Quercetin glycosides induced neuroprotection by changes in the gene expression in a cellular model of Parkinson's disease

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Abstract

Quercetin glycosides, rutin and isoquercitrin are potent antioxidants that have been found to possess neuroprotective effect in diseases like Parkinson's and Alzheimer's disease. In the present study, we have examined the gene expression changes with rutin and isoquercitrin pretreatment on 6-hydroxydopamine (6-OHDA) treated toxicity in rat pheochromocytoma (PC 12) cells. PC 12 cells were pretreated with rutin or isoquercitrin and subsequently exposed to 6-OHDA. Rutin pretreated PC 12 attenuated the *Park2, Park5, Park7, Casp3* and *Casp7* genes which were expressed significantly in the 6-OHDA treated PC12 cells. Rutin up-regulated the *TH* gene which is important in dopamine biosynthesis but isoquercitrin pretreatment did not affect the expression of this gene. Both rutin and isoquercitrin pretreatment up-regulated the ion transport and anti-apoptotic genes *(NSF and Opa1).* The qPCR array data were further validated by qRT-PCR using four primers, *Park5, Park7, Casp3 and TH*. This finding suggests that changes in the expression levels of transcripts encoded by genes that participate in ubiquitin pathway and dopamine biosynthesis may be involved in Parkinson's disease.

Keywords: 6-Hydroxydopamine Parkinson's disease Flavonoids Parkin

Introduction

Parkinson's disease (PD), an example of neurodegenerative disorder is an incurable, debilitating condition that results from progressive loss of a particular type of nerve cells, known as dopaminergic neurons, which are located at *Substantia nigra pars compacta*, an area in human mid brain (Double, 2012). Dopaminergic neurons are essential component of the human brain, which controls the voluntary movement, behavioral processes such as motivation, mood, arousal, reward, sexual gratification and stress (Chinta and Andersen 2005). Therefore, the depletion of dopamine

neurotransmitters results in the appearance of clinical features including bradikinesia, resting tremors, rigidity, hypokinesia and akinesia (Blum *et al,* 2001; Nikam *et al,* 2009). Furthermore, PD also associated with the presence of inclusion body and abnormal protein aggregation in the dopaminergic neurons, which clearly indicates the end stage of the molecular cascade of neuroinflammation process (Ross, 2004).

Several lines of evidence have suggested and proved that free radical damage is one of the contributing factors which results in PD as well as other neurodegenerative diseases. However, it is crucial to realize that reactive oxygen species is (ROS) not only harmful by-product in cellular metabolism but is critical participant in intracellular signaling cascade, cellular senescence and apoptosis (Double, 2012; Tabner *et al,* 2002; Thannickal and Fanburg 2000). In fact, the oxidative damage of cellular components such as protein, enzymes, lipid and DNA by free radical species was primarily caused by imbalance between cellular free radical generation cascade and endogenous antioxidant system. Besides that, studies have demonstrated that free radical species also involved in triggering the proinflammatory caspases including Caspase 1, 3, 8, leading to cellular apoptosis (Chong *et al,* 2002; Pasinelli et al, 2000; Prasad *et al*, 2006; Singh *et al*, 2004).

Quercetin, a flavonol group is ubiquitously found in natural food sources such as vegetables, onion, soy, herbs, tea and grains (Schmalhausen *et al,* 2007). Quercetin exist in two forms; aglycone and glycoside derivatives. Quercetin by itself is an aglycone and the presence of glycoside structure in the quercetin basic chain will form quercetin glycosides (De Oliveira *et al*, 2010). Presence of rutinose and rhamnose in the quercetin side chain will result in the formation of rutin and isoqucercitrin, respectively (Chen *et al,* 2006). Studies have demonstrated the neuroprotective

properties of rutin via activation of endogenous antioxidant enzymes (Magalingam *et al,* 2013), directly scavenging the overwhelming production of free radical species (Yang *et al,* 2008) and reduce lipid peroxidation (Magalingam *et al,* 2013). However, the pharmacological effects of isoquercitrin are not explored extensively as very limited studies have been published related to flavonoid isoquercitrin. Nevertheless, isoquercitrin has been found to have antioxidant properties as it caused activation of natural antioxidant enzymes in neuronal cells (Lee *et al,* 2010; Magalingam *et al,* 2014). Wagner *et al* reported that isoquercitrin displayed an antioxidant role by preventing the generation of (thiobarbituric acid reactive substance (TBARS) and reduce the deoxyribose degradation (Wagner *et al,* 2006).

Although, there is no report on the role of rutin and isoquercitrin in modulating the gene expression in neurodegenerative diseases but Kwon *et al* has recently demonstrated the attenuation of pro-inflammatory gene expression in inflammatory bowel disease (IBD) and carcinogenesis by rutin treatment (Kwon *et al,* 2005). Therefore, the main objective of this study was to investigate the genetic modulation of quercetin glycosides, rutin and isoquercetrin pretreatment on PC12 cells prior exposure to 6-Hydroxydopamine (6-OHDA), a specific dopaminergic neurotoxin. In addition, the data obtained from quercetin glycoside pretreatment against 6-OHDA treated PC12 cells were compared with L-3,4-dihydroxyphenylalanine (L-DOPA) pretreatment against the similar experimental setup. It is prudent to compare the protective effect of emerging antioxidant molecules with L-DOPA as L-DOPA is considered as the cornerstone drug for the treatment of PD.

Materials & Methods

Materials

PC 12 cells were purchased from ATCC (#CRL -1721.1 PC-12 ADH, *Rattus norvegicus*, Manassas, VA, USA), 6-hydroxydopamine, rutin, isoquercitrin, L-DOPA, Poly-L-lysine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Pen-strep, horse serum, and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). RNeasy Mini Kit, QIAShreddar, PARN-124A RT^2 Profiler PCR Array, RT^2 SYBR® Green Fluor qPCR mastermix; RT^2 First strand kit were purchased from Qiagen (Hilden, Germany), Superscript DNA Mastermix (Invitrogen, Carlsbad, CA) and DNA primers were purchased from Bioneer Inc (CA, USA).

Cell Culture, RNA isolation and PCR analysis

PC 12 cells were grown in a humidified incubator with 5% CO_2 at a temperature of 37˚C in DMEM 1X High Glucose medium supplemented with 5% horse serum and 5% Fetal Bovine Serum and Pen strep (100 U/ml). The cells were grown in poly-L-lysine-coated T-75 culture flasks and subsequently harvested, dispersed and plated on a poly-L-lysine-coated 96-well microplate at a density of $1x10^6$cells/ml and incubated overnight in order to facilitate cell adhesion to the flask surface (Magalingam, 2013). The cells were then pretreated for 8 hours with 100µM of rutin, 10µM of isoquercitrin and 100µM of L-DOPA respectively and subsequently treated with 100µM of 6-OHDA for 24 hours. The incubation period and concentration of each antioxidant were obtained from MTT assay, which showed the highest cell viability percentage at 8 hours pretreatment (Magalingam, 2013;

Magalingam, 2014). Following this, total RNA was isolated in order to perform quantitative real time PCR technique.

Total RNA was isolated using RNeasy Mini Kit (Qiagen ,Hilden, Germany). Samples were reverse-transcribed in duplicate using RT^2 First strand kit (Qiagen, Hilden, Germany) using 1 μg of total RNA according to the manufacturer's protocol. RNA sample integrity was assessed (28S:18S ribosomal RNA subunit ratio) by Agilent 2100 Bioanalyser (Agilent Technologies, USA).

The qPCR array analysis was performed using customized rat Parkinson's disease array which consist of 84 genes on real-time iQ5 Optical Module PCR Detection System (BIORAD,US) using RT^2 SYBR® Green Fluor qPCR Mastermix (Qiagen, Hilden, Germany).The cycling protocol consist of 40 cycles involving denaturation at 95 °C for 15 seconds followed by 1 minute at 60 °C for annealing of primers and finally 60 seconds at 55 °C for extension. Three types of background check were carried out including Rat Genomic DNA contamination, reverse transcription control (RTC) and positive PCR control (PPC) which were run in triplicates. The C_T value of rat genomic DNA was greater than 35 in all samples tested. The ΔCT values were calculated according to manufacturer's instruction. The C_T value of each gene of interest were normalized to the average of 5 housekeeping genes in the array which were β-Actin, β-2 microglobulin, Hypoxanthine, Lactate dehydrogenase A and large Ribosomal protein, P1. Then, the $\Delta\Delta C_T$ of each sample was obtained by subtracting the ΔC_T (control sample) from ΔC_T (treatment group) and subsequently the fold change was calculated using the formula $2^{-\Delta C_T}$. Fifteen genes with direct or indirect involvement in the pathogenesis of Parkinson's disease were analyzed (Table 1).

Four candidate genes (Table 2) were chosen from each gene groups (Table 1) to validate the qPCR array data by real-time PCR (q-RT-PCR) using Taq-man technology (Invitrogen, Carlsbad, CA). Purified RNA was processed using SuperScript III Platinum SYBR Green one-step qRT-PCR Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. A total reaction volume was 25µl with 20ng of RNA, and 10µM of forward and reverse primers were carried out for four primers namely *Park 3, Park 5, Casp 3* and *TH* (Table 2). The cycling protocol consist of 40 cycles involving denaturation at 95 °C for 15 seconds followed by 1 minute at 49 °C for annealing of primers and finally 30 seconds at 72 °C for extension. A dissociation curve analysis was performed at the end of the cycle to confirm amplification specificity. All samples were run in triplicates. Fluorescence was detected using real-time iQ5 Optical Module PCR Detection System (BIORAD, US).The *Park3, Park5, Casp3* and *TH* mRNA levels were normalized to GAPDH mRNA, reference gene. Three reactions without template (Non-template control) were also run on each plate to check for any nonspecific amplification.

Statistical Analysis

The relative gene expression of *Park5, Park7, Casp3* and *TH* were normalized with reference gene GAPDH gene in each analyzed sample. The reproducibility of results was determined by performing triplicate measurement for each sample analyzed. The treatment groups (L-DOPA, rutin, isoquercitrin) were statistically compared with 6-OHDA group alone and the 6-OHDA group was compared with negative control group. The normalized data set was compared using a Student's *t*-test and $p<0.05$ was considered as statistically significant values.

Results

Gene expression using qPCR array analysis

PC12 rat pheochromocytoma cells were incubated with 100µM of rutin, 10µM isoquercitrin and 100µM L-DOPA respectively for 8 hours and subsequently treated with 100µM of 6-OHDA. The RNA was isolated and cDNA was synthesized prior to qPCR array analysis. Fifteen genes which expressed a significant up/down regulation between the pretreated group and control group were analyzed (Table 1).

Gene expression of 6-OHDA treated PC12 cells

Figure 1 show the 15 types of mRNA expressions which were modulated in a cellular model of Parkinson's disease. *Atxn2* (ataxin) and *Atxn3* mRNA expression were up-regulated almost 1 fold in 6-OHDA treated PC12 cells. Although, all the *Parkin* genes were activated but *Park5* and *Park7* showed the highest fold change compared to other genes. The pro-apoptotic caspases including *Casp 1, 3 and 7* (caspases) were also up-regulated in 6-OHDA treated PC12 cells. Importantly, *Casp3* and *Casp7* genes showed a more than 1 fold change in comparison with negative control. The oxidative stress generated by 6-OHDA triggered the apoptotic cascade that lead the cells to programmed cell death. The anti-apoptotic genes *Ppid* (Peptidylprolyl isomerase cyclophilin D) gene was down-regulated and *Opa1* (Optic atrophy 1) gene was up-regulated. The genes which control the secretion of dopaminergic neurotransmitter, *Nsg1* (Neuron Specific Gene Member 1) and *TH* (Tyrosine hydroxylase) were down-regulated in 6-OHDA treated PC12 cells. In addition, *Egln1* gene, that code for protein that involved in the membrane ion transport activity was up-regulated and *NSF* (N-ethylmaleimide-sensitive factor) gene that controls the membrane-trafficking events was down-regulated in a cellular model of Parkinson's disease.

L-DOPA pretreatment on 6-OHDA treated PC12 cells

Most of the gene expressions in 6-OHDA induced PC12 cells (Figure 1) were attenuated in 8 hours pretreatment with 100µM L-DOPA as shown in Figure 3. The mRNA expressions which were significantly down-regulated were *Atxn 2, Atxn 3, Park2, Park5, Park7, Stub1, Casp1, Casp3* and *Casp7*. On the other hand, L-DOPA caused the expression of *TH* and *Nsf* genes which was attenuated in PC12 cells treated with 6-OHDA alone. However, the *Ppid, Nsg1* and *Egln1* genes were greatly suppressed in L-DOPA pretreated cells. On top of that, L-DOPA did not affect the *Opa1* gene as it was suppressed in both 6-OHDA induced control cells and L-DOPA treated cells (Fig 1).

Rutin pretreatment on 6-OHDA treated PC12 cells

Rutin demonstrated almost similar gene regulation as L-DOPA with a few exceptions. The 8 hours pretreatment of rutin on 6-OHDA treated PC12 cells caused the down-regulation of *Atxn 2, Park5* and *Park7*.Besides that, rutin also suppressed the pro-apoptotic genes such as *Casp3* and *Casp7*. In addition, rutin has shown a great protective effect by expressing both anti-apoptotic genes, *Opa1* and *Ppid*. But, these effects were absent in L-DOPA pretreated PC12 cells, whereby L-DOPA up-regulated the *Opa1* gene but suppressed the *Ppid* gene. Importantly, rutin caused a 2.5-fold change in *Nsf* mRNA level, which was the highest fold change compared to all other genes. Besides that, the *TH* gene was expressed in rutin pretreated PC12 cells and *Egln1* mRNA was suppressed in the same antioxidant compound.

Isoquercitrin pretreatment on 6-OHDA treated PC12 cells

The pro-apoptotic genes namely *caspase 1, 3* and *7* were up-regulated and *parkin* genes including *Park5, Park7* and *Stub1* were greatly suppressed in isoquecitrin pretreated PC12 cells. The antioxidant potency of isoquercitrin was less eminent as the *TH* and *Ppid* genes were suppressed in isoquercitrin pretreatment although these genes were up-regulated in rutin pretreated cells. Interestingly, the *Nsf* gene which was linked to ion transport pathway and the anti-apoptotic gene *Opa1* were expressed in isoquercitrin pretreated PC12 cells similar to L-DOPA and rutin pretreatment.

Quantitative real time RT-PCR validation

The mRNA expression of four selected genes were studied using q-RT-PCR technique to validate the data obtained from qPCR array analysis. The q-RT-PCR data correlated with qPCR array findings as all the genes up/down regulated in q-RT-PCR were found to have a similar outcome in qPCR array analysis. Figure 2 shows the *Park5, Park7* and *Casp3* were highly expressed and *TH* gene was suppressed in 6-OHDA treated PC12 cells (Fig 2A). *Park5* and *Park 7* genes were down-regulated by all the pretreatment compounds, including L-DOPA, rutin and isoquercitrin (Fig 2 B, C, D). L-DOPA and rutin(Fig 2 B,C) demonstrated a remarkable elevation of Tyrosine hydroxylase enzyme which was absent in PC12 pretreated with isoquercitrin (Fig 2 D). Besides that, PC12 cells pretreated with L-DOPA and rutin(Figure 2 B, C) expressed potent anti-apoptotic effect as the *Casp3* protein was significantly down regulated with $p<0.05$. However, isoquercitrin failed to cause any gene expression changes on *Casp3* and *TH* genes in 6-OHDA treated PC12 cells.

Discussion

Rat Parkinson's disease PCR array is a powerful tool that profiles 84 genes that directly or indirectly involved in the pathogenesis of the disease. In other word, it serves as a screening test to assess the mRNA expression changes between the PD group and neuroprotective agent treated group. In this study, the 100µM of 6-OHDA treated PC12 group had served as control or Parkinson's disease group and the changes of mRNA expressions in this group were compared with mRNA expression level in L-DOPA, rutin or isoquercitrin pretreated group. The qPCR array findings were validated by performing quantitative real time RT-PCR on 4 selected primers from different gene grouping (Little *at al*, 2012).

The exposure of neurotoxin, 6-OHDA on rat pheochromocytoma PC12 cells, was to induce oxidative stress and cell death hence to mimic the natural event in Parkinson's disease (Blum *et al*, 2001; Walkinshaw and Waters, 1994). The survived PC12 cells were subjected to PCR analysis to study the changes in mRNA expression caused by 6-OHDA. Although the array comprises of 84 genes, the gene analysis was narrowed to 15 genes to focus on the major mRNA expression changes caused by neuroprotective agents under study. Besides that, the genes were grouped according to their functions for superior data presentation (Table 1).

The Parkin substrates, *Atxn2* and *Atxn 3* genes which were up-regulated in 6-OHDA treated PC12 cells have a close association with Spinocerebellar ataxia types 2 (SCA2) and 3 (SCA3) are autosomal dominantly inherited, which occur due to CAG repeat expansions in the coding regions of the genes (Albrecht *et al*, 2004; Tang *et al*, 2000). *Atxn 2* has been reported to play a vital role in RNA metabolism and translational control (Van de Loo *et al*, 2009) as well as component of stress granules (Ralser *et al*, 2005). Stress granules are untranslated mRNAs which forms transient cytoplasmic

inclusions that are produced in response to environmental stress such as heat shock, osmotic imbalance or oxidative stress (Dewey *et al*, 2012). A recent study has demonstrated that *Atxn2* protein was a component of stress granules which are highly regulated during oxidative stress (Nonhoff *et al*, 2007). On the other hand, *Atxn 3* involved in proteasome mediated protein degradation as a deubiquitinating enzyme that controls the flow through the ERAD (Endoplasmic reticulum associated degradation) pathway during oxidative stress. Hence, PC12 cells which were treated with 6-OHDA activated the *Atxn 2* and *Atxn 3* genes, as the genes were still functional and able to generate proteins to reverse the accumulation of toxic protein to protect the cells from undergoing apoptosis (Zhong and Pittman, 2006).

Furthermore, we have demonstrated the activation of *Parkin* genes, namely *Park2, Park5, Park7,* and *Stub1* in an oxidative stress condition. Mounting evidence suggests that *Parkin* proteins as part of ubiquitin-proteosome pathway, a system that acts as a quality control to dispose damaged, misshapen, and excess proteins (Chan *et al*, 2011; Imai *et al*, 2000). The up-regulation of *Parkin* mRNA expressions (i.e *Park2, Park5, Park7*) in 6-OHDA treated PC12 cell model posits the role of *Parkin* proteins as an E3 ubiquitin-protein ligase to eliminate the misfolded protein from neuronal cells as a consequence of oxidative damage where it is considered a normal recovery process (Albrecht *et al*, 2004). Since, the mutation of *Parkin* genes is linked to absence of E3 ubiquitin-protein ligase that causes accumulation of these misguided proteins in dopaminergic neurons and results in neuronal death as well as motor dysfunction, which give rise to Parkinson's disease (Deng *et al*, 2008; Zhang *et al*, 2000). In detail, *Park2* gene encodes for *Park2* protein, an E3 ligase related with E2 ubiquitin conjugating enzymes such as UbsH7, enhances ubiquitination of CyclinE, CDCrel and Synphilin-1, which are subsequently targeted for degradation by proteasomes (Koziorowski *et al*, 2010). Besides

the localization of *Park2* protein at microtubules is an important factor for its ubiquitin ligase activity toward misfolded substrates. *Park5* or *UCHL1* gene provides instruction to produce ubiquitin carboxyl-terminal esterase L1 enzyme and UCHL1 is a member of deubiquitylating enzymes (DUB) that reverse the action of ubiquitylation reactions in ubiquitin-proteosome pathway (Chen *et al*, 2012; Kahle *et al*, 2009). *Park7* mRNA or also known as of *DJ-1* gene is a ubiquitous redox-responsive neuroprotective protein with diverse functions. During oxidative stress, *DJ-1* proteins were up-regulated to control the redox signaling kinase pathways and regulates transcriptional of antioxidative genes. On the other hand, *Stub1* (STIP1 homology and U-Box containing protein 1), also known as CHIP (C terminus of HSC70-Interacting Protein) interacts with cytosolic chaperone Hsc/Hsp70 to demonstrate intrinsic E3 ubiquitin ligase activity and promotes ubiquitylation (Jiang *et al*, 2001). Thus, the expression of *Park2, Park5, Park7* and *stub1* genes in our cell model of Parkinson's disease occurs as a response to this stressful event to eliminate the misfolded protein molecules.

Oxidative stress ensued by 6-OHDA has up-regulated the pro-apoptotic genes such as *Casp1, Casp3 and Casp7* (Figure 1) which are the members of the caspase (cysteine aspartate protease) family of proteins, and have been shown to be the executioner proteins in apoptosis process (Dodel *et al*, 1999). *Casp1* activation was mediated by inflammation, a multiprotein complex which promotes the secretion of pro-inflammatory cytokines such as interleukin 1β and IL-18. Furthermore, *Casp3* by itself is activated *by caspases 8, 9,* and *10* and functions in cleaving and activating *Casp6* and *Casp7* which leads to cell apoptosis. Treatment of PC12 cells against 6-OHDA has caused oxidative stress and activated the caspase genes that subsequently lead the cells to a programmed cell death (Franchi *et al*, 2009; Lamkanfi and Kanneganti, 2010; Xu *et al*, 2001).

Many lines of evidence have linked the implication of oxidative stress and mitochondrial dysfunction as promoting the factors to neuronal apoptosis. The neuro stressor, 6-OHDA has caused the inhibition of *Opa1* and stimulation of *Ppid* genes in the PC12 cell model. The *Opa1* and *Ppid* genes function in regulating the normal physiology of mitochondrial membrane (Lin and Beal, 2006; Russell *et al*, 2002). *Opa1* or officially known as "optic atrophy 1" functions in shaping and morphology maintenance of mitochondria through the fusion process (Szlarz and Scorrano, 2012). Several studies have displayed *Opa1* and PARL (Presenilin associated rhomboid like) plays a major role as heat shock response. During heat shock or oxidative stress, long form *Opa1* protein was lost and results in mitochondrial fragmentation (Sanjuan *et al*, 2012). This process triggered the mitochondrial recovery or mitochondria conditioning that stimulate the accumulation of soluble intermembrane form of *Opa1* oligomer and subsequently caused the suppression the cytochrome-c release. The inhibition of cytochrome c release from mitochondria compartment serve as cellular resistance to apoptosis stimuli (Gottlieb, 2006). However, in this study the *Opa1* mRNA expression was inhibited due to execution of apoptosis pathway by activation of *Casp 1*, *3* and *7* proteins that overwhelmed the mitochondria recovery by *Opa1* protein. On the other hand, the *Ppid* (peptidylprolyl isomerase D) gene that encodes cyclophilin D protein plays a distinct role in regulating the permeability transition pore of mitochondria (Matas *et al*, 2009). The *Opa1* gene was down-regulated as 6-OHDA neurotoxin was capable of causing profound mitochondrial fragmentation preceding the breakdown of mitochondrial membrane potential and release of cytochrome c heme protein (Gomez-Lazaro *et al*, 2008).

Apart from that, *Nsg*1 and *Th* genes which are responsible in the catalysis of the rate limiting step in catecholamine biosynthesis in the dopaminergic neuron were down-regulated in 6-OHDA treated PC12 cells. *Nsg1* or Neuron-Specific Gene Family member 1 is a type of protein preferentially located in the brain, neuroendocrine glands and spinal cord. The role of this gene was not clearly defined, but

studies have shown that *Nsg1* protein function in neurons and germ-cells chemotaxis and endocytotic machinery (Rengaraj *et al*, 2011). Moreover, a recent study has reported that *Nsg1* protein was involved in trafficking of neuron-glia cell adhesion molecule (NgCAM). On the other hand, *TH* mRNA provides instruction for the expression of tyrosine hydroxylase enzyme which convert amino acid tyrosine to dopamine neurotransmitter (Daubner *et al*, 1992; Yap *et al*, 2008). In this study, the suppression of *TH* mRNA in 6-OHDA treated PC12 suggested that exposure of neuronal cells to oxidative stress caused suppression of *TH* mRNA level as well as tyrosine hydroxylase enzyme concentration. Subsequently, this event caused disruption of dopamine biosynthesis and resulted in dopamine depletion and motor function dysfunction as observed in a rat model of Parkinson's disease (Sherman and Moody, 1995).

The mRNA expressions level of ion transport proteins, *NSF* (N-ethylmaleimide-sensitive factor) was down-regulated and in contrary the *Egln1* mRNA expression was highly expressed in 6-OHDA treated PC12 cells. *NSF* plays a crucial role as vesicular transport and membrane fusion (Whiteheart *et al*, 2001). Soluble *NSF* attachment proteins (SNAP) bind SNARE (soluble NSF attachment protein receptor proteins) to form SNAP-SNARE complexes (Sollner *et al*, 1993). NSF disassembles the SNARE complexes to recycle the monomers to facilitate the membrane fusion process. The 6-OHDA caused inhibition of *NSF* mRNA expression and caused depletion of free *NSF* which subsequently disabled the vesicle fusion machinery essential for restoration of synaptic damage and progressively leads to synaptic dysfunction and neuronal death (Liu and Hu, 2004). Egl nine homolog 1 (*Egln1*) gene is an important oxygen sensor in cells that negatively control the activity of *HIF-1A* (Hypoxia-inducible factor-1 alpha). *HIF-1A*, a transcriptional complex activated at hypoxic condition via inhibition of *Egln1* gene expression and subsequently activates the anti-oxidative genes that promote alterations in cellular metabolism (Baik *et al*, 2013). The data presented in this study consistent with

the theory that oxidative stress caused by 6-OHDA on PC12 cells, which was actually a hypoxic state inhibited the egln1 mRNA expression level and studies have demonstrated that the inhibition of *Egln1* is associated with protective measures exhibited by cells to limit glucose oxidation and reactive oxygen species production (Baik *et al*, 2013).

Although the main objective of this study was to assess the mRNA expression changes caused by antioxidant bioflavonoids rutin and isoquercitrin, but L-DOPA was included in this study to compare and contrast the mRNA expression changes between these flavonoids and the gold standard treatment of Parkinson's disease (Jin *et al* 2010). L-DOPA pretreatment and subsequent treatment with 6-OHDA showed an absolute contrary gene expression changes than PC12 cells exposed to 6-OHDA alone. Our findings show that L-DOPA pretreatment down-regulated most of the genes in *Ataxin*, *parkin* and *caspase* groups. To ensure the validity of PCR array data, the mRNA expression changes of *Park 5, 7, Casp3* and *TH* were performed on q-RT-PCR assay. Here, we show that the qPCR array data correlated well with q-RT-PCR findings (Figure 2).

Several studies have demonstrated that, activation of *Casp3* appears to be the major factor for the execution of apoptosis in neuronal cells (Robertson *et al* 2000). In our study, we have demonstrated L-DOPA at low concentration protected the PC12 cell via suppression of *Casp1*, *3* and *7* which are the crucial players in inducing apoptosis cascade. In addition, we showed that the protective effect of L-DOPA was closely associated with activation of Tyrosine hydroxylase enzyme activity. Essentially, the presence of L-DOPA in the experimental system has activated the *TH* mRNA level to generate more tyrosine hydroxylase enzyme for the rate limiting biosynthesis of dopamine neurotransmitter from the oxidation of L-DOPA. Hence, this study has demonstrated the recovery of the dopamine

biosynthesis pathway in L-DOPA pretreatment of 6-OHDA treated PC12 experimental model has contributed to PC12 cells survival through suppression of *Park2, Park7* and *Park5* genes.

Besides that, the neuroprotective effect caused by rutin and isoquercitrin pretreatment were almost similar as L-DOPA pretreatment with few exceptions. The antioxidant mechanism exhibited by rutin to protect the 6-OHDA induced PC12 cells was dissimilar from the protective mechanism by L-DOPA pretreated cells. In our preliminary study, rutin has activated the internal antioxidant enzymes and attenuated lipid peroxidation treated by 6-OHDA to protect PC12 neuronal cells (Magalingam *et al* 2013). The suppression of free radical damage has contributed to cell survival via activation of protective genes such as *TH, Nsg1* and *NSF* genes as well as down-regulation of pro-apoptotic genes including caspases and *parkins*.

However, the neuroprotective effect of isoquercitrin pretreatment was less potent compared to rutin pretreatment as isoquercitrin regulated fewer genes compared to rutin. We suggest that rutin and isoquercitrin executed different protective pathways although both are grouped as quercetin glycosides. Jung *et al* have proven that isoquercitrin pretreatment of neuronal cells activated antioxidant enzymes and suppressed free radicals induced lipid peroxidation (Jung *et al* 2010). In our gene expression study, quercetin 3-glucoside or isoquercitrin activated the *Nsg1, Opa1* and *Nsf* to protect the cells from undergoing apoptosis. However, the caspases like *Casp1, Casp3* and *Casp7* genes were not down regulated in isoquercitrin pretreatment against 6-OHDA induced PC12 cells and these data have been validated using qRT-PCR analysis using *Casp3* primer sequence. Interestingly, isoquercitrin pretreatment has attenuated the *Parkin* gene expression, namely *Park5, park7* and *Stub1*.

The isoquercitrin has a great ability to decrease free radical induced misfolded protein synthesis hence Parkin proteins were not synthesized (Jung *et al* 2010; Silva *et al* 2009).

In conclusion, the results of the present study suggested that quercetin glycosides rutin and isoquercitrin have significant antioxidant and neuroprotective effects against 6-OHDA induced neurotoxicity. Rutin modulated several protective genes to prevent the neuronal cell death, particularly suppressing the *Park 5, Park 7,* and *Casp 3* genes and up-regulating the *TH* gene. On the other hand, isoquercitrin suppressed the expression of *Park 5* and *Park 7* genes but did not affect the expression levels of *Casp3* or *TH* genes. Rutin and isoquercitrin could be effective therapeutic agents in protecting dopaminergic neurons in PD. These quercetin glycosides may be used along with L-Dopa therapy to protect against the neurodegeneration in PD.

Conflict of Interest: There is no ethical/legal conflicts involved in the article.

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Table 1

The grouping and types of genes analyzed using quantitative real time PCR technique

Gene Grouping	Types of Genes
Parkin Substrate	*Atxn2* [van de Loo, 2009], *Atxn3* [Chou, 2008]
Parkin Complex	*Park2*[Chen, 2012], *Park5*[Day, 2010], *Stub1*[Chen,2012], *Park7*[Raman,2013; Bandopadhyay,2004]
Pro-apoptosis	*Casp1* [Asahi,1999], *Casp3*[Gruden, 2013], *Casp7* [Lamkanfi, 2010]
Anti-apoptosis	*Opa1* [Graya,2013], *Ppid* [Bourke,2013]
Dopaminergic	*Nsg1* [Rengaraj, 2011] , *TH* [Asmus, 2008]
Ion transport	*NSF* [Yu, 2011; Whiteheart, 2001], *Egln1* [Baik, 2013, Buroker, 2012]

Table 2

Primers used in RT-PCR analysis

Gene ID	Gene name	Size	Primer sequence
nm017237	*Park5* (5’) *Park5* (3’)	267	CAAGTGTTTCGAGAAGAACGA AGAGCCACTGCGGAGAA
nm057143	*Park7* (5’) *Park7* (3’)	261	AAAACACAGGGACCATACGA CTTCTCCACACGGCTCTCT
nm012922	*Casp3* (5’) *Casp3* (3’)	233	ATTACCCTGAAATGGGCTTG CACACACACAAAACTGCTCCT
nm012740	*TH* (5’) *TH* (3’)	238	GACATTGGACTTGCATCTCTG GCTGGTAGGTTTGATCTTGGT
nm017008	*GAPDH* (5’) *GAPDH* (3’)	240	AGGTGAAGGTCGGAGTCAAC AGGGATCTCGCTCCTGGAA

Figure Legends:

Figure 1 *: Histogram showing the base 2 logarithm of the fold change in the expression levels of 15 genes that expressed a significant up/down regulation between the rutin, L-DOPA and isoquercitrin as pretreated group against 6-OHDA treated cells as control group using customized Rat Parkinson's disease PCR array. The x-axis represents the genes of quantitative real-time PCR analysis and the y-axis represents the fold changes of 15 genes.*

Figure 2*: Histograms showing the base 2 logarithm of the fold change of validation study of selected mRNA expression of 4 selected genes, namely Park5, Park7, Casp3 and TH using Quantitative reverse transcription-PCR (q-RT-PCR) assay and compared with PCR array data.* ***A****:6-OHDA treated PC12 cells;* ***B****:L-DOPA pretreatment and exposure to 6-OHDA (L-DOPA + 6-OHDA);* ***C****:Rutin pretreatment and exposure to 6-OHDA (Rutin + 6-OHDA);* ***D****:Isoquercitrin pretreatment and exposure to 6-OHDA (Isoquercitrin + 6-OHDA). Values are represented as mean ± SEM, (n=3) and bars with *indicates P <0.05. The normalized data set from pre-treated groups were compared with normalized data obtained from 6-OHDA treated PC12. While the 6-OHDA treated group was normalized from PC12 cells grown in complete culture media alone.*

Figure 1

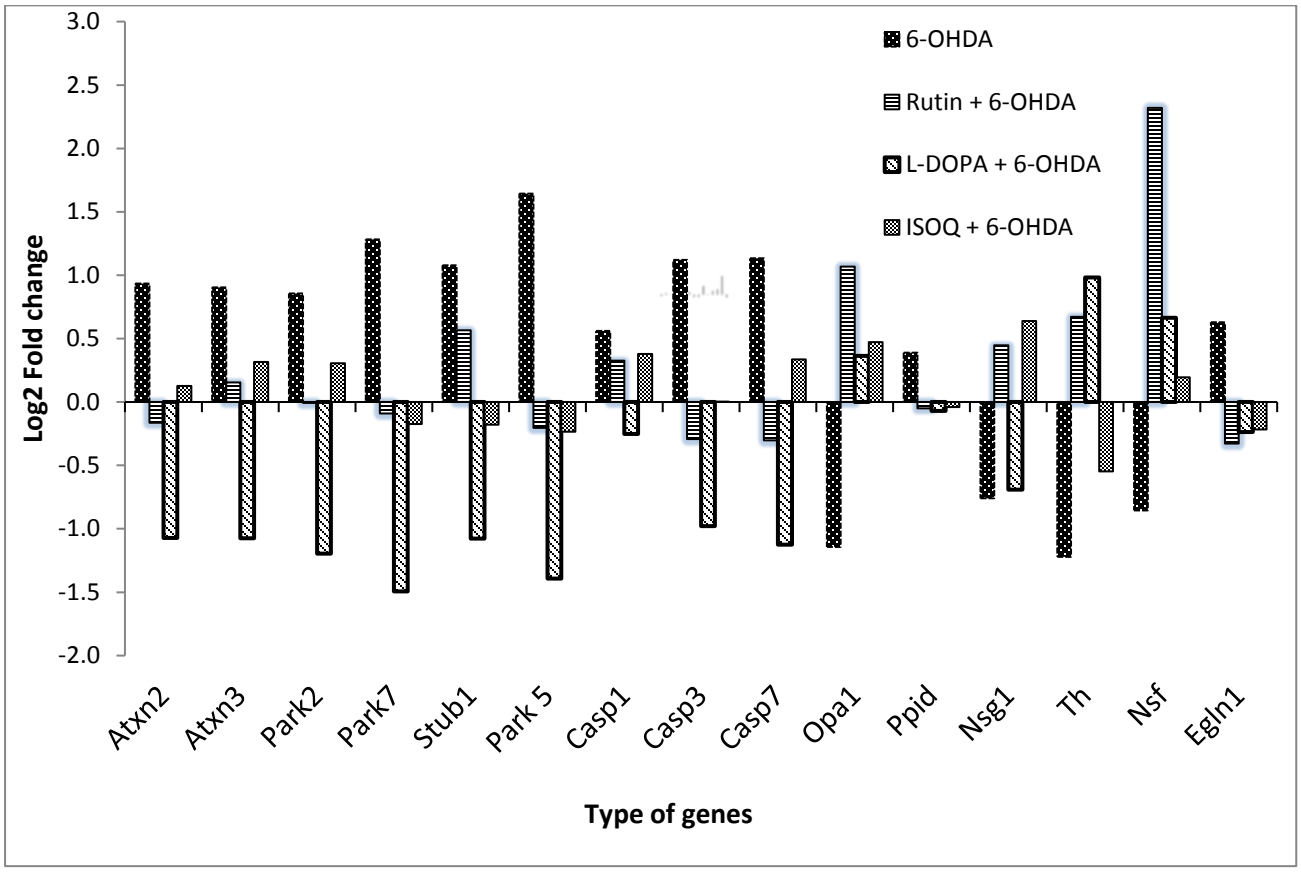
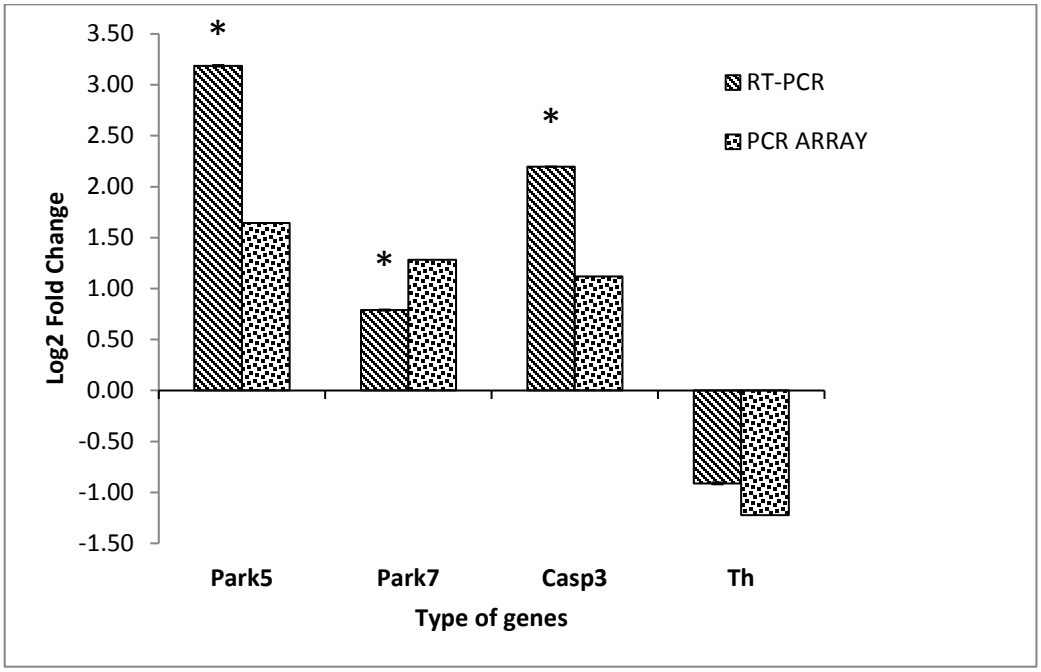
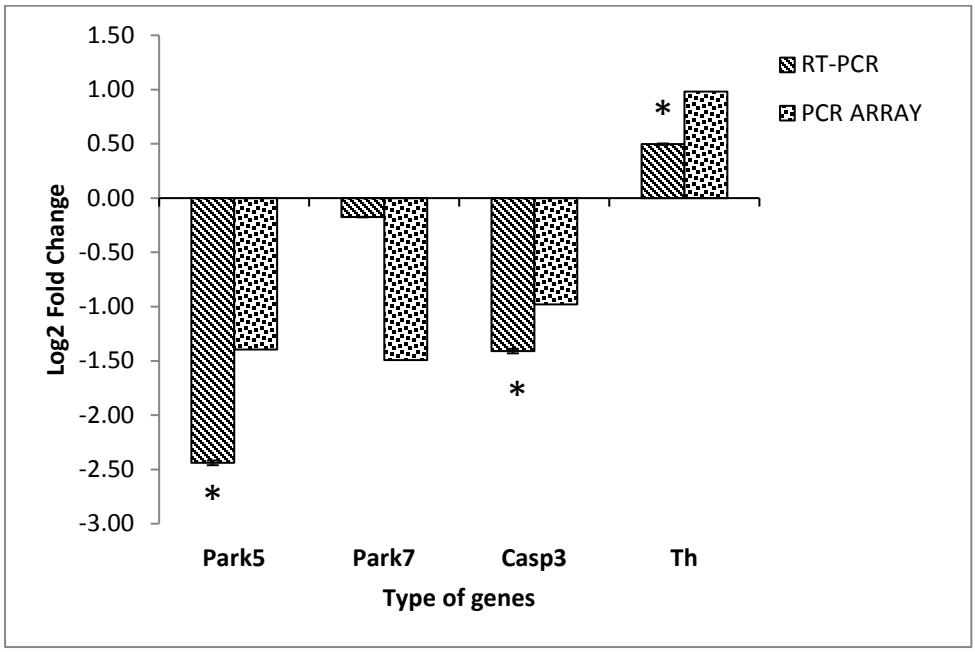


Figure 2

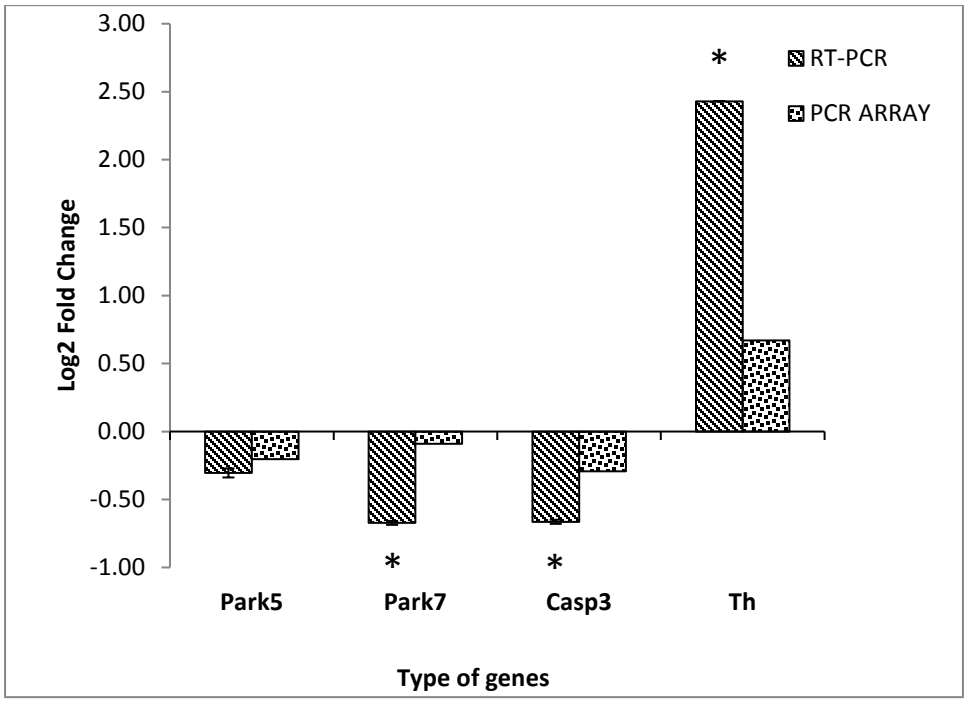
A.



B.



C.



D.