate nNOS over-expression and thus excessive NO production. The NO formed readily diffuses to neurons where it can react with superoxides and produces toxic peroxynitrite which exert deleterious effect over several biomolecules (Salvemini et al. 2006). NO and its toxic metabolite ONOO- directly impair the mitochondrial respiratory chain complexes thus leading to energy failure and ultimately cell death (Stewart and Heales 2003). Curcuminoids has been reported to be the specific peroxynitrite inhibitor even better than standard antioxidants (Reddy and Lokesh 1992). Furthermore, withanolides administration has also exerted significant effect on carbonyl content. It is evident from the recent studies that standard root extract of *Withania somnifera* has potential effect over modulation on protein translation and neuro-regenerative properties. Recent study has reported that, *Withania somnifera*, induces growth regulators including phosphor inOSitide-3 kinase (PI3K) and up-regulation of PI3K induces ribosomal p70 S6 kinase (p70s6k) to regulate translation of proteins and cell growth (Surendran 2009). Moreover, deprenyl treatment did not exert any significant effect over carbonyl content. Based upon the present studies it is speculated that withanolides and curcuminoids exhibit neuroprotective effect either by attenuating nNOS expression or by direct scavenging of toxic peroxynitrite radicals.

From the present findings we can speculate that curcuminoids scavenging properties are may be due to the presence of polyphenols as a major skeleton and they can neutralise the oxidising state of the deleterious highly active molecules as super oxides, hydroxyl ions and carbonyl content. Moreover, consistence to our findings other studies have demonstrated the protective effects of plant phenolic against brain damage in PD (Sun et al. 2008). These studies have used either a single compound such as curcumin, resveratrol, EGCG, or a complex mixture of extracts from grape, blueberry and green tea exerted neuroprotective effect over various PD
models (Weinreb et al. 2004; Mercer et al. 2005; Chen et al. 2007; Masuda et al. 2006). The neuroprotective effects of these phenolic compounds are attributed in-part through the free radical scavenging, iron/metal chelating, and their anti-oxidant properties. Cumulative evidences suggest that phenolic compounds can target specific signalling pathways and interact with specific proteins, including aggregation of α-synuclein (Masuda et al. 2006; Ramassamy 2006; Vafeiadou et al. 2007). In a recent study curcumin possess the ability to scavenge oxygen derived free radicals, which implicated its potential as a neuroprotective agent (Sharma, 2009). Other studies also suggest that curcumin possess immense potential as a therapeutic candidate for the prevention and treatment of neurological disorders. Several studies including, cerebral edema, a cause of increased intracranial pressure after acute brain injury, was significantly controlled by pre-treatment as well as post treatment with curcumin (Thiyagarajan, 2004). Similarly, administration of curcumin after inducing cerebral ischemia indicated neuroprotection activity against stroke (Longa, 1989). Moreover, curcumin has a potential to increase the cholinergic activity of neurons in streptozotocin- induced dementia in rats (Awasthi, 2010). Effects of curcumin on the pathophysiology of Alzheimer's disease (AD) have been studied and several groups have shown its ability to inhibit Aβ-plaque formation (Lim, 2001; Perl, 2010; Yang, 2004). Also, curcumin has been proposed as a potential candidate to treat PD (Jagatha, 2008; Zbarsky, 2005). Liu and co-workers (2011) showed that curcumin can inhibit mitochondrial cell death against PD. Moreover, chronic stress and or acute toxic insult may be a risk factor for the onset of depression that induces neurodegeneration in hippocampal neurons. It has shown that curcumin administration can increase hippocampal neurogenesis in chronically stressed rats (Xua, 2007). Taken together, it can be assume that one of the possible mechanism underlying the neuroprotective effect of curcuminoids against MPTP neurotoxicity, as shown in the present study, may involve its catechol-like structure, since it is known that catechol-containing/polyphenols/alkaloids compounds are potent radical scavengers.
In addition to mitochondrial dysfunction and oxidative stress, inflammation has also been implicated for dopaminergic degeneration in PD (Depino et al. 2003; Teismann and Schulz 2004; Minghetti, 2004). In the present study, the up-regulated expression of GFAP protein indicate the hyper-activation of microglial cells on MPTP induction which is in accordance with the previous studies (O Callaghan et al. 1990). Microglial activation seems to be major drive in PD progression, since robust microglia activation has been observed in humans as well as in primates and rodents after MPTP intoxication (Przedborski and Vila, 2003, Langston et al. 1999; Liu and Hong, 2003). Furthermore, SNpc has been hypothesized to be particularly at higher risk for inflammation as it has the highest density of microglia in the brain (Lawson et al. 1990). Therefore, suppression of glial activation might be a promising therapeutic approach in PD treatment and management (Yokoyama et al. 2008; Schintu et al. 2009). Moreover, microglial hyper activation has been readily associated with toxic inflammatory reactions, such as the release of pro-inflammatory mediators, including cytokines, prostaglandins and free radicals (Liu and Hong, 2003; Teismann et al. 2003). Pertaining to it, in the present study we have observed a significant elevation in the levels of IL-1β and TNF-α, however, IL-6 level was found to be unaltered in the striatum of MPTP-induced group (Ojha et al. 2012). Consistent with present findings previous studies also reported elevated levels of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 in the striatum of MPTP-induced mice brain (Mogi et al. 1994; Blum-Degen et al. 1995; Muller et al., 1998). Furthermore, activated glial cells expressing pro-inflammatory cytokines, as TNF-α, IL-1β and IFN-γ were over expressed in the substantia nigra (SN) of PD patients (Hunot et al. 1996; Hirsh et al. 1998). It is evident that the inflammatory cytokines, along with other factors released from the dying dopaminergic cells, seem to amplify and sustain the neuro-inflammation resulting to irreversible destruction of SN dopaminergic neurons (Orr et al. 2002).

The present study also demonstrated significant up-regulation of iNOS protein expression in MPTP-induced mice brain (Ojha et al. 2012) which was in compliance with other published
reports (Liberatore et al. 1999; Iravani et al. 2002). It is evident that iNOS expression usually remains dormant and only gets expressed in presence of toxic insult, usually in presence of elevated pro-inflammatory cytokines (Liberatore et al. 1999; Dehmer et al. 2000). Furthermore, iNOS exerted cytotoxic effect through NO mediated neurotoxicity, which was intricately associated with PD pathogenesis. Excessive NO interacts with superoxide anions to form reactive nitrogen species (RNS), which leads to extensive cellular injury culminating into dopaminergic cell death (Dawson et al. 1993; Przedborski et al. 1996; Nagatsu and Sawada 2007). The present study demonstrated a significant elevation in NO generation, as quantified by the increased total nitrite content in MPTP-induced mice brain (Liberatore et al. 1999). Collectively, the excessive generation of pro-inflammatory cytokines, iNOS protein expression and NO content potentially culminating to dopaminergic degeneration as suggested unequivocally by present as well as various pre-clinical and clinical studies (Allan and Rothwell, 2001; Fisher et al. 2001; Gayle et al. 2002; Liu et al. 2002; Ma and Ma, 2002; Sriram et al. 2002; Ojha et al. 2012). It has been suggested that after the initial toxic insult, activation of microglia concurred which potentially gets amplified with disease progression thereby leading to dopaminergic cell death (Gao et al. 2003b).

The present study demonstrated significant down regulation of glial fibrillary acidic protein expression in the striatum and substantia nigra of withanolides treated groups as compared to MPTP-alone group. Consistent with present findings, recent study conducted by Bargagna-Mohan and co-workers (2010) have demonstrated that withaferin-A targets intermediate filaments glial fibrillary acidic protein and vimentin in a model of retinal gliosis through covalent modification in cysteine residue of GFAP. Furthermore, the data of the present study suggested that withanolides pre-treatment, significantly improved IL-1β and TNF-α levels in treated group however, no alteration was observed in the IL-6 level as compared to diseased control. Consistent to present findings, recent study conducted by UjlaMinhas and co-workers (2011) have reported that root extract of *Withania somnifera* inhibited over expression of pro-
inflammatory cytokines particularly IL-6 and TNF-α on pristane-induced model of systemic lupus erythematosus (SLE). A significant suppression of iNOS protein expression with concomitant reduction in total nitrite content was observed followed by withanolides treatment when compared MPTP-alone group. In another study, withaferin A was reported to attenuate iNOS expression and nitric oxide production through Akt inactivation and reduction in LPS-induced activity of NF-κB in cell line as a dose dependent manner (Oh et al. 2008). From the present findings it was hypothesized that the neuroprotective activity of withanolides was also attributed to its potent immunomodulatory activity which may be in-part due to suppression of NF-κB activity.

Concurrently, pre-treatment with curcuminoids significantly attenuated GFAP expression and IL-1β and TNF-α level in the striatum of MPTP-induced group. In the recent study, Yang and co-workers (2008) have indicated that curcumin exerted neuroprotective effect specifically through attenuating microglial activation and NF-κB mediated signalling cascade in LPS and MPP⁺ induced dopaminergic degeneration in neuron-glia cultures. The present in-vivo results were in accordance with the previous published report and hypothesize that curcuminoids might have prevented dopaminergic neurodegeneration through the inhibition of microglial mediated inflammatory cascades in MPTP model of PD. Interestingly; we have noticed that inhibition of TNF-α on curcuminoids treatment was significantly associated with the improvement of motor coordination in rota-rod assessment (Ojha et al. 2012). Pertaining to it, cumulative pre-clinical and clinical findings have suggested the involvement of TNF-α in dopaminergic neurodegeneration (Hunot et al. 2001). Deficiency of TNF-α receptors was reported to suppress the microglial activation and reduced susceptibility to MPTP-induced neurotoxicity (Sriram et al. 2006). Therefore, based upon the correlation studies, it is postulated that the neuroprotective effect of curcuminoids in the MPTP-induced dopaminergic degeneration was attributed to the inhibition of microglia mediated exacerbated pro-inflammatory cytokines generation, including
The present immunoblot data observed a significant down-regulation of iNOS protein expression with concomitant reduction of total nitrite content in the striatum of curcuminoids treated group. Recent studies reported that curcumin act as a potent iNOS inhibitor by modulating the inflammatory response through down-regulation of the mRNA production for pro-inflammatory cytokines and iNOS (Nanji et al. 2003; Yang et al. 2008). In addition, our previous published report and other studies demonstrated the potent NO scavenging activity of curcuminoids equivalent to the standard antioxidant vitamin E (Sreejayan and Rao 1997; Rastogi et al. 2008). Therefore, it is suggested that curcuminoids treatment might have protected the dopamine depletion either by inhibiting the iNOS protein expression and/or ONOO· mediated oxidative modifications. Moreover, the present study results also revealed that curcuminoids exerted better effect than standard deprenyl over inflammatory biomarkers in MPTP model of PD. Thus, collectively present data suggest that curcuminoids prevents dopaminergic neurodegeneration by attenuating the microglial activation, inhibited pro-inflammatory cytokine generation, down-regulated iNOS expression and scavenge the reactive nitrogen species in the MPTP induced mice brain.

Therefore, the present study demonstrated the pleiotropic protective mechanism of action of withanolides and curcuminoids against MPTP induced dopaminergic degeneration. Our observations suggest that both withanolides and curcuminoids possess immense potential as a putative drug candidate and warrants further research to extrapolate preclinical findings into clinical studies for better treatment and management of PD.