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Iron status, immune system, and expression of brain divalent metal transporter 1 and dopamine receptors D1 interrelationship in Parkinson's disease and the role of grape seed and green coffee bean extracts and quercetin in mitigating the disease in rats

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ABSTRACT

Introduction: Parkinson's disease (PD) is a neurodegenerative disease with a prevalence of 1% in the elderly worldwide. The aim of the research is to study the interrelationship of iron status, the immune system including inflammatory cytokines, brain divalent metal transporter 1 (DMT1), and dopamine receptors D1 (DRD1) in a PD rat model. The potential protective effects of grape seed and green coffee bean ethanol extracts and quercetin were also studied.

Methods: Phenolic and flavonoid contents of grape seed and green coffee bean and *in vitro* free radicals scavenging activities of the extracts and quercetin were determined. Male rats were divided into five groups. Group 1 served as normal control (NC), group 2 represented Parkinsonian control (PC). Groups 3, 4, and 5 were the test groups treated by daily oral green coffee bean, grape seed extracts, and quercetin, respectively. PD was induced by rotenone in groups 2 to 5. Brain oxidative stress, DMT1 and DRD1 expressions, and histopathology were assessed. Parameters of the immune system, represented by plasma interferon-gamma (IFNγ) and CD4, and brain tumor necrosis factor-alpha (TNF-α) along with iron status were also determined.

Results: Phenolic and flavonoid contents of green coffee bean were high compared to grape seed ($P<0.05$). Quercetin experienced the highest in-vitro free radicals scavenging activities. Iron deficiency anemia, together with elevated IFNγ, TNF-α, DMT1 expressions, and brain malondialdehyde (MDA), were demonstrated in PC compared to NC ($P<0.05$). Also, reduction in CD4 and brain reduced-glutathione (GSH) ($P<0.05$) were noticed in PC with brain histopathological alterations. Different treatments showed variable improvements in the majority of parameters ($P<0.05$) and brain histopathology.

Conclusion: Iron deficiency anemia might result from cytokine elevation in PD. Reduced DRD1 and altered immune system including cytokines together with increased brain DMT1 might induce neurodegeneration in PD. Different treatments showed variable neuroprotective effects through modulation of inflammation, oxidative stress, immune system, iron status, DMT1, and DRD1.

Implication for health policy/practice/research/medical education:
Iron deficiency anemia, reduction in brain DRD1, an immune system alteration, an increase in brain DMT1, and oxidative stress might be involved in the pathogenesis of PD. Nutraceuticals represented by grape seed and green coffee bean ethanol extracts and quercetin might be implicated in the prevention of PD.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. The prevalence of the disease is 0.3% in the general population and 1% in people over 60 years old (1). The disease is characterized by dopaminergic neuronal degeneration and cell death in the substantia nigra with accumulation of Lewy bodies (intra-cytoplasmic inclusion) consisting of ubiquitin and α-synuclein. These histopathological hallmarks generate cardinal symptoms represented by bradykinesia, tremors, rigidity, and postural instability. Lewy bodies also present in other parts of the central nervous system, which lead to non-motor symptoms like depression, sleep disorders, constipation, and dementia. The symptoms of PD only appear after the loss of more than 70% of dopaminergic cells (2). Brain inflammation mediated by microglial activation might be involved in the pathogenesis of PD. Microglia in a normal state serve the role of immune surveillance, while on exposure to neurotoxins like rotenone, an abnormal stimulation occurs, resulting in microglial activation leading to immune disorder and elevated production of inflammatory cytokines (3). The activated microglia secrete pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) that trigger intracellular death-related signaling pathways. Microglial cells are divided rapidly and produce pro-inflammatory, and cytotoxic reactive oxygen and nitrogen species, which are deleterious and lead to progressive degeneration of dopaminergic neurons (4,5).

Complex I inhibition is linked to Parkinsonian motor deficits through inducing mitochondrial dysfunction, neuroinflammation, and degeneration of dopaminergic neurons of substantia nigra (6). Rotenone, which induces the PD model in rodents, inhibits complex I resulting in the aforementioned changes in addition to high oxidative stress and activated mitochondrial-dependent apoptosis pathways with the formation of cytoplasmic inclusions similar to Lewy bodies (7). Therefore, rotenone-induced PD in rats is suitable for testing new therapeutic agents.

The neurotransmitter dopamine (DA) plays a significant role in not only motor function but also cognitive and neuroendocrine (8). DA receptors are classified into two subfamilies (D1 and D2). The dopamine receptor D1 (DRD1) subfamily is expressed in different brain regions like the cortex, hippocampus, amygdala, substantia nigra, and striatum (9). DRD1 regulates neuron growth, mediates some behaviors, and controls the DRD2 mediated events (10). DRD1 stimulates neurons by activating cyclic AMP-dependent protein kinase. DRD1 agonists have profound anti-parkinsonian effects and can produce cognitive enhancement. It was reported that DRD1-deficit mice increased locomotor activity, which clarified that D1 receptors exert distinct and complex physiological effects in locomotor activity (11). DRD1 agonists directly activate DRD1 and bypass the presynaptic synthesis of DA. DRD1 agonists have neuroprotective activity induced by scavenging free radicals, reducing DA synthesis, release, and metabolism, and exerting anti-apoptotic effects. There is experimental and clinical evidence that activation of DRD1 is beneficial in the treatment of PD (12).

Iron and immunity have close link since the innate immune system (monocytes, macrophages, microglia, and lymphocytes) responds to any insult like tissue injury by controlling iron flux, mediated by proteins involved in iron homeostasis like hepcidin and ferroportin. In addition, lymphocytes play a vital role in adaptive immunity. Effector molecules can arrange the inflammatory response by mobilizing a variety of cytokines, neurotrophic factors, chemokines, and reactive oxygen and nitrogen species (13). Therefore, it is important to correlate the possible pathological pathways of the PD with the patients' ferrokinetic state.

Iron deficiency anemia was reported to increase PD risk, and a high iron intake might reduce such risk. Meanwhile, increased iron deposition is noted in the substantia nigra of PD patients. Few studies have suggested the occurrence of iron deficiency anemia after the onset of PD. Patients in the late stage of PD have a lower serum iron and ferritin than control (14-17). Therefore, a question is raised whether an iron deficiency is a risk factor or a consequent result of PD. Iron accumulation in dopaminergic and glial cells in PD patients may contribute to the generation of oxidative stress, protein aggregation, and neuronal death. The mechanism involved in iron accumulation is unclear; however, increased brain DMT1 expression might have a critical role in iron-mediated neurodegeneration in PD (18). This complexity denotes the fine balance required in iron homeostasis since this metal is indispensable for many cell functions but highly toxic when appearing in excess.

According to previous researches, drugs used for treating PD have significant limitations. Therapies for PD are limited since they target the final disease pathologies but not the actual cause of disease, which is not suitable for managing disease onset or progression. Therefore, it is mandatory to search for new approaches to PD therapy. Bioactive antioxidant compounds in plant foods might have anti-inflammatory and neuroprotective effects in PD in which high oxidative stress is implicated in the nigrostriatal dopaminergic neuronal loss. These effects are expected to consequently ameliorate the pathological changes in PD, including iron status in blood and brain, immune parameters, including inflammatory cytokines, in addition to DRD1. Green coffee beans and grape seed alcohol extracts were selected from such plant food to study their beneficial health effect in PD. It has been reported that coffee consumption is inversely related to tremor severity in male PD patients (19). However, the relationship between coffee consumption and tremors in the general population is controversial in PD (20). Therefore, the therapeutic effect of coffee in PD patients

still needs investigation. As quercetin was reported to be the compound of therapeutic effect in the coffee beans and not caffeine (21), it is worthy of studying the remedial effect of quercetin in PD patients. On the other hand, the grape seed was demonstrated previously to contain phenolic compounds with reported antioxidant and anti-inflammatory effects (22) with probable therapeutic efficiency on PD. The present study was designed to study the changes of brain DMT1, DRD1, and oxidative stress and to investigate the interrelation of iron status and immune responses along with the brain histopathological changes in a rat model of PD. Another important aim is to investigate the role of grape seed and green coffee bean extracts and quercetin as neuroprotectors and modulators of the pathological state of the PD rat model.

Materials and Methods

Plant materials

Grape seeds (*Vitis vinifera,* family Vitaceae) from the Egyptian variety were separated from the fruits and dried in a hot air oven at 40ºC. Arabica green coffee beans (*Coffea arabica* L., family Rubiaceae) were purchased from Saudi Arabia.

Main chemicals

Rotenone was purchased from Abcam, USA for induction of PD. Quercetin was obtained from Molekula, Wessex House, Shaftesbury, Dorset, UK. All chemicals used in the study were of high grade and purity.

Animals

Male Wistar rats of body weight ranging from 110-130 g were purchased from the Animal House of National Research Centre, Egypt. Rats were kept individually in stainless steel cages at ambient temperature (25°C ± 2), with a 12 hours light/dark cycle. Food and water were supplied *ad libitum*.

Preparation of extracts

A known weight of dried grape seeds and coffee beans were extracted separately by absolute ethanol using continuous extraction apparatus (Soxhlet). The solvents were evaporated from each extract at a temperature not exceeding 40ºC under reduced pressure. The yield of each extract was weighed.

Total phenolics, total flavonoids, and free radical scavenging activity

Green coffee beans powder and grape seeds (0.1 g) were extracted separately with 2.0 mL of 80% aqueous ethanol overnight on a platform shaker. The mixtures were centrifuged, and the residues were re-extracted in an ultrasonic water bath for 5 minutes. The mixtures were centrifuged and the supernatants obtained from each seed type were combined.

Total phenolics were determined colorimetrically in the green coffee beans and grape seeds according to the modified Folin-Ciocalteu micro method (23). Absorbance was measured at 760 nm using a UVPC spectrophotometer. Gallic acid (GA) was used as a standard for the calibration curve. Total phenolic content was expressed as mg GA equivalent/g dry coffee bean and as mg GA equivalent/100g dry grape seed.

Total flavonoids were determined in the green coffee beans and grape seeds (24). Quercetin was used as a standard for the calibration curve. Total flavonoids content was expressed as mg quercetin equivalent (QE)/g dry coffee bean and as mg QE/100 g dry grape seed.

DPPH free radical scavenging activities of green coffee bean, grape seed, and quercetin were measured according to a previously described method (25) with some modifications; the absorbance was recorded at 515 nm. Blank was run in parallel in an identical manner without test samples. The percentage of DPPH radical-scavenging activity was calculated as follows:

[(A control – A sample)/A control] × 100, where A control was the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test sample.

Preparation of rotenone for treating rats

An amount of 125 mg rotenone was dissolved in 1 ml dimethylsulfoxide (DMSO). Then, 40 µL of the previously prepared solution was diluted in 1960 µL olive oil and mixed by a vortex. This reagent was freshly prepared 2-3 times/week and kept in the dark in refrigerator. Before injection, the solution was taken off from the refrigerator and mixed by a vortex for uniformity (26). The vehicle of the previously prepared solution was prepared without rotenone to be used for the control group.

Preparation of doses of grape seed and coffee bean extract and quercetin

The extracts and the quercetin were prepared individually in distilled water.

Preparation of diet

A balanced diet consisting 12% casein, 10% sunflower oil, 68.5% corn starch, 5% wheat bran, 3.5% mineral mixture, and 1% vitamin mixture was prepared and fed to rats all over the experiment.

Design of animal experiment

Forty rats were divided into five groups, eight rats each. Group 1 served as normal control (NC), group 2 represented the Parkinsonian control group (PC). Groups 3, 4, and 5 were the test groups. All rats were maintained on a balanced diet all over the experimental period, which lasted 4 weeks. Rats of groups 3, 4, and 5 were treated orally from the first day of the experiment with green coffee ethanol extract as 500 mg/kg, grape seed ethanol extract as 500 mg/kg, and quercetin as 100 mg/kg on a daily basis

till the end of the experiment. At the start of the fourth week, rats of groups 2 to 5 were treated subcutaneously with rotenone as 2.5 mg/kg rat body weight/day for 7 consecutive days for induction of the PD model (27,28). Both control groups were given daily oral doses from distilled water throughout the experiment. Rats of the NC group were injected subcutaneously by the same quantity of the vehicle used for the preparation of rotenone on a daily basis in the last week of the experiment. Rats' body weights were monitored weekly during the experiment. At the end of the experiment, body weight gain was calculated. Blood samples were obtained from fasted rats in heparinized tubes and centrifuged at 3000 rpm for 15 minutes to obtain the plasma. Plasma iron and total iron-binding capacity (TIBC) were determined as previously reported (29,30). Transferrin saturation percentage was calculated using the following formula: (Plasma iron/TIBC) × 100. Plasma soluble transferrin receptor (sTfR), ferritin, IFN-γ, and CD4 were assessed using ELISA kits supplied from Sunlog Biotech Co. LTD, China. The brain was excised, immediately put on ice, and cut into two longitudinal sections. One section was used for the assessment of malondialdehyde (MDA) (31), reduced glutathione (GSH) (32), and TNF-α, adopting the method of ELISA kit purchased from Sunlog Biotech Co. LTD, China. Inter and intra-assay CV of all the aforementioned ELISA Kits were < 12 and < 10, respectively. However, the sensitivity was acceptable and differed according to the assayed parameters. Gene expressions of brain divalent metal transporter 1 (DMT1) and dopamine receptor D1 (DRD1) were assessed using real time polymerase chain reaction (RT-PCR). The other section of the brain was fixed in 10% formalin for histopathological examination (33) using hematoxylin and eosin.

Gene expression analysis of brain DMT1 and DRD1 by RT-PCR

Total RNA was isolated from brain tissue with PureLink® RNA Mini Kit (Ambion® Life Technologies™), according to the manufacturer's instructions. The cDNA was synthesized from 1.5 µg of total RNA in 20µL reaction with RevertAid first strand cDNA synthesis kit (Thermo Fisher® Invitrogen™), according to the manufacturer's instructions. The RT-PCR was performed with a Rotor-Gene® MDx instrument. The RT-PCR reaction mixture (25 µL) contained 1 µL template cDNA, 1× the EvaGreen® PCR master mix (HOT FIREPol® EvaGreen® qPCR Mix Plus, Solis BioDyne™) and 0.2 µM of the primer pairs. Primers pairs sequences used for DMT1 and DRD1 are presented in Table 1. PCR reactions were performed using the following protocol: 50°C for 2 minutes, 95°C for 12 minutes, 45 cycles of 15 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C, melting curve program (60-95°C). PCR water was used instead of cDNA templates as a negative control. The relative expression of the target genes was calculated using $2^{-\Delta\Delta CT}$ method (34). The target gene expression was normalized to the expression of the house-keeping gene GAPDH.

Statistical analysis

The data were expressed as mean ± standard error (SE) and analyzed with one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison tests using the SPSS statistical program. Differences were considered significant at $P \leq 0.05$. The normality of the data was confirmed using Kolmogorov-Smirnov and Shapiro-Wilk tests.

Results

Results showed that total phenolics and flavonoids in green coffee beans were 254.5 ± 0.346 GA/g and 84.58±0.356 QE/g, respectively (Figure 1A). The grape seed content of phenolic compounds was 67.898 ± 1.00 mg GA/100g, while the flavonoid content was 47.955 ± 0.772 mg QE/100g (Figure 1A). These results demonstrated significantly high values of phenolic and flavonoid contents of green coffee beans compared to grape seeds ($P < 0.05$). Total phenolics and flavonoids in the alcohol extract of grape seed were 87.7 mg GA/100 g and 65 mg QE/100 g extract, respectively. Percentage DPPH scavenging activities of green coffee bean extract, grape seed extract, and quercetin were 87.558 ± 0.145, 78.923 ± 0.306, and 90.975 ± 0.169, respectively ($P < 0.05$) (Figure 1B). The yields of extracts from green coffee beans and grape seeds were 12.01 and 14.05 g/100 g dry sample, respectively.

Table 2 demonstrates a significant reduction in final body weight and body weight gain in rats of the PC group compared to those of the NC group. The different treatments produced an insignificant increase in final body weight compared to the PC group. Rats receiving green coffee and grape seeds extracts showed a significant increase in body weight gain, while quercetin treatment

Table 1. Primers used for real-time polymerase chain reaction amplifications

Target genes	Sequences	Product size (bp)
DMT1	FW (5′-TTTGGCTTTCTCATCACTATCATGGC -3′) RW(5′-ATTGGCTTCTCGAACTTCCTGCTTATTGGC-3′)	248
DRD1	FW (5′-TCCTTCAAGAGGGAGACGAA-3′) RW(5′- CCACACAAACACATCGAAGG-3′)	168
GAPDH	FW (5′- GTATCGGACGCCTGGTTACC-3′) RW(5′- CGCTCCTGGAAGATGGTGATGG-3′)	202

DMT1: Divalent metal transporter 1, DRD1: Dopamine receptors D1, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

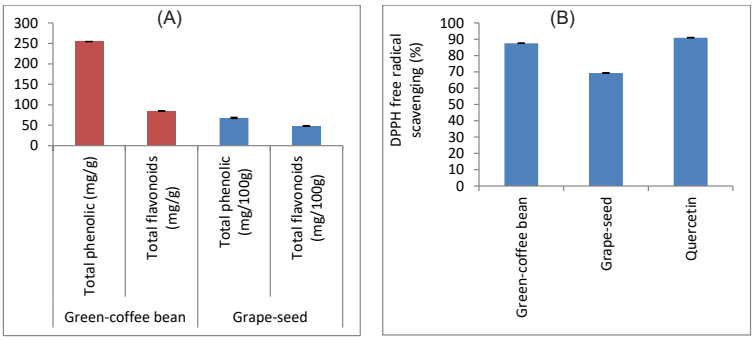

Figure 1. Total phenolics and flavonoids in green coffee bean and grape seed (A). DPPH free radical scavenging activity % of green coffee bean and grape seed extracts and quercetin (B).

demonstrated only an insignificant increase compared to the PC group. All test groups showed a significant reduction in final body weight and body weight gain compared to the NC group.

Results in Table 3 indicate a significant reduction of plasma iron, % transferrin saturation, and ferritin with a significant increase in sTfR and TIBC in the PC group compared to the NC group. When calculating sTfR/log ferritin, it could be noticed that it was significantly high in the PC group compared to the NC group. Treatment with different nutraceuticals produced a significant increase in plasma iron compared to the PC group. Soluble transferrin receptors were significantly reduced on treatment with grape seed extract and quercetin but not coffee extract compared to the PC group. Only green coffee bean extract produced a significant increase in ferritin compared to the PC group. TIBC showed a significant reduction in the test groups compared to the PC group, while the levels were still significantly high compared to the NC group. The percentage of transferrin saturation demonstrated a significant increase on different treatments compared to PC. The levels of % transferrin saturation in the test groups were significantly lower compared to the NC group. The values of sTfR/log ferritin were shown to be significantly reduced on different treatments compared to the PC group; quercetin was superior in this respect.

Table 4 reveals a significant increase in brain MDA and reduction in GSH compared to the NC group, indicating elevated oxidative stress in the PC group. The different treatments showed a significant reduction in MDA with a concomitant increase in GSH compared to PC; both parameters matched the NC.

Immune system-related parameters (Table 5) showed significant increases in INFγ and TNF- α with significant reductions in CD4 in PC compared to NC, while different treatments demonstrated significant improvements of

Table 2. Initial and final body weight and body weight gain of different experimental groups

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
NC	$121.7^{a} \pm 2.67$	$230.7^{b} \pm 5.73$	$109.00^{d} \pm 4.43$
PC	$121.7^{a} \pm 3.93$	$165.0^{a} \pm 6.86$	$41.71^{a} \pm 3.71$
Green coffee bean extract	$121.7^{a} \pm 4.31$	$184.3^{a} \pm 5.75$	$62.67^{c} \pm 2.49$
Grape seed extract	$121.3^{a} \pm 3.87$	$174.8^{a} \pm 5.84$	$53.50^{bc} \pm 2.95$
Quercetin	$121.2^{a} \pm 2.6$	$172.5^{a} \pm 5.28$	$51.33^{ab} \pm 2.88$

NC: Normal control, PC: Parkinsonian control.
In each column same letters mean non-significant difference while different letters mean significant difference at $P < 0.05$. Data are expressed as mean values ± standard error.

Table 3. Plasma iron status of different experimental groups

Groups	Iron (µg/dL)	TIBC (µg/dL)	%Transferrin saturation	sTfR (ng/mL)	Ferritin (ng/mL)	sTfR/log ferritin
NC	$60.04^{b} \pm 5.49$	$65.5^{a} \pm 1.35$	$91.97^{d} \pm 8.75$	$0.42^{a} \pm 0.02$	$81.17^{d} \pm 1.54$	$0.22^{a} \pm 0.006$
PC	$31.88^{a} \pm 6.05$	$274.93^{f} \pm 0.32$	$11.89^{a} \pm 2.61$	$0.69^{b} \pm 0.01$	$66.00^{a} \pm 1.81$	$0.38^{d} \pm .006$
Green coffee bean extract	$74.44^{b} \pm 8.6$	$128.01^{b} \pm 0.8$	$58.14^{c} \pm 0.7$	$0.62^{cb} \pm 0.03$	$71.67^{b} \pm 1.85$	$0.33^{c} \pm 0.009$
Grape seed extract	$72.76^{b} \pm 8.47$	$154.04^{c} \pm 0.29$	$47.24^{c} \pm 0.44$	$0.59^{c} \pm 0.04$	$70.67^{ab} \pm 1.41$	$0.32^{bc} \pm 0.006$
Quercetin	$64.3^{b} \pm 8.09$	$218.14^{e} \pm 13.06$	$29.58^{b} \pm 3.6$	$0.56^{c} \pm 0.02$	$70.17^{ab} \pm 1.76$	$0.3^{b} \pm 0.008$

NC: Normal control, PC: Parkinsonian control, TIBC: Total iron binding capacity, sTfR: Soluble transferrin receptors.
In each column same letters mean non-significant difference while different letters mean significant difference at $P < 0.05$. Data are expressed as mean values ± standard error of the mean.

such parameters. INFγ showed restoration to the normal level on different treatments, while TNF-α only matched NC level on receiving quercetin. There was a significant reduction in CD4 when the rats given different treatments were compared to the NC group.

DMT1 gene expression (Table 6) was significantly up-regulated in PC compared to NC. Treatments with green-coffee bean extract, grape seed extract, and quercetin produced significant ($P<0.05$) down-regulation of the mRNA expression of DMT1 compared to the PC group by 5.6, 35, and 33 fold-change, respectively. The mRNA expression of DRD1 was significantly down-regulated in PC compared to NC. The level of DRD1 was significantly up-regulated after treatment with green-coffee bean extract, grape seed extract, and quercetin compared to PC.

The histopathological changes in brain tissues of different groups are demonstrated in Figure 2. The brain tissues from the control group (Figure 2, A1 and A2) exhibited normal architecture in both cortexes with well-organized nerve cells and striatum. The neurons were placed in neat rows with normal rounded basophilic nuclei. The granular cells looked rounded in shape and demonstrated large circular vesicular and prominent nucleoli. In the PC group (Figure 2, B1-B4), the most consistent findings in brain tissue sections were those indicating severe degenerative alterations, including pyknotic nuclei surrounded by marked cytoplasmic vacuolations associated with neuronophagia, satellitosis, and focal gliosis. The proliferation of glial cells and focal hemorrhage in the white matter were also present. Also, there was leukocytic infiltration and congested capillaries with red blood cells. However, treatment of rats with coffee bean extract (Figure 2, C1-C3) and quercetin (Figure 2, E1-E3) showed a reduction in the histopathological changes with some alterations still present, as seen in Figures 2, C2 and C3 and Figure 2, E2 and E3. Sections from the rats treated with grape seed extract (Figure 2, D1-D4) revealed almost normal neuronal cells of the brain tissue with only a few histopathological changes such as minimal vacuolation, pyknotic, and apoptotic nuclei.

Table 4. Brain malondialdehyde and reduced glutathione of different experimental groups

Groups	MDA (nmol/g tissue)	GSH (mmol/g tissue)
NC	$72.68^{a}\pm8.04$	$78.29^{b}\pm3.08$
PC	$191.17^{b}\pm5.93$	$33.26^{a}\pm1.28$
Green coffee bean extract	$58.36^{a}\pm6.81$	$90.34^{b}\pm10.14$
Grape seed extract	$57.51^{a}\pm14.55$	$80.44^{b}\pm6.09$
Quercetin	$84.2^{a}\pm12.37$	$95.63^{b}\pm15.06$

NC: Normal control, PC: Parkinsonian control, MD: Malondialdehyde, GSH: Reduced glutathione.
In each column same letters mean non-significant difference while different letters mean significant difference at $P<0.05$. Data are expressed as mean values ± standard error of the mean.

Table 5. Plasma INFγ and CD4 and brain TNF-α of different experimental groups

Groups	INFγ (pg/mL)	TNF-α (ng/g tissue)	CD4 (pg/mL)
NC	$30.43^{b}\pm0.82$	$14.83^{a}\pm1.14$	$715.33^{c}\pm4.44$
PC	$33.10^{a}\pm0.61$	$31.33^{b}\pm1.45$	$638.33^{a}\pm4.01$
Green coffee bean extract	$30.00^{b}\pm1.36$	$22.83^{c}\pm1.30$	$670.33^{b}\pm2.84$
Grape seed extract	$30.47^{b}\pm0.41$	$23.67^{c}\pm1.47$	$665.00^{b}\pm3.72$
Quercetin	$29.83^{b}\pm0.46$	$17.17^{a}\pm1.58$	$668.50^{b}\pm4.10$

NC: Normal control, PC: Parkinsonian control, INFγ: Interferon gamma, TNF-α: Tumor necrosis factor-alpha, CD4: Cluster of differentiation 4.
In each column same letters mean non-significant difference while different letters mean significant difference at $P<0.05$. Data are expressed as mean values ± standard error of the mean.

Table 6. The relative expression of DMT1 and DRD1 genes in brain tissue of different experimental groups

Groups	Relative expression	
	DMT1	DRD1
NC	0.020 ± 0.001^{a}	1.106 ± 0.062^{a}
PC	1.011 ± 0.105^{b}	0.013 ± 0.001^{b}
Green coffee bean extract	0.179 ± 0.028^{a}	0.208 ± 0.009^{c}
Grape seed extract	0.029 ± 0.003^{a}	0.187 ± 0.016^{c}
Quercetin	0.030 ± 0.003^{a}	0.365 ± 0.014^{d}

NC: Normal control, PC: Parkinsonian control, DMT1: Divalent metal transporter 1, DRD1: Dopamine receptors D1.
The mRNA expression of DMT1 and DRD1 is normalized with housekeeping gene (GAPDH), values are represented as means ± SE, the similar letters in the same column mean non-significant difference while different letters mean significant difference at $P<0.05$.

Discussion

As PD is the second most common neurodegenerative disorder affecting the elderly (1), the study of its pathogenesis must be completely understood to search for new effective therapies. An important issue in the present research was to study the interrelationship of iron status, the immune system, including inflammatory cytokines, oxidative stress, and expression of brain RDR1 as postulated biomarkers in rotenone-induced PD model in rats. Histopathological brain changes of such a model were examined. In addition, the efficiency of nutraceuticals represented by green coffee bean and Egyptian grape seed extracts and quercetin in ameliorating the aforementioned PD pathological changes were studied.

The hypothesis that immune system alteration is involved in PD pathogenesis has been raised for many years. Observation of changes in immunoglobulin synthesis, lymphocyte population in blood, and acute phase protein production that occur in patients with PD suggested stimulation of the immune system that may result in PD. T-cell activation that leads to the production of INF-γ in PD further supported this hypothesis. Strong evidence suggests that an immune system mechanism is involved in neuronal damage in PD patients because, in

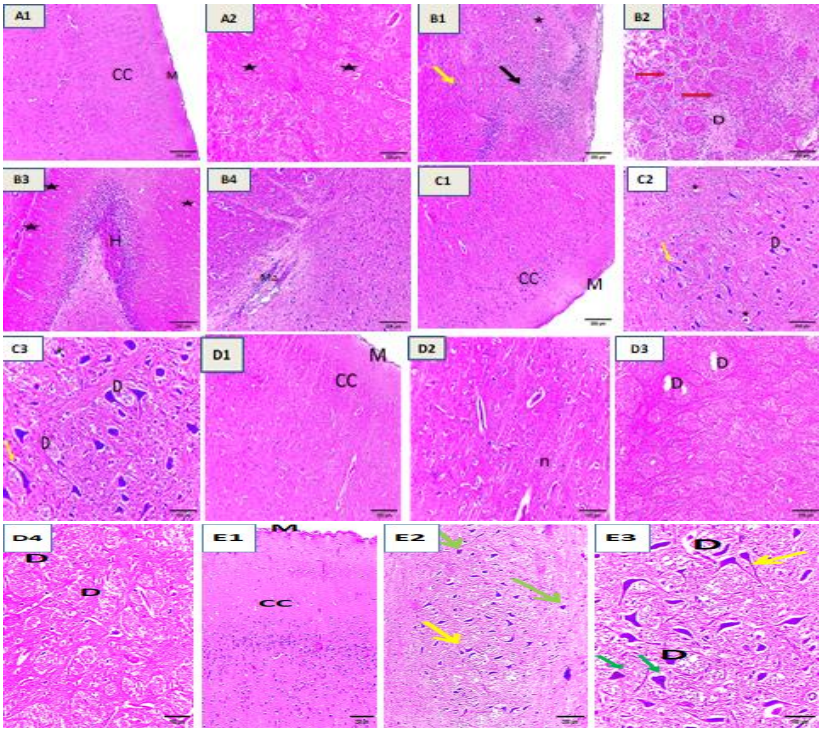

Figure 2. Histopathological examination of brain tissue [Stained with hematoxylin and eosin (H&E)]. **A1 and A2:** Brain sections of NC rats (Low power). **A1.** The cerebral cortex of a normal healthy rat displayed normal meninges (M) and cerebral cortex (CC) with intact neurons showing normal and prominent nuclei, **A2**. Brain section of normal healthy rat demonstrated perikaryon as well as normal myelination (black star). **B1-B4**: Brain sections of PC rats (Low power), **B1**. Brain section showed shrunken, atrophied and degenerated neurons associated with bodies simulate Lewy bodies (yellow arrow) and neuronophagia in which the degenerated neurons appeared shrunken and surrounded by microglia cells (black arrow), and neuropil vacuolation (asterisks). **B2.** The brain section demonstrated severe accumulation of neuroglial cells (red arrow) associated with demyelination. **B3.** Rat brain section displayed hemorrhage in the hippocampal region (H), as well as congestion of the blood vessels in the midbrain and striatum. **B4.** Rat brain sections demonstrated cerebral malacia (Ma) seen as an area of tissue loss with a modest amount of residual gliosis associated with degenerating and (or) apoptotic neurons. **C1-C3**: Brain sections of rats treated with coffee bean extract where C1 and C2 represent low power while C3 is high power. **C1.** The brain section of rats displayed a reduction in karyopyknotic neuronal cells of the cerebral cortex (Low power). **C2&C3**: Rat brain displayed demyelination of nerve fibers (D) with Lewy bodies (yellow arrow), neuronal swelling, chromatolysis and neuropil vacuolation (asterisks) **D1-D4:** Brain sections of rats treated with grape seed extract where D1 and D3 represent low power while D2 and D4 are high power. **D1&D2.** Rat brain sections demonstrated a marked reduction in lesions of degenerating and (or) apoptotic neurons. **D3&D4. Brain sections** displayed some demyelination of nerve fibers (D) with neuropil vacuolation. **E1-E3:** Brain sections of rats treated with quercetin where E1 and E2 represent low power while E3 is high power. **E1.** Rat brain demonstrated moderate degeneration and necrosis of neurons as fewer lesions and tissue damage appeared to be minimized as well as reduced vacuolar spaces around the pyramidal cells in the cerebral cortex. **E2&E3**. Rat brain displayed demyelination of nerve fibers (D), neuronal swelling, chromatolysis and nuclear margination (green arrow). **NC**: Normal control, **PC:** Parkinsonian control

the damaged region of the brain, there is an indication of inflammation, increased cytokines, and components of complement (35). However, another assumption is that immune system involvement might be a result of the injured brain region in PD patients that may induce stimulation of the immune system. Therefore, stimulation of the immune system might be a result of the disease, not a cause. The present results demonstrated an elevated INF-γ and a reduced CD4 in plasma together with increased TNF-α in the PD control group, emphasizing an alteration in the immune system.

High oxidative stress induces dopamine reactive oxygen species and oxidized dopamine metabolites are toxic to nigral neurons in PD patients. Recently the involvement of neuro-inflammatory processes in nigral degeneration has also gained increasing attention. Various pathways could link dopamine-dependent oxidative stress and microglial activation and ascribing a pathogenic trigger to the chronic inflammatory response characteristic of PD (36). An immune-reaction-associated inflammatory process in the brain may be reflected in the depletion of CD4 and increased IFN- γ in the peripheral immune system in PD patients (37), as seen in the present research.

It was reported that mitochondrial dysfunction-induced-elevated oxidative stress, inflammation, and environmental toxicity have important roles in PD pathogenesis (38). These processes contribute to neurodegeneration via lipid peroxidation that leads to the production of active aldehydes, including MDA (39). Rotenone, an insecticide and herbicide, is reported to reproduce neurochemical, neuropathological, and behavioral features of PD in rats (40) that simulate human PD changes. In the present study, rotenone treatment demonstrated a marked increase in brain MDA that has a role in dopaminergic neuronal loss.

In addition, the PD control group showed brain GSH reduction indicating elevated oxidative stress in the brain. Also, the high level of brain TNF-α, an inflammatory cytokine, has been demonstrated in rotenone-treated rats. Activation of microglia by rotenone might be responsible for TNF-α release, which might play a role in sustaining dopaminergic degeneration. The alterations induced by rotenone in the present study agreed with a previous study (41).

In rats with iron deficiency anemia, hemoglobin level and serum iron were lower, while TIBC was higher than normal rats (42). A reduction in the percentage transferrin saturation and ferritin together with an increase in soluble transferrin receptors indicate a state of iron deficiency anemia (43,44). Also, it was reported that elevated sTfR/log ferritin is an efficient index of iron deficiency anemia whether the subject suffers from inflammation or not (44). The aforementioned changes in iron status were demonstrated in the present research pointing to the occurrence of iron deficiency anemia in the PD rat model, supporting the hypothesis that iron deficiency anemia is a result of PD. This result did not negate that iron deficiency anemia might be a risk factor for PD, which needs further prospective studies. On the other hand, the up-regulation of DMT1 in the PD control group in the present study might reflect the deposition of iron in the brain, which might participate in neurodegeneration in PD through the generation of oxidative stress, neuronal apoptosis, and protein precipitation in the brain (18). The accumulation of iron in brain tissue might have a role in the occurrence of iron deficiency anemia in PD through an imbalance in iron homeostasis in other parts of the body.

The cross-talk between iron homeostasis and the immune system is crucial to be understood in PD. It is important to study the mutual effect between iron status and immunity. Immune activation can result in alteration in iron homeostasis that may impair erythropoiesis and contribute to immunopathology (45). Both cytokines and acute-phase protein may be important factors for the pathogenesis of iron deficiency anemia in chronic diseases since they lead to retention of iron within the macrophages and hypoferremia (46). On the other hand, there is a recent concept concerning the role of iron in immunity (47). The iron proteins that influence innate immunity include transferrin receptors, ferritin, hepcidin, lactoferrin, siderocalin, haptoglobin, hemopexin, Nramp1, and ferroportin (48). Soluble transferrin receptors showed elevated level, while ferritin, a protein that serves to store iron in the tissue, was reduced in the present study in the PC group. Ferritin was expected to increase as an acute phase reactant during inflammation; however, the concomitant presence of anemia might abolish this effect. It is worthy of mentioning that iron loading and depletion can adversely affect the immune system.

Rotenone induces several histopathological changes in the brain resembling pathological human manifestation of PD (49), as could be seen in the present study. Also, the reduction in body weight gain in the PD control group in the current study might be due to non-motor symptoms like gastrointestinal complaints frequently reported in such disease (50).

Dopamine D1 receptors were down-regulated in the PC group in the present study, reflecting the reduction in dopamine neurotransmitter. The reduction in brain DRD1 resulted in the inhibition of neuron growth and could not control DRD2 mediated events (10). DRD1 deficiency stimulated locomotor activity in mice that manifested in PD (11).

Multiple lines of evidence suggest that consumption of plant food rich in phenolic compounds may exert a neuroprotective effect, which may result in a lower risk of neurodegenerative disease, including PD (19,51). The mechanisms underlying phenolic compounds include suppressing oxidative stress via mitochondrial respiratory chain function (52) and alleviating inflammatory responses associated with glial activation (53). The present study investigated the possible health benefits of coffee bean and grape seed ethanol extracts rich in phenolic compounds and the quercetin flavonoid on the PD model in rats.

The current research showed reduced brain oxidative stress reflected in reduced MDA and increased GSH along with reduced inflammation as could be seen from reduced TNF-α and IFN-γ on the administration of coffee bean or grape seed extracts and quercetin. The *in vitro* DPPH scavenging activity % of coffee bean or grape seed extracts and quercetin supported the *in vivo* inhibition of oxidative stress in the present study. Iron status was improved on supplementation of coffee bean extract except for soluble transferrin receptors. It was also improved by grape seed extract and quercetin except for ferritin. The improvement of iron status might be due to the perfection of the immune system manifested by the level of CD4, TNF-α, and IFN-γ together with the up-regulation of brain DRD1. The down-regulation of brain DMT1 on the administration of coffee bean extract, quercetin, and grape seed extract might inhibit iron accumulation in the brain with a consequent reduction in oxidative stress and inflammation that might produce neuroprotection. The reduction in body weight gain induced by rotenone was improved by treatment with coffee bean extract and grape seed extract in the present study, which might be ascribed to improved non-motor activity induced by rotenone, while quercetin did not improve body weight gain. Histopathological changes induced by rotenone was improved with variable degrees on treatment with the tested nutraceuticals; grape seed extract was superior, followed by quercetin, while coffee bean extract showed the least improvements.

The present research showed that phenolic and flavonoid contents of green coffee beans were high compared to grape seeds. It was reported that caffeine treatment, an essential flavonoid component of coffee beans, inhibited

brain oxidative stress induced by rotenone, which was accompanied by improved brain histopathological changes. Caffeine offered neuroprotection and ameliorated neurochemicals in the rat PD model (41). Controversial results on the effect of caffeine on PD are present in literature (54,55). However, quercetin, not caffeine, was reported as the major neuroprotection in coffee beans (21). Coffee drinkers were significantly less likely to have PD compared to nondrinkers (56). The reported bioactive coffee components are quercetin, chlorogenic acid, and caffeine that showed previously to prevent neurotoxicity through reducing INF-γ, and inflammatory biomarkers represented by TNF-α and Interleukin 6 released from activated microglia and astrocytes. Coffee bioactive constituents were also demonstrated to reduce the nuclear factor kappa of the activated B cells. Quercetin was shown to reduce oxidative/nitrative damage to DNA, protein, and lipids with a concomitant increase of GSH in specific brain cells, and it was indicated that quercetin was the major neuroprotective component in coffee against PD (21). The presence of quercetin in green coffee bean extract of reported anti-inflammatory and antioxidant effect (57) could have a therapeutic efficiency towards PD. It has been shown that coffee is a rich source of bioactive compounds called β-carbolines' alkaloids, which may reduce the risk of PD through exhibiting a wide spectrum of biochemical effects, including the antioxidant, neuroprotective, and anti-inflammatory effects (58).

The grape seed extract was shown to prevent rotenone-induced defects in mitochondrial respiration in a dopaminergic cell line (59). The presence of phenolic compounds in grape seed alcohol extract might impart an antioxidant, anti-inflammatory, and immunoregulatory activity (60), thereby improving PD. Grape-derived polyphenols suppress presynaptic oxidative stress and inflammation in neurodegenerative diseases (22). The antioxidant activity of grape seed was demonstrated to be attributed to flavonoids and 3 different flavan-3-ols (flavanols) represented by catechin, epicatechin, epicatechin gallate, and its polymers in addition to procyanidins and tannins (61,62).

The mechanism underlying the anti-parkinsonian effect of the studied plant extracts and quercetin might reside in the reduction of inflammation and oxidative stress that could be direct or through regulation of the immune system. Thereby they may reduce iron accumulation in the brain and improve brain tissue injury, especially by reducing DMT1. These effects could exert anti-apoptotic action in the brain. The present tested extracts and the flavonoid quercetin might act as DRD1 agonists. Therefore, they can possess an anti-parkinsonian effect and impart neuroprotective activity. Improvements of iron status denoted in plasma could be ascribed to the aforementioned described changes on the treatment by the extracts and quercetin.

The strength of the present study may lie in searching and finding out an alteration in iron status and immune system that were interrelated to each other and to PD pathology; the current research might be among the rare studies that focus on these points. However, the limitation may reside in not studying in-depth the other causes of immune system alterations in PD, like the changes in colonic microbiota that have been recently shown to have an intimate relation to immunity and inflammation. Therefore, prospective researches must be implemented in such an area. In addition, a clinical study is needed to confirm the results of the present research in human.

Conclusion

The present study showed brain high oxidative stress and DRD1 down-regulation along with an intimate interrelationship between immune system represented by CD4, TNF-α, and IFN-γ and iron status in both brain and plasma in rotenone-induced Parkinsonian model in rats. The same rat model showed severe changes in brain histopathology. Quercetin, as well as coffee bean and grape seed extracts, exhibited neuroprotective activity in rotenone-induced Parkinsonism. The effect of such nutraceuticals has been suggested to arise from their anti-inflammatory and antioxidant effect related to immune-regulation action. The up-regulation of DRD1 and down-regulation of DMT1 are among the anti-Parkinsonian effects of such agents. The tested nutraceuticals improved iron deficiency manifested in rotenone-induced Parkinsonism along with the brain histopathological changes with variable degrees. These nutraceuticals must be investigated thoroughly in clinical trials to be used in PD.

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The work was completely carried out in National Research Centre, Egypt.

Authors' contributions

ASY proposed and designed the study and wrote the manuscript with interpretation of the results. Animal experiment and subsequent biochemical analyses and statistics of such parts were carried out by AESK and MRS. MHB carried out the molecular biology part with the implementation of phenolic, flavonoids, and DPPH tests and their statistical analysis and wrote the first draft of such parts. RAA determined INF-γ and its statistical analysis. All authors read and approved the final version and agreed to publish it.

Conflict of interests

None to be declared

Ethical considerations

Handling and care of animals were carried out according to the Medical Research Ethics Committee, National Research Centre, Cairo, Egypt (Registration No. 19175)

and followed the recommendations of the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No-85-23, revised 1985).

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