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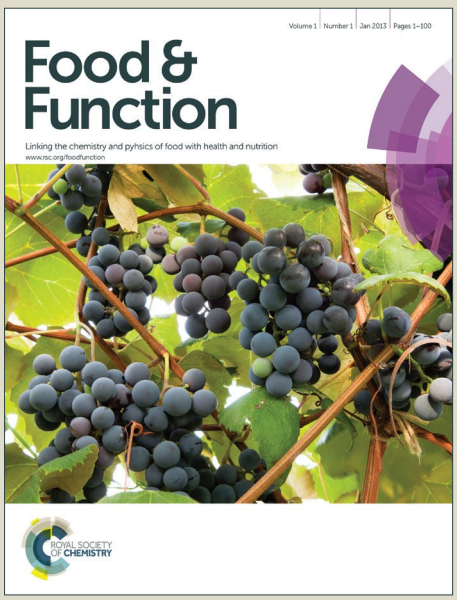

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Title:

Amelioration of apoptotic events in the skeletal muscle of intra-nigrally rotenone infused Parkinsonian rats by *Morinda citrifolia* – Up-regulation of Bcl-2 and blockage of cytochrome C release

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Abstract

Parkinson's disease is a progressive neurodegenerative movement disorder with the cardinal symptoms of bradykinesia, resting tremor, rigidity, and postural instability, which lead to abnormal movements and lack of activity which in turn cause muscular damage. Even though studies have been carried out to elucidate the causative factors that lead to muscular damage in Parkinson's disease, studies related to apoptotic events that occur in the skeletal muscle and a therapeutical approach to culminate the muscular damage has not been extensively studied. Hence, this study evaluates the impact of rotenone induced SNPc lesion on skeletal muscle apoptosis and the efficacy of ethyl acetate extract of Morinda citrifolia in safeguarding the myocytes. Biochemical assays along with apoptotic markers

studied by immunoblot and Reverse Transcription–Polymerase Chain Reaction in the current study revealed that supplementation of Morinda citrifolia significantly reverted the alterations in both biochemical and histological parameters in rotenone modeled PD rats. Treatment with Morinda citrifolia also reduced the expression of pro-apoptotic proteins Bax, caspase-3 and caspase-9 and blocked the release of cytochrome c from mitochondria induced by rotenone. On the other hand, it augmented the expression of Bcl2 both transcriptionally and translationally. Hence, this preliminary study paves a way to show cast that the antioxidant and anti-apoptotic activities of Morinda citrifolia can be exploited to alleviate skeletal muscle damage induced by Parkinsonism.

Keywords: Apoptosis, Morinda citrifolia, Oxidative stress, Parkinson's disease, Skeletal muscle.

Abbreviations: LD- Levodopa; MCE- Ethyl acetate extract of Morinda citrifolia fruit; PD- Parkinson's disease; ROS- Reactive oxygen species**;** Rot Ind – Rotenone Induced rats; SNPc- Substantia Nigra Pars Compacta; VTA- Ventral tegmental area

1.0 Introduction

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder that results primarily from the death of dopaminergic neurons in the substantia nigra disturbing the output from the basal ganglia to the lower motor neurons and altering the pattern of activation of skeletal muscles.[1] The skeletal muscle has no intrinsic spontaneous activity because it lacks the ion channels like cardiac muscles, which are responsible for spontaneous membrane depolarization[2], disruption in the neuromuscular signalling leads to increased resistance to passive movements[3], and an increased activity in antagonist muscles[4] which features the

cardinal symptoms of Parkinson's disease like bradykinesia, resting tremor, rigidity, and postural instability.[5] These abnormal movements and lack of activities in turn leads to muscular damage.

Pharmaceutical treatments for Parkinson's either increase the levels of dopamine, or mimic its action. Thus, mainstream pharmaceutical treatments cannot be expected to address the underlying cause of disease progression - neurodegeneration. In this regard, if a neuroprotective drug used for treating PD not only safeguards the substantia nigral neurons from degeneration, but also the effector organs from further lesions, the efficacy of the treatment shall be doubled. Recent investigations have clearly established the therapeutic effects of natural compounds in neurodegenerative diseases, and studies are being carried out to determine the complex molecular events associated with the therapeutic effects of these natural compounds.

Morinda citrifolia popularly known as "Noni" is a medium sized ever green tree distributed throughout the tropics in Southern and Northern hemispheres.[6] All parts of the plant, including leaves, fruits, bark and roots contain several pharmacologically active components, namely, anthraquinones, flavonoids, iridoids and oligosaccharides, exhibiting therapeutic potentials such as anticancer, antiviral, antibacterial activities and are used to treat scopolamine, β-amyloid, streptozotocin induced memory impairment in animals.[7-11]

However, the literature evidence for the skeletal muscle degeneration at the molecular level in rotenone model of Parkinsonism and the therapeutic efficacy of *Morinda citrofolia* in combating the rotenone induced PD in rats with reference to skeletal muscle is vague. Therefore, the current study evaluates the efficacy of

Morinda citrofolia in protecting the skeletal muscles of Parkinsonian rats induced by stereotaxical infusion of rotenone.

2.0 Materials and Methods

2.1 Chemicals, Solvents, and Extraction

Scopoletin, rotenone and bovine serum albumin were procured from Sigma-Aldrich, USA. All other chemicals used were of analytical grade obtained from Merck Chemical Supplies (Darmstadt, Germany), Sisco Research Laboratories (SRL, India), and S.D. Fine Chemicals, Mumbai, India. The fruits of *Morinda citrifolia* (Noni) were collected from NCRN farm, Shalavakkam, Tamilnadu. Authentication of plant material was done by Prof. P. Jayaraman of Plant Anatomy Research Centre, Tambaram, Chennai. Before starting the extraction, the fruits were washed twice and were shade dried for 7 days. Extraction was carried out according to the method followed by Mohd *et al.* with slight modifications.[12] The fruit samples collected were grounded to powder and was extracted with ethyl acetate in a shaker at room temperature for 3 days. The samples were filtered and the filtrates were oven dried to yield a green viscous mass. *In-vitro* studies were carried out to determine the antioxidant property[13] of ethyl acetate extract of *Morinda citrifolia* fruit (MCE) and a pilot study were carried to determine the optimal dose of MCE *in-vivo*. The optimal dose was determined to be 150mg/kg body weight, fixed based on the potential degree to scavenge the free radicals and to boost the antioxidant status.

2.2 Quantitation of Phyto-constituents in MCE

Scopoletin, quercetin and rutin in MCE was quantitated by high performance liquid chromatography using a C-18 column.[14] MCE (150mg) was dissolved in methanol (2ml). The internal standard solution (scopoletin, quercetin

and rutin were dissolved in methanol at the concentration of 1mg/mL) was prepared and both the MCE and standard solutions (20µl) was subjected for HPLC analysis. The mobile phase consisted of 0.1% phosphoric acid (A) and methanol (B), and a gradient elution was programmed as follows: A/B: 60/40 (0–10 min) and 30/70 (30–40 min). The flow rate was 1.0 mL/min and detection wavelength was set at 340 nm.

2.3 Animals and Surgical Procedures

Healthy male Sprague-Dawley rats were used throughout the study. The animals were procured from Central Animal House Facility, Dr. ALM PGIBMS, University of Madras, Taramani Campus, Chennai - 600 113, India. All experiments were performed with animals approved by the Institutional Animal Ethical Committee **(IAEC No. 01/09/12)** in compliance with the relevant laws and institutional guidelines. The animals were housed under conditions of controlled temperature (25 ± 2°C) with 12/12 h light/dark cycle and were given food and water *ad libitum*.

The animals were divided into five groups consisting of six animals each. Group I rats served as control, while Group II to Group V rats were subjected to stereotaxic surgery. Group III, IV and Group V rats were stereotaxically infused with rotenone to induce Parkinsonism. Briefly, rats were anaesthetized with ketamine hydrochloride and xylazine (80 mg/kg and 10mg/kg; i.p.) and placed on a small animal stereotaxic frame (Stoelting, IL, USA). Rotenone dissolved in DMSO was infused into the right Ventral Tegmental Area (VTA, AP: 5.0 mm; L: 1.0 mm; DV: 7.8 mm) and into the right Substantia Nigra Pars compacta (SNPc, AP: 5.0 mm; L: 2.0 mm; DV: 8.0 mm) each 6µl at a flow rate of 0.2 µl/min using a Hamilton 26 gauge needle.[15] The infusion needle was left in place for additional five minutes for complete diffusion of the drug. In sham operated controls (Group II), the rats received 6 µl of the vehicle

(DMSO and PEG in the ratio of 1:1) instead of rotenone during stereotaxic surgery. After post-operative recovery i.e. after two weeks, Group IV rats were treated with Levodopa (LD, 10mg/kg with 25mg/kg benserazide[16]) for the next 30 days. While Group V rats were pre-treated with MCE for 30 days prior to stereotaxic surgery and the treatment was continued after recovery for a further period of 30 days.

At the end of the experimental period, rats were anesthetized with ketamine (22 mg/kg, i/m) and blood samples were collected via cardiac puncture into the anticoagulant-contained and anticoagulant-free test tubes. Skeletal muscles (Gastrocnemius muscle) were excised immediately, immersed in ice-cold physiological saline, and weighed. Small sections from each tissue were kept aside for histological studies. A 10% tissue homogenate was prepared by using Tris-HCl buffer (0.01 M, pH 7.4), followed by centrifugation at 12,000 rpm for 10 min. The supernatant was used for the analysis of various parameters. The part of tissue was immediately used for the isolation of mitochondria for further analyses and the rest of the tissue was stored at -80^{o}C for gene and protein expression studies. Protein concentration of the tissue homogenate was determined by the standard method of Lowry *et al.* [17] using bovine serum albumin as standard.

2.4 Histopathological studies

Histology of skeletal muscle was studied using haematoxylin and eosin (H and E). A portion of skeletal muscle tissue was fixed in 10% buffered formalin. The washed tissues were dehydrated in the descending grades of isopropanol and finally cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 µm thickness, stained with Haematoxylin and Eosin (H&E). The tissue sections were then viewed under light microscope (Nikon microscope ECLIPSE E400, Japan) for histopathological changes.

2.5 Skeletal muscle marker enzymes (Serum / Tissue)

Skeletal muscle marker enzymes, such as Creatine Kinase (CK), Lactate Dehydrogenase (LDH), and Aspartate Transaminase (AST), in serum were analyzed using commercial kits from Spinreact in semi-auto analyser (Rx Monza, Randox, UK). The enzyme assays in skeletal muscle were carried out using standard protocols as described previously.[18-20]

2.6 Oxidative Stress Markers

Oxidative stress markers such as hydrogen peroxide, nitric oxide, lipid peroxides and protein carbonyls were assayed as described previously.[21-24]

2.7 Assessment of Antioxidant Defense Systems

The enzymatic antioxidants superoxide dismutase, catalase, Glutathione Peroxidase, Glutathione reductase and non-enzymatic antioxidant reduced Glutathione were assayed using standard protocols as described elsewhere.[25-29]

2.8 Isolation of skeletal muscle Mitochondria

Mitochondria of the skeletal muscle were isolated by the method of Ernster and Nordenbrand.[30]

2.9 Gene Expression Studies by Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the skeletal muscle tissue using total RNA isolation reagent (TRIZOL, Invitrogen, Carlsbad, CA, USA). Oligonucleotide primer sequences of the selected genes for reverse transcription–polymerase chain reaction (RT–PCR) were synthesized by Sigma–Aldrich (St. Louis, MO, USA) and Eurofins Genomics (India). The amplified products were separated by electrophoresis on 2% agarose gel and identified by ethidium bromide staining.

Specificity was confirmed by the size of the amplified products with reference to 100 bp DNA ladder (Bio vision, USA) and the band intensities were quantified by Quantity One Software (Bio-Rad, USA).

PCR Primer sequences and conditions used

S.No	Gene Name	*Primer sequence (5'- 3')*		Annealing temperature ° C	Amplic on size
1	**Bax**	*F*	*GCAGACGGCAACTTCAACTG*	59	158 bp
		R	*TGGATCCAGACAAACAGCCG*		
2	**Bcl2**	*F*	*CGTCAACAGGGAGATGTACCCC*	60	164 bp
		R	*ACTGCCAGGAGAAATCAAACAGA*		
3	**Caspase 3**	*F*	*GCTGGACTGCGGTATTGAGA*	59	108bp
		R	*TAACCGGGTGCGGTAGAGTA*		
4	**Caspase 9**	*F*	*CAGGCATTGTCATGTGTGCC*	59	155 bp
		R	*GAGACGCAGTCCAGATCCAG*		
5	**β-actin**	*F*	*GCCATGTACGTAGCCATC*	59	375 bp
		R	*GAACCGCTCATTGCCGAT*		

2.10 Immunoblotting

Samples containing 50–100 µg of proteins were separated by SDS-electrophoresis on 10–12% polyacrylamide gels and transferred to polyvinylidene difuoride membranes. The membranes were incubated with specific primary antibodies and the antibodies used were Bax, Bcl2 (Abcam 1:1000 dilution), Caspase-3, Caspase-9 (Cell Signaling Technology, 1:1000 dilution), and Cytochrome C (SantaCruz biotech, 1:1000 dilution). To verify the uniformity of protein load and transfer efficiency across the test samples membranes were re-probed with β-actin (Cell Signaling Technology, 1:1000 dilution). Immuno-reactive bands were developed with Immobilon Western-Chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA) and visualized by using an enhanced chemiluminescence system (Chemi-Doc, BioRad, USA) and presented in comparison to β-actin expression.

2.11 Statistical Analysis

The results are expressed as mean ± standard error mean (SEM). Differences between groups were analyzed by one-way analysis of variance (ANOVA) using the SPSS software package for Windows (Version: SPSS 20.0). Post hoc testing was performed for inter-group comparisons using the least significant difference (LSD) test; significance at p values <0.05 has been given by respective symbols in tables and figures.

3.0 Results

3.1 Characterization of MCE

Fig 1 shows the HPLC chromatogram of the ethyl acetate extract of *Morinda citrifolia* fruit. Rutin (2.6µg) was found to be in higher concentration followed by quercetin (1.4µg) and scopoletin (0.6µg) in 1mg of MCE. Therefore, a dose of MCE given to rats (150 mg/kg body weight) contains **392µg of rutin, 210µg of quercetin and 90µg of scopoletin** (Table 1).

3.2 Skeletal muscle integrity

To determine the effect of MCE on maintaining the integrity of skeletal muscle, the marker enzymes for skeletal muscle damage were assayed using standard protocols and the results are displayed in Table 2. The results portray that the activities of marker enzymes were significantly ($p<0.05$) lowered in the skeletal muscle of PD animals with the maximum decrease being observed with the CK accounting to about 36% followed by AST and LDH by 30% and 27% respectively, and elevated in the serum of rotenone induced animals when compared with that of the control rats. Administration of MCE or LD to the PD rats resulted in the significant decrement in the activities of CK, LDH and AST in the serum.

3.3 Histoarchitecture of Skeletal Muscle

Figure 2 shows the histopathological studies of skeletal muscle tissue in the rats stereotaxically infused with rotenone treated with or without *Morinda citrifolia* extract. Control muscle architecture seems to be normal as the muscle fibre was compact and the nucleus is in the periphery, same can be observed in sham control as well. However the fasciculation of muscle was seen in rotenone induced rats with rarefactions in the fascicle. Treatment with MCE or LD showed a remarkable recovery in terms of compactness of the muscle bundle and seems to be robust. Our results suggest that MCE prevents skeletal muscle damage as determined from the histopathological observations and marker enzymes.

3.4 Oxidative Stress Markers

Tissue damage is associated with redox imbalance in which there will be a shift towards the oxidant/pro-oxidant state. Hence, in the present study we evaluated various oxidative stress markers such as hydrogen peroxide, nitric oxide, lipid peroxides and protein carbonyls in the skeletal muscle to determine the effect of MCE against rotenone induced oxidative stress (Fig 3). The results reveal that there was a significant ($p<0.05$) increase in these oxidative stress markers (hydrogen peroxide, nitric oxide, MDA and protein carbonyls) in Group III skeletal muscle tissue on comparison with the control rats. MCE was capable of bringing back the levels of these oxidative stress markers by an average of 15% in rotenone administered rats, which signifies the potent radical scavenging property of *Morinda citrifolia*.

3.5 Enzymatic and Non-enzymatic Antioxidant status

Next, we tested whether MCE supplementation could boost the antioxidant status by assessing both the enzymatic (Superoxide Dismutase (SOD), Catalase,

Glutathione Peroxidase (GPx) and Glutathione reductase (GR)) and non-enzymatic (GSH) antioxidants in the skeletal muscle of PD rats. A ubiquitous decrease ($p<0.05$) in the activities of all the antioxidant enzymes (Fig 4) was observed in the skeletal muscle of these rats when compared to control rats. However, on supplementation with MCE or LD, a significant augmentation in the activities of all these enzymes was observed ranging from 27% to 40% with a maximum improvement in the GR. Non-enzymatic antioxidants showed a similar pattern as that of the enzymatic antioxidants. There was a decrement in the levels of non-enzymatic antioxidant GSH by about 16% in Group-III rats when compared with the control rats. MCE supplementation significantly increased the levels of GSH by about 13% when compared to Group III rats.

3.6 Protein and mRNA expression of Pro-apoptotic and Anti-apoptotic proteins

Intrinsic apoptotic pathway involves many different proteins, among which the Bcl2 family proteins and caspases play a major role. Hence, to elucidate the mechanism by which MCE acts as an anti-apoptotic drug we analyzed the mRNA and protein expression of pro-apoptotic Bax, caspase-3, caspase-9 and anti-apoptotic Bcl2 in the skeletal muscle of control and stereotaxically rotenone infused PD rats treated with or without MCE/LD (Figs. 5-8)**.** The mRNA and protein expression of Bax, Caspase-3, and Caspase-9 were significantly elevated in the skeletal muscle of rotenone induced Parkinsonian rats as compared to control animals, whereas the Bcl2 expression was significantly reduced. An increase of 79% in Bax/Bcl2 ratio was observed in the skeletal muscle of rats stereotaxically infused with rotenone when compared to that of the control rats; however, on co-treating with MCE or LD, the ratio was declined to 35% when compared with Group-III rats. The observed results dictate that MCE treatment has significantly inhibited the up-regulation of

the mRNA and protein expression of Bax and down-regulation of Bcl2 both at transcriptional and at the translational levels induced by rotenone. The mRNA and protein levels of caspase-3 and caspase-9 were also significantly reduced on co-treatment with MCE when compared to rotenone alone infused PD rats.

3.7 Cytochrome C release from Mitochondria

Western blot analysis of cytosolic and mitochondrial cytochrome C levels in the skeletal muscle of all the groups was performed, and the results are shown in Fig 9. Simultaneous increase of cytochrome C in the cytosolic fraction and a concomitant decrease in the mitochondrial fraction were observed in rats administered with rotenone when compared with the control rats. On treating the rats with MCE or LD, a concurrent decline in the levels of cytochrome c in cytosol and a sequential improvement in the levels of the same in skeletal muscle mitochondria were observed when compared to that of the rotenone alone administered rats. Decline in the cytosolic levels of cytochrome c suggests that MCE may prevent the mitochondrial damage and thereby blocking the release of cytochrome c into the cytosol and preventing the initiation of early apoptotic events.

4.0 Discussion

Studies pertaining to skeletal muscle damage in Parkinsonian rats and the impact of drug treatments at the molecular level are less pronounced in the literature. Hence the current study was designed to study the impact of rotenone induced lesion on the skeletal muscle and to evaluate the therapeutic efficacy of MCE against PD induced skeletal muscle damage.

GC-MS analysis of MCE (data not shown) showed the presence of many phyto-constituents along with scopoletin, rutin and quercetin which is well supported by literature evidences.[31-33] Hence HPLC analysis was carried out to

quantitate the levels of these phytoconstituents in the MCE and the results were depicted in Fig 1 and Table 1. The levels of quercetin were found to be 1.41 µg/mg of MCE which is 2-folds higher than that of the ethanolic extract of *Morinda citrifolia* fruit[32] and the levels of scopoletin and rutin were found to be 0.6µg and 2.61µg/mg of MCE which is 10-folds and 30-folds higher respectively than that of the fruit at different ripeness stages.[33]

Levels of skeletal muscle enzymes in serum are markers of the functional status of muscle tissue. An increase in the activities of these enzymes may represent an index of cellular necrosis and tissue damage following acute and chronic muscle injuries.[34] A significant increase was observed in the activities of creatine kinase (CK), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) in the serum, and a simultaneous decrease in the activities of these enzymes was observed in the skeletal muscle of stereotaxically rotenone infused rats.

Serum CK is usually elevated after the intramuscular injection, strenuous exercise, trauma, muscular injuries, acute psychosis, and restraint.[35] Takubo *et al.* have suggested that rigidity in PD patients might have been the cause for the elevated serum CK; however, they have not observed a correlation between serum CK and rigidity.[36] On the other hand, serum CK levels have been reported to be elevated in other conditions associated with rigidity like neuroleptic malignant syndrome and catatonia.[37, 38] Levodopa administration had reverted the levels of CK in serum to normal limits. Wei and Chen have reported that the withdrawal of levodopa led to increased serum CK levels in PD patients and administration of the same has brought back the levels to normal.[37] In this regard, MCE is also shown to bring back the levels of CK to normal limits suggesting the efficacy of MCE in safeguarding the muscular tissue. Similar results have been obtained by Anugweje

et al. who have demonstrated that Noni juice supplementation had a significant reduction in the magnitude of exercise induced elevation in serum creatine kinase levels of athletes.[39]

Muscle LDH activity is related to muscle fibre composition.[40] Lesions of the basal ganglia affect the expression of myofibrillar proteins in the muscle fibres.[1] Hence, rotenone induced lesion in SNPc may have altered the muscle fibre composition as evidenced by the morphological changes in the skeletal muscle and thereby damaging the muscular tissue which in turn might have led to decrease in the activity of LDH in the skeletal muscle and elevated levels in serum. In chronic muscle injury the serum levels of both AST and ALT are found to be increased, with AST as a major marker enzyme in muscle injury.[41] All the enzyme activities were comparatively elevated in the skeletal muscle and lowered in the serum on treating to the rotenone induced Parkinsonism rats with MCE.

In the present study, the levels of free radicals, hydrogen peroxide and nitric oxide were significantly elevated in the skeletal muscle of rotenone infused Parkinsonian rats. The consequence of the resulting increase in the oxidative stress is reflected as the increased levels of lipid peroxides and protein carbonyls in the skeletal muscle of these rats. Bashkatova *et al.* have reported that chronic administration of rotenone increases levels of nitric oxide and lipid peroxidation products in cerebral cortex and striatum of rats.[42] Increased reactive oxygen species (ROS) production in skeletal muscle has been associated with many conditions like aging, diet and physical inactivity.[43] PD induced physical inactivity might have been the cause for increased ROS production, as exercise is shown to strengthen the weakened muscle and the damaged brain in 6-OHDA induced PD rats.[44] Hence, the observed increase in ROS in the skeletal muscle might have been due to motor

inactivity induced by rotenone. Further muscle dysfunction occurs via elevated oxidative stress i.e. increase in free radicals and the inadequate support from antioxidant defense system leading to a framework of cellular damage[45] and thereby further deterioration of muscular activity in PD patients. On the other hand simultaneous administration of MCE reduced the levels of free radicals. Ethyl acetate extract of noni fruit is shown to reduce oxidative and nitrosative stress and also improved brain energy metabolism in streptozotocin induced memory impairment in mice.[11] Ethanolic extract of *Morinda citrifolia* is shown to reverse the scopolamine induced memory loss in mice by culminating oxidative stress.[10] Shaw *et al.* have reported the antioxidant property of scopoletin stating that the compound is capable of scavenging superoxide anion and thereby culminating the free radical mediated secondary complications.[46] Thus, MCE supplementation to PD patients might be beneficial in reducing the free radical mediated secondary complications such as formation of lipid peroxides and protein aggregates. From these observations, it is promising that MCE might act as a protective agent that could ameliorate sarcopenia in PD cases.

To prevent or delay oxidation reactions, cells have a variety of antioxidant enzymes and low molecular weight antioxidants as a defense against the deleterious effects of ROS overproduction and oxidative damage.[47] Elevated ROS production, in the absence of increased antioxidant defenses, would lead to oxidative damage and oxidative stress.[48] In the present study, there was a significant decline in the activities of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and as well as the non enzymatic antioxidant glutathione in the skeletal muscle on intra-nigral infusion of rotenone when compared to those of control rats. Saravanan *et al.* have established that the

administration of rotenone leads to decrease in GSH level and changes in the activities of antioxidant enzymes in the striatum and SNPc.[49] MCE treatment restored the rotenone-induced decrease in GSH level and changes in antioxidant enzyme (SOD, catalase, GPx and GR) activities in the skeletal muscle. The ethyl acetate extract of *Morinda citrifolia* fruit is shown to boost the antioxidant enzymes such as SOD, GPx, and GR in β-amyloid induced cognitive dysfunction in mice.[9] This antioxidant effect of *Morinda citrifolia* may be attributed to the presence of a large number of phyto-constituents in the ethyl acetate fraction of Noni with antioxidant properties such as scopoletin, quercetin, and rutin which have been reported to have potent antioxidant and neuroprotective actions. [8, 11, 50]

Under normal physiological conditions, ROS serve as "redox messengers" in the regulation of intracellular signalling, whereas excess ROS may induce irreversible damage to cellular components leading to cell death by promoting the intrinsic apoptotic pathway through mitochondria.[51] On the other side, regular physical activity may represent an effective strategy to decrease apoptotic signalling, and possibly muscle wasting and dysfunction, during aging and disease.[52] As there was a decrease in the physical activity and an elevation in ROS levels in PD rats, the activation of various apoptotic proteins involved in intrinsic pathway of apoptotic signalling and the effect of MCE on these proteins in the skeletal muscle were analysed by studying the mRNA and protein expression.

Key members of the Bcl-2 super families (proapoptotic Bax, Bak, Bad, Bim, and Bid; antiapoptotic Bcl-2, Bcl-XL, and Bcl-w) of proteins are the major players in mitochondrial outer membrane permeabilization and thereby mediate intrinsic pathway of apoptosis.[53] In the present study, the Bax/Bcl2 ratio was altered and a stipulated increase in the ratio (79%) was observed in the skeletal muscle of PD rats

which clearly shows that the pro-apoptotic Bax level was significantly elevated both at the transcriptional and at the translational levels, whereas the Bcl2 level was significantly decreased. Our findings are in coherence with the previous findings, where rotenone administration significantly decreased the basal level of Bcl-2 and increased the Bax protein expression and the ratio of Bcl-2/Bax was also decreased by 40% in rotenone-treated SH-SY5Y cells.[54] Li *et al.* have demonstrated that ROS is capable of inducing apoptosis by regulating the phosphorylation and ubiquitination of Bcl2 family proteins resulting in increased pro-apoptotic protein levels and decreased anti-apoptotic protein expression.[55] Hence, the ROS generated in skeletal muscle might have been the reason for enhanced apoptosis. Co-administration of MCE significantly augmented the levels of Bcl2 and down-regulated the protein and mRNA levels of Bax. This is the first report stating the anti-apoptotic activity of scopoletin enriched MCE, unlike the literature which reports it to be an apoptosis promoting factor in many cancer cell lines.[56-58] However, one of the closely related family members of *Morinda* genus namely *Morinda officinalis* is shown to possess anti-apoptotic activity.[59] Probably, the antioxidant activity of the phyto-constituents scopoletin, quercetin and rutin present in the MCE would have been responsible for this anti-apoptotic activity.

Stimulation of the intrinsic mitochondrial apoptotic pathway by ROS and mitochondrial DNA damage promote outer membrane permeabilization and mitochondria-to-cytosol translocation of different proteins including cytochrome c.[60] Bax has been shown to induce cytochrome c release and caspase activation both in vivo and in vitro.[61,62] Bcl-2 was found to be capable of inhibiting Bax-induced apoptosis and it has also been shown that it is capable of blocking spontaneous

cytochrome c release in cell-free extracts and in cells treated with apoptosis-inducing agents.[63]

As the Bax/Bcl2 ratio is altered in the current scenario we further investigated the levels of cytochrome c in mitochondria and cytosol. The cytosolic level of cytochrome c was significantly elevated in the skeletal muscle of PD rats when compared to that of the control rats. Our results are coherent with the previous studies carried out by Li *et al.* who have reported that rotenone administration decreased mitochondrial cytochrome c levels and increased cytosolic cytochrome c levels, indicating a typical release of cytochrome c from mitochondria to the cytosol.[64] Co-treatment with MCE resulted in decreased levels of cytosolic cytochrome c in the skeletal muscle when compared with rats infused with rotenone which might be attributed to the mito-protective effect of *Morinda citrifolia* which blocks the release of cytochrome c.

Apoptosis is characterized by the activation of a family of cysteine dependent aspartate-directed proteases termed caspases that are responsible for the initiation and execution of apoptotic cell death. The activation of initiator caspases (caspase-8 and -9) results in the cleavage and activation of downstream effector caspases (caspase-3, -6, and -7), which are responsible for the selective and limited proteolysis of multiple cellular proteins involved in the morphological and biochemical changes associated with apoptosis.[65] When cytochrome c is released from mitochondria, it stimulates cell death by interacting with Apaf-1, recruiting and activating procaspase-9.[66] Increase in both the mRNA and protein levels of caspase-3 and caspase-9 was reported in the present study in the rats infused with rotenone. The increase in the protein as well as mRNA levels of caspase-9 and 3 may be attributed to the release of cytochrome c to the cytosol in rotenone infused rats as the

release of cytochrome c is the upstream for the activation of caspase-9, caspase-8, and caspase-3.[67] Li *et al.* have reported that rotenone is shown to induce both caspase 9/3-independent and -dependent cell death.[68] Co-supplementation of MCE brought down the protein expression of both caspase-3 and caspase-9 which might be due to the blockage of cytochrome c release by MCE. MCE not only inhibits the activation of caspases but it also down-regulates the mRNA levels of both the caspase-3 and caspase-9 thereby decreasing the levels of pro-caspases in the skeletal muscle tissue.

5.0 Conclusion

In conclusion, administration of rotenone has increased the oxidative stress in the skeletal muscle as evidenced by increase in the level of free radicals and macromolecular damages and decrease in the antioxidant status. This in turn causes loss of skeletal muscle integrity and mitochondrial damage. Mitochondrial damage further leads to the release of cytochrome c into the cytosol which activates the downstream caspase cascade and leads to cell death. Supplementation of *Morinda citrifolia* has significantly augmented the antioxidant status by scavenging the free radicals and thereby culminating the rotenone induced oxidative stress. MCE also prevents cytochrome c release by maintaining the integrity of mitochondria. It also augments the anti-apoptotic Bcl2 both at the transcriptional and at the translational level thereby alleviating the rotenone induced apoptosis. Hence this preliminary study paves a way to show cast that the anti-apoptotic activity of MCE, which might be due to the presence of scopoletin and other phyto-constituents, and can be exploited to alleviate skeletal muscle damage induced by Parkinsonism. However, further studies are warranted for translating it.

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Conflict of interest

The authors declare that they have no potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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Fig 1: HPLC Chromatogram of scopoletin (a), quercetin (b) and rutin (c) in the ethyl acetate extract of *Morinda citrifolia* fruit

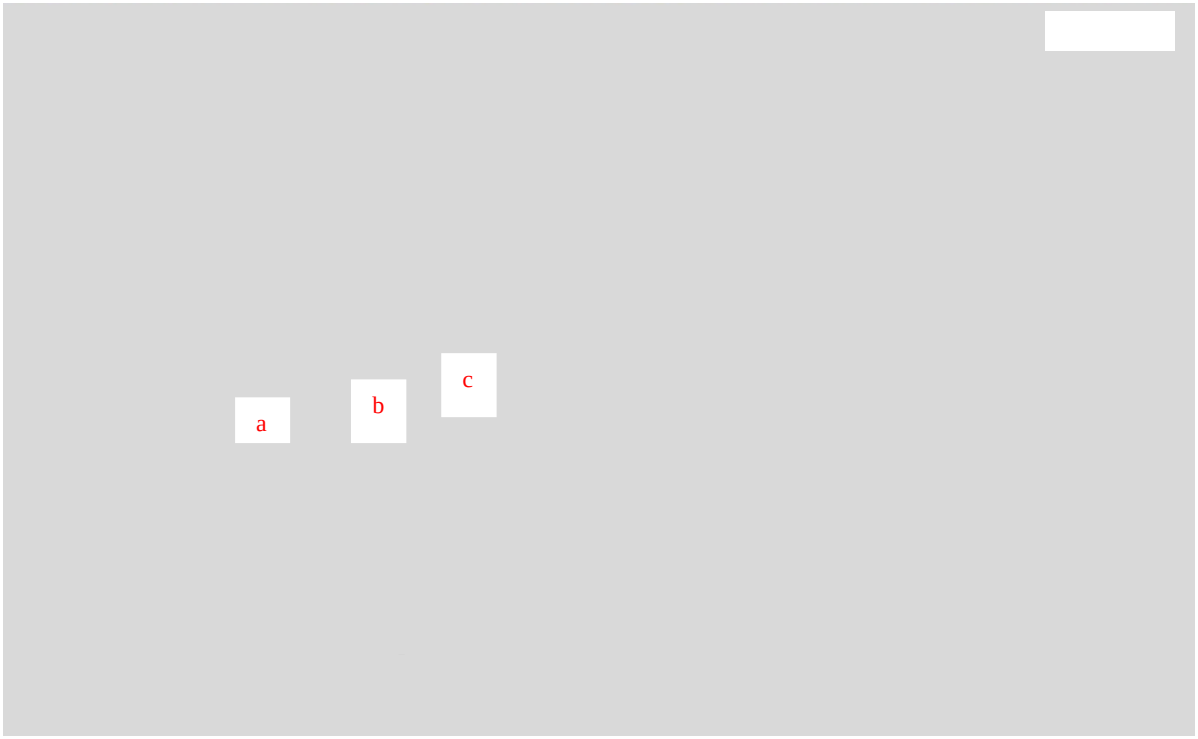


Table 1 – Concentrations of phenolic constituents in MCE

Compounds	µg/mg ± S.D.
Rutin	2.61 ± 0.18
Quercetin	1.41 ± 0.096
Scopoletin	0.60 ± 0.048

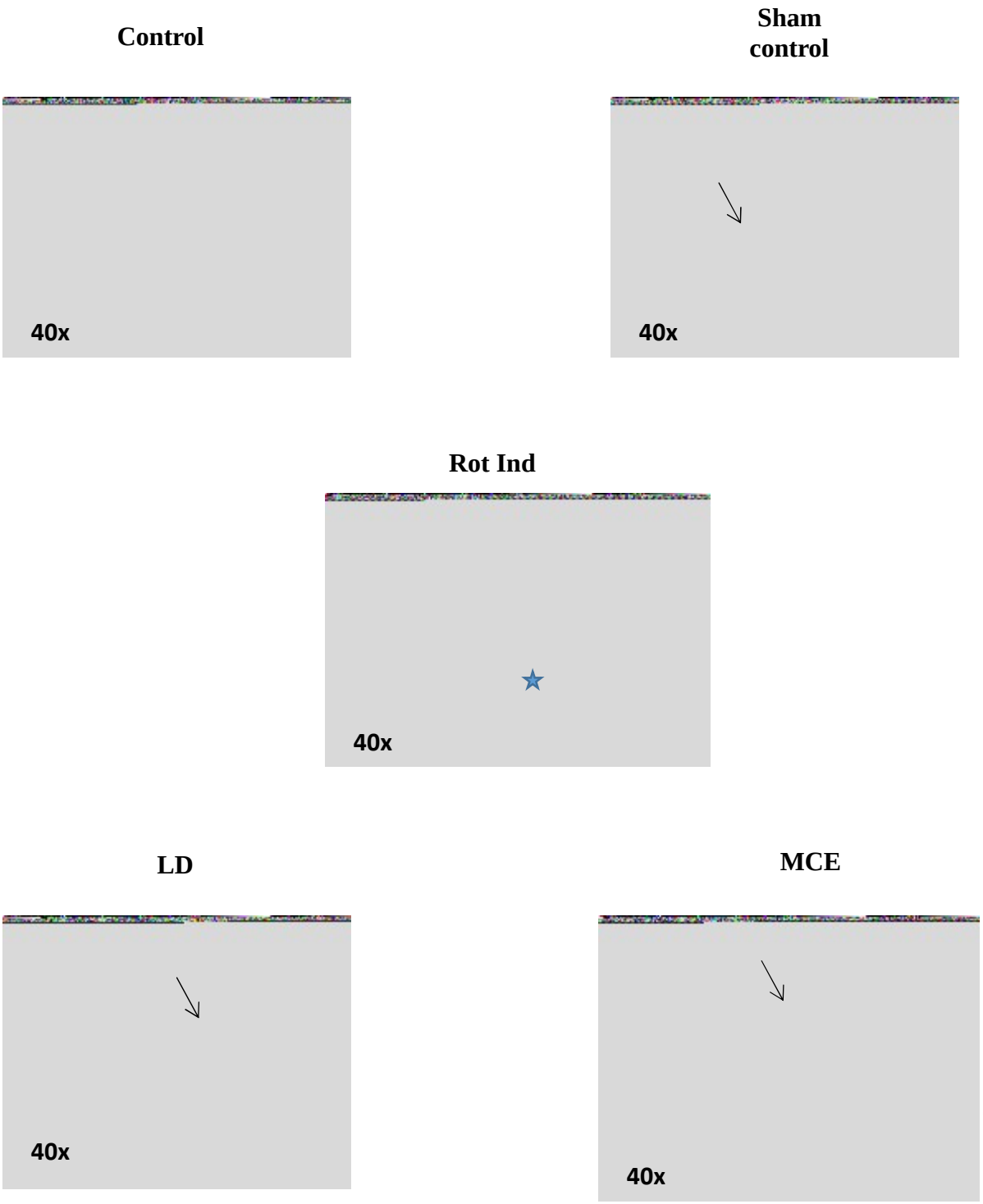
Values are expressed as mean ± S.D.

Table 2: Effect of MCE/LD on the activities of marker enzymes in the skeletal muscle and serum of rotenone induced Parkinsonian rats

Groups	Control	Sham Control	Rotenone Induced	LD treated	MCE treated
Skeletal Muscle					
CK	20.51 ± 0.96	20.41 ± 0.94	12.92 ± 0.47[a]	17.58 ± 0.86[c]	18.23 ± 0.61[c]
LDH	45.85 ± 1.69	45.04 ± 1.97	33.25 ± 1.43[a]	38.15 ± 1.57[c]	38.95 ± 1.35[c]
AST	32.95 ± 1.72	31.94 ± 1.17	22.97 ± 1.02[a]	26.97 ± 1.08[c]	26.99 ± 0.88[c]
Serum					
CK	62.38 ± 2.10	61.82 ± 3.27	86.95 ± 3.83[a]	72.06 ± 3.44[c]	72.49 ± 3.34[c]
LDH	207.27 ± 6.43	206.11 ± 7.16	294.97 ± 10.49[a]	257.37 ± 9.33[c]	253.65 ± 9.70[c]
AST	128.18 ± 4.62	129.94 ± 4.86	180.69 ± 6.09[a]	156.02 ± 7.01[c]	154.44 ± 8.14[c]

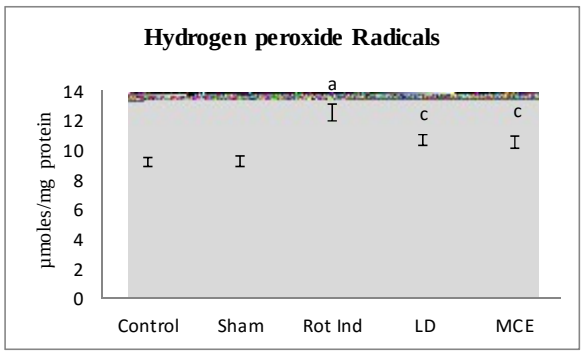
Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'c' represents Rot Ind vs LD, MCE.

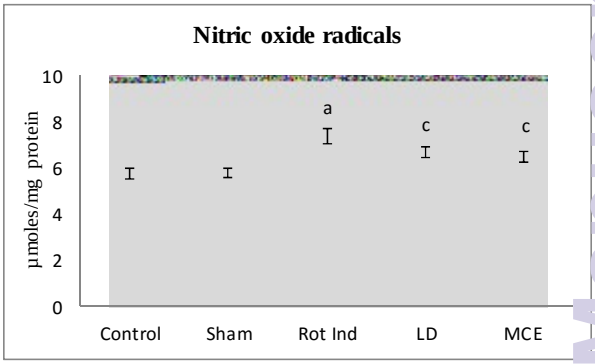
Fig 2: Effect of MCE/LD on the histo-architecture of Skeletal muscle in rotenone induced Parkinsonian rats

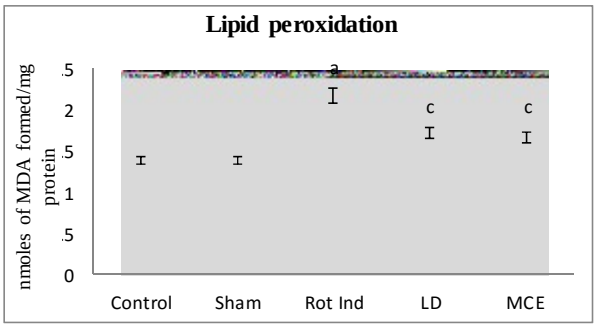


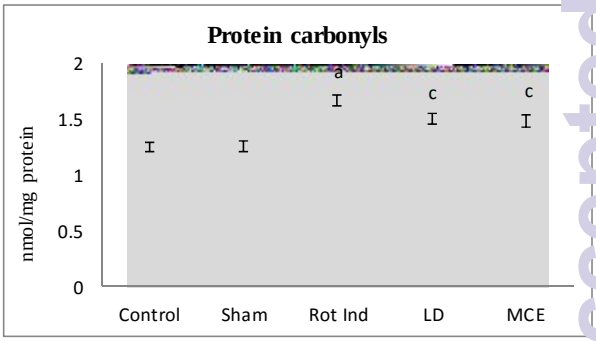
The black arrows represent prominent nucleus situated in the periphery, whereas star represents the less compact muscle fascicle and rarefaction.

Fig 3: Effect of MCE/LD on Free radicals and Macromolecular damage in the Skeletal muscle of rotenone induced Parkinsonian rats



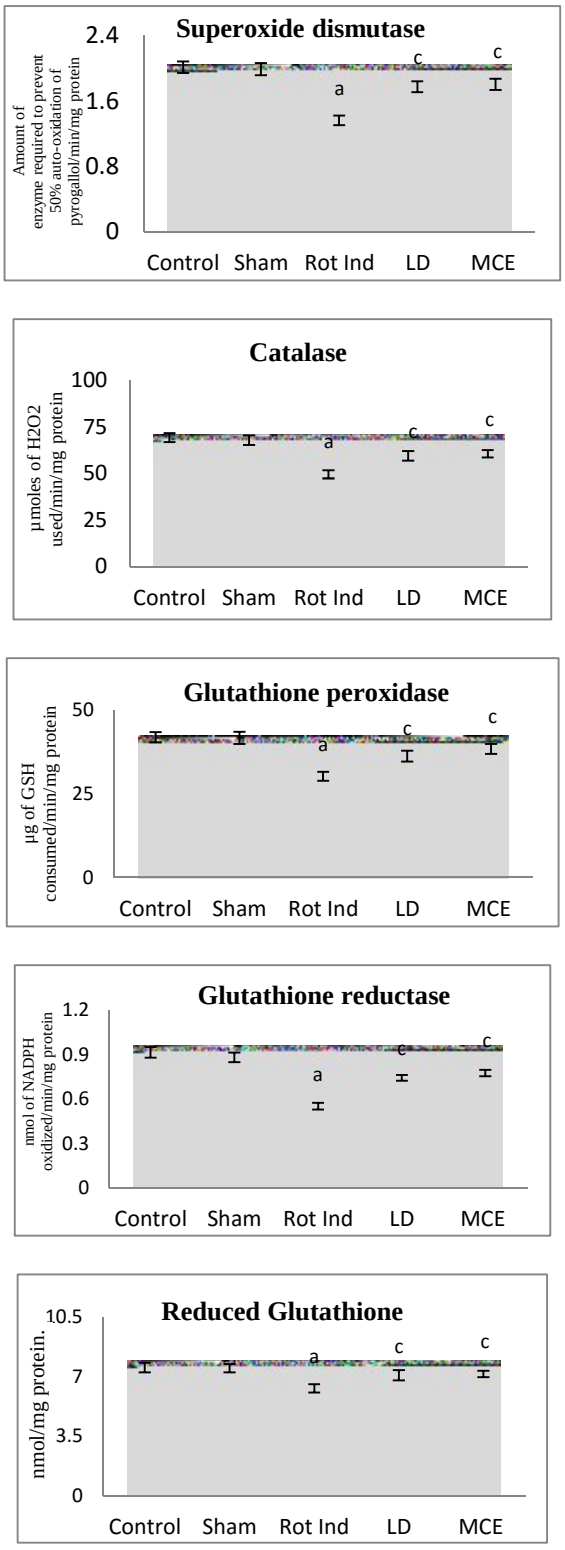






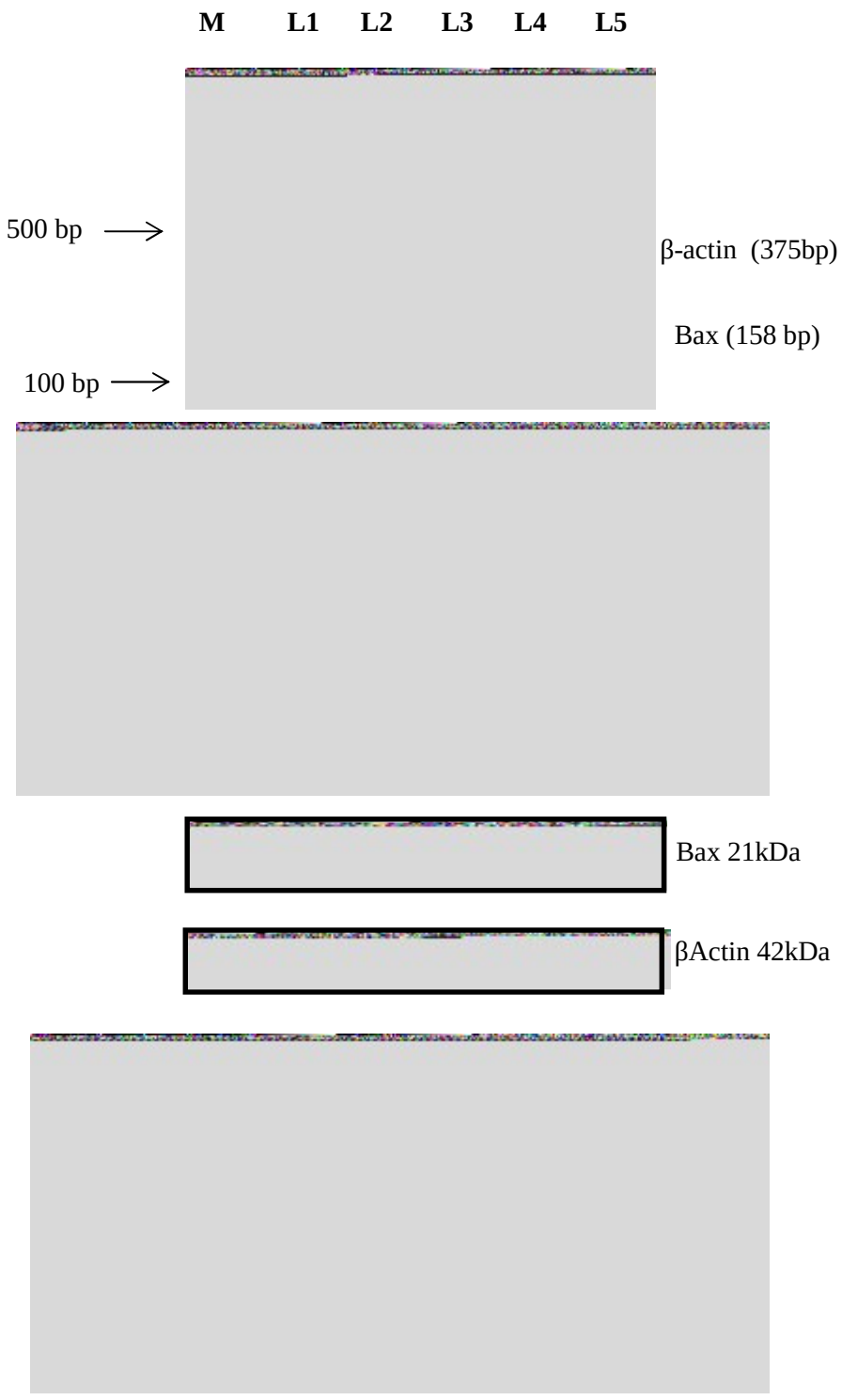
Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'b' represents Sham Control Vs other groups 'c" represents Rot Ind vs LD, MCE.

Fig 4: Effect of MCE/LD on Enzymatic and Non-enzymatic antioxidants in the Skeletal muscle of rotenone induced Parkinsonian rats



Values are expressed as mean ± SEM for six animals in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'b' represents Sham Control Vs other groups 'c" represents Rot Ind vs LD, MCE.

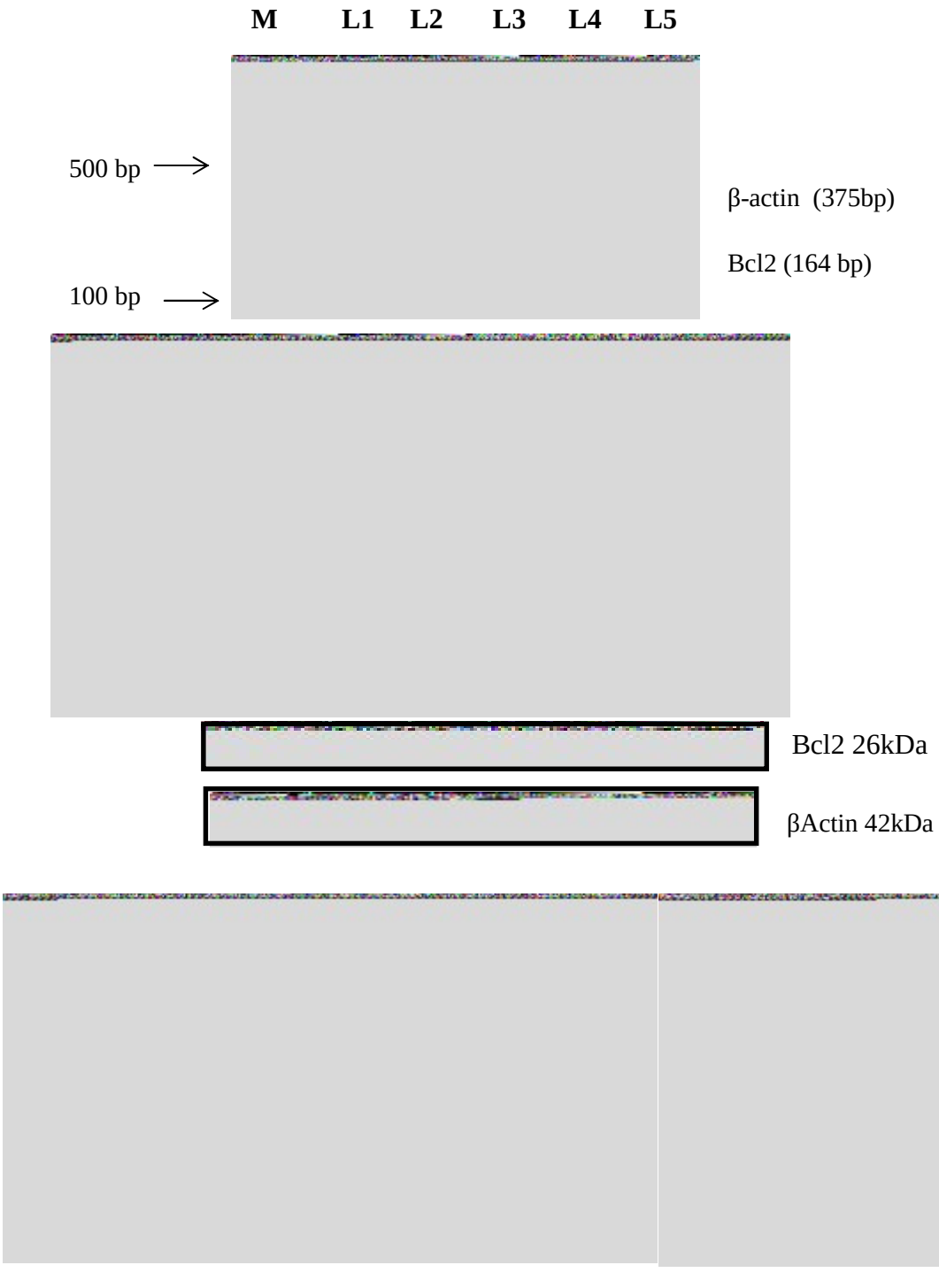
Fig 5: Effect of MCE/LD in the mRNA and Protein expression of Bax in the Skeletal muscle of rotenone induced Parkinsonian rats



M: Marker L1: Control L2: Sham L3: Rot Ind L4: Rot+LD L5: Rot+MCE

Values are expressed as mean ± SEM for three experiments in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'c' represents Rot Ind vs LD, MCE.

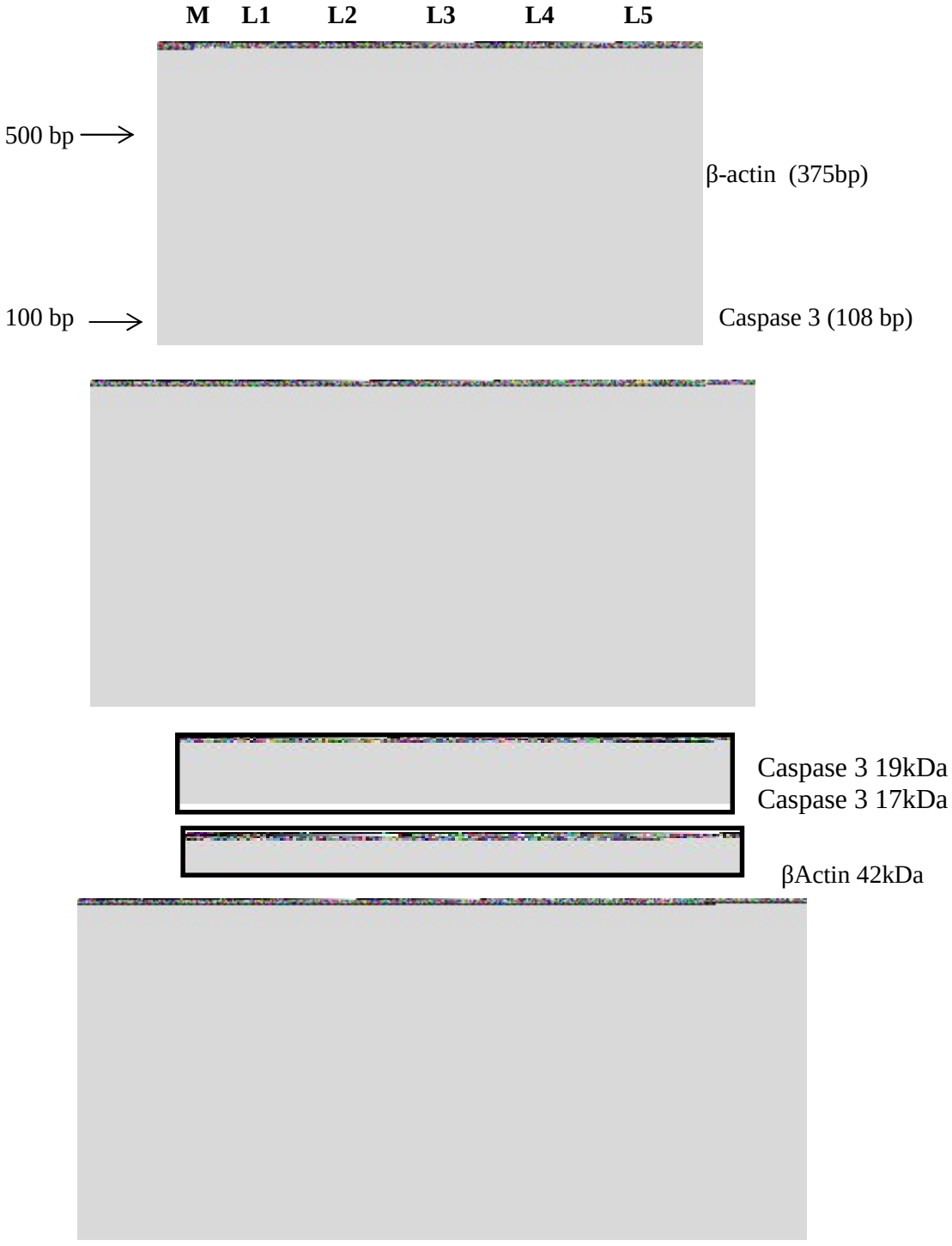
Fig 6: Effect of MCE/LD in the mRNA and Protein expression of Bcl2 in the Skeletal muscle of rotenone induced Parkinsonian rats



M: Marker L1: Control L2: Sham L3: Rot Ind L4: Rot+LD L5: Rot+MCE

Values are expressed as mean ± SEM for three experiments in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'c' represents Rot Ind vs LD, MCE.

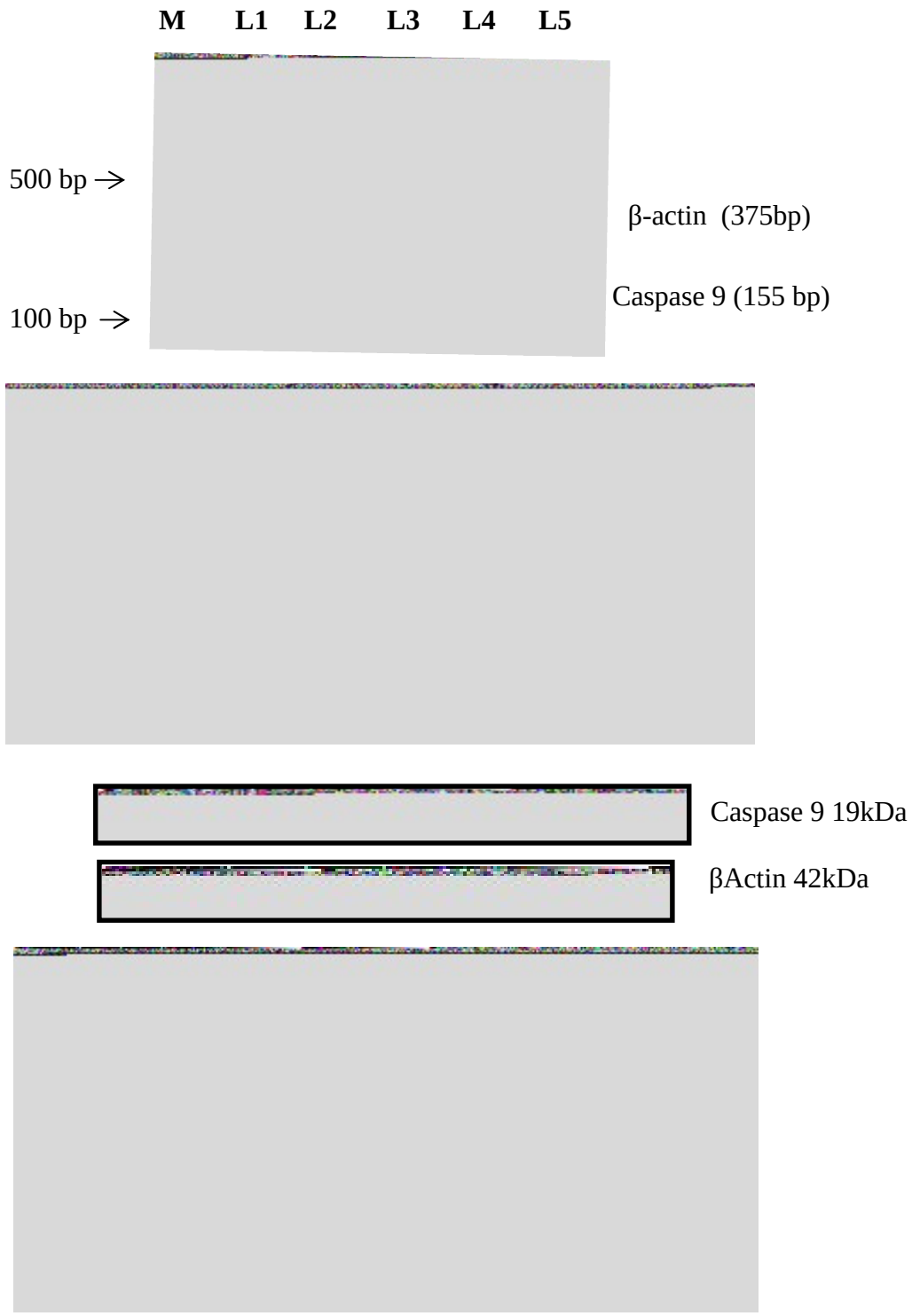
Fig 7: Effect of MCE/LD in the mRNA and Protein expression of Caspase-3 in the Skeletal muscle of rotenone induced Parkinsonian rats



M: Marker L1: Control L2: Sham L3: Rot Ind L4: Rot+LD L5: Rot+MCE

Values are expressed as mean ± SEM for three experiments in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'c' represents Rot Ind vs LD, MCE.

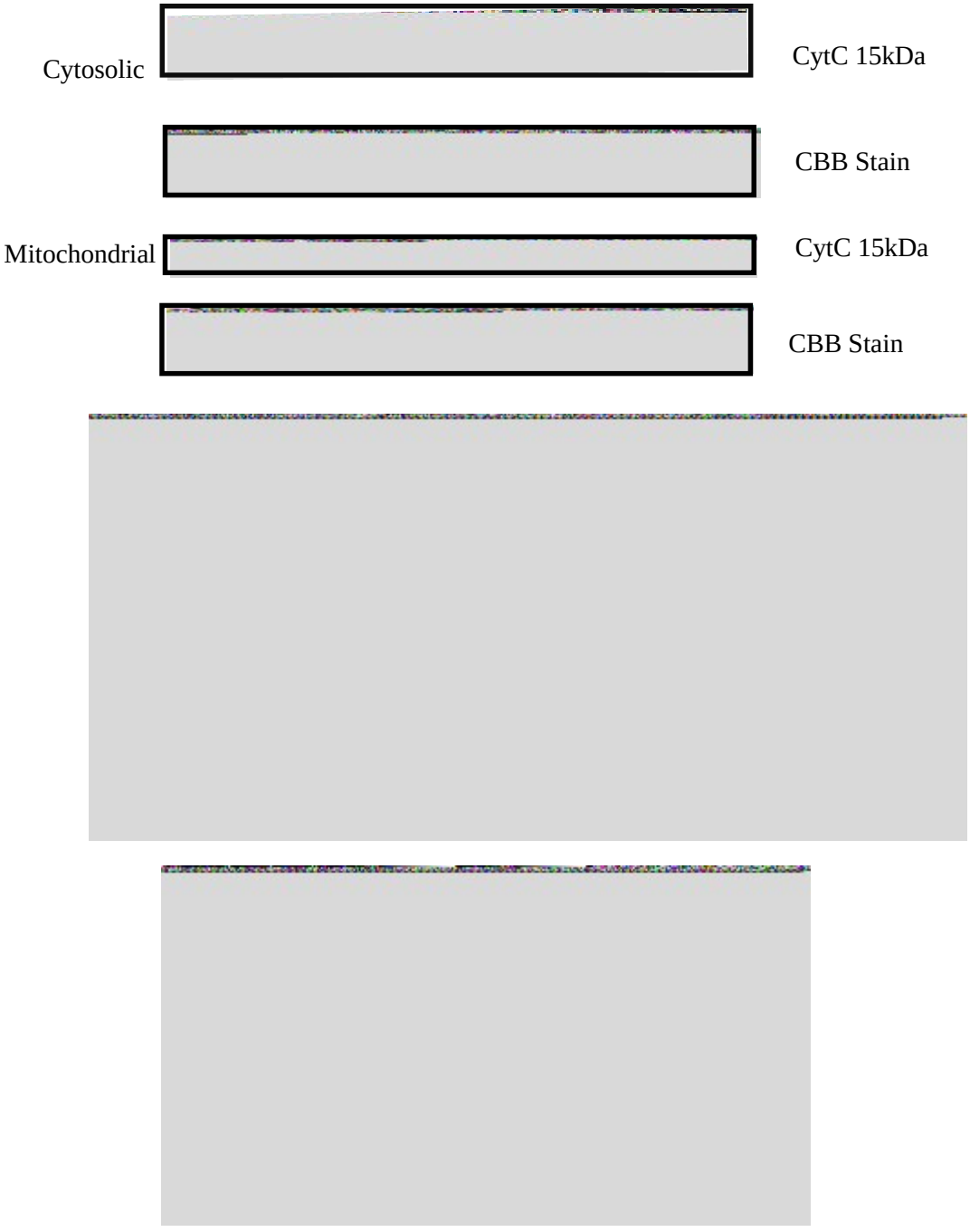
Fig 8: Effect of MCE/LD in the mRNA expression of Caspase-9 in the Skeletal muscle of rotenone induced Parkinsonian rats



M: Marker L1: Control L2: Sham L3: Rot Ind L4: Rot+LD L5: Rot+MCE

Values are expressed as mean ± SEM for three experiments in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'c' represents Rot Ind vs LD, MCE.

Fig 9: Effect of MCE/LD in the Protein expression of Cytosolic and Mitochondrial Cytochrome C in the Skeletal muscle of rotenone induced Parkinsonian rats



Values are expressed as mean ± SEM for three experiments in each group. Values are statistically significant at the level of $p<0.05$ where 'a' represents Control Vs other groups, 'c' represents Rot Ind vs LD, MCE.

GC-MS Characterization and Quantitation of Scopoletin, rutin and quercetin by HPLC

Extraction with Ethyl acetate - MCE

Mitochondrial damage ↑

Rotenone

↑ CytC release

SM membrane integrity CK, LDH & AST ↓

↑ ROS

↓ Bcl-2

↓ Antioxidants SOD, CAT , GSH

↑ Bax/ Cas3/Cas9

– Sites blocked by MCE

Apoptosis