Behavioral, Biochemical and Histopathological effects of Standardised Pomegranate extract with Vinpocetine, Propolis or Cocoa in a rat model of Parkinson’s disease

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\textbf{ABSTRACT}

\textbf{Introduction}: Parkinsonism is a neurodegenerative disorder. Pomegranate (POM) has been previously shown to have a dopaminergic neuroprotective effect against Parkinsonism.

\textbf{Objective}: The aim of the current study is to investigate the possible effect of POM in combination with each of vinpocetine, propolis, or cocoa in the treatment of parkinsonism disease even without being given as adjuvant to L-dopa.

\textbf{Methods}: Rats were divided into seven groups, one normal and six RT model groups. One of the RT groups (2.5 mg/kg/48 h/10 doses sc), for 20 days served as non-treated Parkinsonism model, whereas the others were treated with either L-dopa (10 mg/kg, p.o./day) or with POM (150 mg/kg, p.o./day) together with each of the following: vinpocetine (VIN) (20 mg/kg, p.o./day), propolis (300 mg/kg, p.o./day), cocoa (24 mg/kg, p.o./day). Motor and cognitive performances were examined using four tests (catalepsy, swimming, Y-maze, open field). Striatal dopamine, norepinephrine, serotonin, GABA, glutamate, acetylcholinesterase, GSK-3\textbeta, BDNF levels were assessed as well as MDA, SOD, TAC, IL-1\textbeta, TNF-\alpha, iNOS, and caspase-3. Also, histopathological examinations of different brain regions were determined.

\textbf{Results}: Treatment with L-dopa alone or with all POM combination groups alleviated the deficits in locomotor activities, cognition, neurotransmitter levels, acetylcholinesterase activity, oxidative stress, and inflammatory markers as well as caspase-3 expression induced by RT.

\textbf{Conclusion}: Combinations of POM with each of VIN, propolis, or cocoa have a promising disease-modifying antiparkinsonian therapy even without being given as an adjuvant to L-dopa.

\textbf{Introduction}

Neurodegenerative diseases (NDD) were reported to affect millions of people each year all over the world. But yet the complex multifactorial mechanisms of parkinsonism are not fully understood so far. It has been proposed previously that manganese can cause severe neurological damage (Botsford, George, & Buckley, 2018). Parkinsonism is defined as the
presence of bradykinesia, rest tremor and rigidity. The exact etiology of parkinsonism remains elusive. Age is the most significant risk factor for the development of parkinsonism. In addition to that, complex interactions between environmental and genetic factors can be a predisposing factor to parkinsonism. It was previously demonstrated that the progression of neurodegeneration such as the case in parkinsonism is associated with decreased antioxidant levels and increased oxidative damage to proteins, DNA and lipids (Ballance, Qin, Chung, Gillette, & Kong, 2019). Unquestionably, the injurious effects of the inflammatory response are associated with augmentation of reactive oxygen species (ROS) and oxidative damage that was assessed by inhibition of defense mechanisms such as superoxide dismutase (SOD) and reduced glutathione (GSH). Oxidative stress can then result in induction of the gene expression of a battery of distinct pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β).

Rotenone (RT) is a powerful inhibitor of complex I of the mitochondrial electron transport chain. It’s a popular insecticide and pesticide. Rotenone can impair mitochondrial oxidative phosphorylation, and cause calcium dysregulation. It may also generate an excessive amount of ROS, resulting in extreme oxidative stress that leads to dopaminergic neuron loss as seen in PD (Liu-T, Luo-C, Huang, & Lu-T, 2018).

Natural products are needed as a substitute for chemical therapeutics for their safe toxicity profiles. There is ample evidence that oxidative stress and inflammation are involved in the pathogenesis of Parkinson’s disease (PD); therefore, the use of natural agents modulating both oxidative stress and inflammation have been proposed as a mainstream choice to counteract PD.

The pomegranate (POM) (Punica granatum L.) fruit is an abundant source of ellagitannins (ETs), which contribute to pomegranate’s antioxidative, anti-inflammatory, and anti-apoptotic properties. Pomegranate’s neuroprotective properties have been studied extensively. The inhibition of oxidative stress, as well as a decrease in the development of proinflammatory cytokines and apoptotic proteins, has been linked to neuroprotective effects in various neurological diseases, especially neurodegenerative diseases including Alzheimer’s disease (AD) and PD (Ginsberg et al., 2018; Kujawska, Jourdes, & Kurpik, 2019).

Vinpocetine (VIN) is a phosphodiesterase (PDE) inhibitor with antioxidant and anti-inflammatory properties that have been shown to have a neuroprotective effect. These properties, in addition to its cognitive-enhancing effects, make it an excellent candidate for the treatment of neurodegenerative diseases like PD and AD. VIN also has a major effect on the brain via improving cerebral circulation and metabolism (Hirsch & Hunot, 2009; Hirsch, Hunot, & Hartmann, 2005; Medina, 2010).

Cocoa products have been recognized as a rich source of flavonoids, especially flavanols. Disturbances in cerebral blood flow and oxidative stress have been linked to a variety of health problems in both typical and atypical aging, including AD, vascular dementia, PD, and acute neurological conditions including stroke (Sokolova, Pavlova, Klosterhalfena, & Enck, 2013). Potent antioxidant activity and endothelium-dependent vasodilation capacity of cocoa flavonoids can eventually protect against neuronal and oxidative damage, which can lead to neurological disease, cognitive and functional decline (Safari et al., 2015).

Propolis has been shown to have anticancer, antibacterial, anti-inflammatory, antioxidant, antimicrobial, antifungal, and tumoricidal activity. Propolis has been shown to have neuroprotective effects in a variety of neurological disorders, including cerebral ischemia,
neuronal apoptosis, encephalomyelitis, and PD, which may be due to its high antioxidative capacity to scavenge free radicals and resulted in the survival of more dopamine neurons (Hassanzadeh, Rahimmi, & Hassanzadeh, 2014).

Therefore, this study aimed to investigate the efficacy of POM together with each of VIN, propolis, cocoa, or L-dopa using rotenone-induced PD rat model.

**Materials and Methods**

**Animals**

Adult male albino rats, weighing approximately 300–340 g at the beginning of the experiment, were obtained from the Nile Co. for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Animals were kept under the same adequate environmental conditions and provided with their daily dietary requirements consisting of standard diet pellets (El-Nasr Chemical Co., Abu Zaabal, Cairo, Egypt) and water was given ad-libitum. Rats were housed in stainless-steel cages, three per cage and kept at the animal house (at the facility of Faculty of Pharmacy, Al-Azhar University “girls”). Animal experiments followed the national institute of health guidelines for the care and use of laboratory animals (NIH publications No. 8023, revised 1978). Animal experiments were usually carried out at a fixed time around 9 am-2 pm. All experimental procedures of the study were approved by the animal care and use committee of the Faculty of Pharmacy, Al-Azhar University (ethical approval number 212).

**Chemicals and Reagents**

All of the chemicals and reagents used in the present study were of high analytical grade. Rotenone was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), as a white to dark beige powder. It was suspended in sunflower oil to a concentration of 2.5 mg/ml. Pomegranate extract, standardized to 40% ellagic acid polyphenol, was purchased as a gray-brown powder from Holland & Barrett, UK. It was dissolved in 10% tween 80 saline solution. However, L-dopa was obtained as Sinemet tablets from Global Napi Pharmaceuticals, Cairo, Egypt. Each tablet was grinded and suspended in 10% tween 80 saline solution to a final concentration of 10 mg L-dopa/ml saline. Finally, all other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

**Experimental Design**

All rats were equally divided into seven groups (10 rats/each) and treated with different drugs for 20 days as follows: **Group I**: Normal healthy control group. **Group II**: Rats were treated with RT (2.5 mg/kg/48 h/10 doses sc), for 20 days served as non-treated parkinsonism model (Bhide et al., 2013). **Group III**: Rats were treated with RT (2.5 mg/kg/48 h/10 doses sc), and L-dopa (10 mg/kg, p.o./day) for 20 days (Kaur, Jabbar, Athar, & Alam, 2006). **Group IV**: Rats were treated with RT (2.5 mg/kg/48 h/10 doses sc), POM (150 mg/kg, p.o./day) (Ishola, Akinyede, Adeluwa, & Micah, 2018) and VIN. (20 mg/kg, p.o./day) for 20 days (Messaoudi, Bisson, Nejdi, Rozan, & Javelot, 2008). **Group V**: Rats were treated with RT (2.5 mg/kg/48 h/10 doses sc), POM (150 mg/kg, p.o./day) and cocoa (24
mg/kg, p.o./day) for 20 days (Messaoudi et al., 2008). **Group VI:** Rats were treated with RT (2.5 mg/kg/48 h/10 doses sc), POM (150 mg/kg, p.o./day) and propolis (300 mg/kg, p. o./day) for 20 days (Usman, Bakar, & Mohamed, 2018). **Group VII:** Rats were treated with RT (2.5 mg/kg/48 h/10 doses sc), POM (150 mg/kg, p.o./day) and L-dopa (10 mg/kg, p.o./day) for 20 days.

At the end of this study, all rats were tested for the behavioral tests 1 day after the last injection of RT, according to their increasing invasiveness as the following; catalepsy test in the first day, swimming test in the second day, open-field test in the third day, and finally, Y maze test in the fourth day. After the last behavioral experiment, rats were sacrificed by decapitation after anesthesia using diethyl ether (Merck) and the striatum of eight brains from each group were excised and cleaned with ice-cold saline and preserved immediately at −80°C for further biochemical analysis. They were homogenized in saline, the striatal tissue homogenates were used to assess brain neurotransmitters [Dopamine (DA), Norepinephrine (NE), Serotonin (5-HT), glutamate and gamma-aminobutyric acid (GABA)]; oxidative stress markers [malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (TAC)]; inflammatory mediators [Interleukin 1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS)]; apoptotic marker (caspase-3), acetylcholine esterase (ACHE) activity; brain-derived neurotrophic factor (BDNF); and glycogen synthase kinase 3 beta (GSK-3β). Finally, two brains from each group were preserved in 10% formaldehyde solution for further histopathological examinations.

**Behavioral Experiments**

**Catalepsy Test (Grid Test)**

Catalepsy test is an index for the presence or absence of parkinsonian-like symptoms such as “bradykinesia, akinesia, and rigidity” in rats. The grid test involves placing the rat into an abnormal posture and measuring the time taken by the rat to correct this posture. This period of time is used to estimate the severity of catalepsy (Alam, Mayerhofer, & Schmidt, 2004; Sanberg, 1988).

**Swimming Test**

The swimming test was performed according to the method described and modified by (Alder & Zbinden, 1983; Ali, Hamed, & El-Sayed, 1992; Hamed & El-Sayed, 1991; Vorhees, Klein, & Scott, 1982). The test was carried out in a glass tank apparatus that was half-filled with water and held at a temperature of (26°C – 27°C). The ramp was placed at one side of the glass tank and the swimming began in the center of the opposite side of the tank. Individual rats were positioned at the starting point and watched until they reached the ramp with their forepaws for a maximum time of 3 minutes. The following parameters were used to assess the behavior of rats in the swimming apparatus:

1. **Swimming time:** This is the time it takes to swim from the beginning to the end of the ramp. It was measured in seconds using a stopwatch.

2. **Swimming direction score:** It ranged from (0–4):
   i. When the animal swims straight from the starting point to the ramp, it is given score (4).
ii. When the animal reaches the ramp either through right or left direction, it is given score (3).

iii. When the animal reaches the ramp from both the right and left directions, it is given score (2).

iv. When the animal swims in all directions and in the middle but eventually reaches the ramp within the 3 min, it is given score (1).

v. When the animal swims in all directions and passively floats in the water but does not reach the ramp within 3 min, it is given score (0).

The measured parameters were recorded as a measure of muscular strength, neuromuscular coordination as well as awareness and vigilance.

**Open Field Test**

It is the most widely used method for determining changes in behavioral activities like locomotor activity and exploratory behavior. Behavioral measures including locomotor activity, latency and rearing are sensitive to varying degrees to DA loss in the striatum. The apparatus is formed of square wooden box (80 × 80 × 40 cm) with red side and white floor divided into 16 equal squares 4 × 4 by black lines. The test was conducted in a quiet room with white light. Using a stopwatch and video camera, the following parameters have been recorded over the course of 3 min: latency time (the time taken by rat to start moving), ambulation frequency (the number of squares crossed by the animal during 3 min period), rearing frequency (the number of times the rat stood stretched on its hind limbs with and without forelimbs support). Finally, grooming time was measured as time spent scratching the rat’s face, licking forelimbs, fur, and genitals during a 3 min period (Cunha & Masur, 1978; Hamed, Khayyal, Mansour, & Al-Ansary, 2006; Van den Buuse & De jong, 1989; Vorhees, 1974).

**Y-Maze Test for Assessment of Short-term Spatial Working Memory**

The Y-Maze test was used to enhance the activity of various brain regions, including the hippocampus, basal forebrain, septum, and prefrontal cortex (Kilari, Rao, Sreemanthula, & Kola, 2015). The results of this test are thought to represent spatial working memory, which is a type of short-term memory. The Y maze test is used to assess the ability of rodents to explore new environments as rats normally tend to discover the new maze arm rather than returning to one that was previously visited. The spontaneous alternation was thought to be a simple and fast test of spatial working memory, devoid of worry, reward, or reinforcers (Sarter, Schneider, & Stephens, 1988). The apparatus is made of black wood and consists of a three-arm maze in the form of a capital Y, with each arm spaced at a 120° angle. During the test, each rat was positioned in the center of the Y maze, then the sequence of entries into the three arms was recorded over a period of 8 min if the hind paws of the rat were fully within the arm. The ability to alternate requires the rat to remember which arms have been visited previously. Every experiment was scored, and the percentage of spontaneous alternation was calculated using the following equation:

**Spontaneous alternation (%)** = (Actual alternations/Maximum alternations − 2) x 100.

Actual alternations are described as consecutive entries into each of the three arms, without repetition.

Maximum alternations are the total number of arm entries minus two.
To prevent olfactory cues, the maze was cleaned with a 10% ethanol solution and dried until the next animal was examined (Teixeira, Souza, Menezes, Carmo, & Fonteles, 2013).

**Biochemical Investigations**

**Determination of Neurochemical Parameters (NE, DA, 5-HT, Glutamate, GABA)**

NE, DA, 5-HT, glutamate and GABA levels were determined in the striatal tissue homogenates using Rat Norepinephrine ELISA Kit (MyBioSource, San Diego, USA, Cat. No. MBS269993), as described by Yu and Dayan (Yu & Dayan, 2005), Rat Dopamine ELISA Kit (MyBioSource, San Diego, USA, Cat. No. MBS026032), Rat serotonin ELISA Kit (MyBioSource, San Diego, USA, Cat. No. MBS9362408), and Rat Glutamate ELISA kit provided by (MyBioSource, San Diego, USA, Cat. No. MBS756400) respectively, according to the manufacturer’s instructions. Finally, Rat GABA ELISA Kit (MyBioSource, Inc., San Diego, USA, Product Number MBS269152), according to the methodology of Ben-Ari et al. (Ben-Ari, Gaiarsa, Tyzio, & Khazipov, 2007).

**Determination of ACHE Activity**

It was measured in the striatal tissue homogenate using a commercially available kit supplied by Sigma-Aldrich Co. (St. Louis, MO, USA), Product Number MAK119, according to the methodology of Ellman et al. (Ellman, Courtney, Andres, & Featherstone, 1961).

**Determination of BDNF**

It was assessed in the striatal tissue homogenate using ELISA Kit supplied by (MyBioSource, Inc., San Diego, USA, Product Number MBS494147), according to the methodology of Kovalchuk et al. (Kovalchuk, Hanse, Kafitz, & Konnerth, 2002).

**Assessment of Oxidative Stress Markers (MDA, TAC, SOD)**

The content of MDA (Cat. No. MD 25 28), activity of TAC (Cat. No. TA 25 12), and SOD (Cat. No. SD 25 20), in striatal tissue homogenate were carried out using commercially available kits provided by Biodiagnostic, Giza, Egypt, according to the manufacturers’ instructions.

**Assessment of Brain Inflammatory Mediators [IL-1β, TNF-α, iNOS]**

IL-1β, TNF-α and iNOS were determined in striatal tissue homogenate using ELISA Kit obtained from RayBiotech, Inc., USA, IL-1β (Cat. No. ELR-IL1b), TNF-α (Cat. No. RTA00, SRTA00, PRTA00), and iNOS (Cat. No. E0837r), according to the manufacturer’s instructions.

**Determination of Apoptotic Marker (Caspase-3)**

The level of caspase-3 gene expressions was calculated in the striatal tissue homogenate using beta-actin (β-actin) as a housekeeping gene using quantitative real-time-Polymerase Chain Reaction (qRT-PCR) via SYBR Green Kit (Fermentas, USA) (Livak & Schmittgen, 2001; Pfaffl, 2001). The relative gene expression of caspase-3 was calculated according to Applied Biosystem software using the comparative Ct method. The gene-specific primer pairs are presented in table (1).
Determination of Glycogen Synthase Kinase 3 Beta (GSK-3β)

It was assessed in the striatal tissue homogenate using Rat Glycogen Synthase Kinase 3β ELISA kit supplied by (MyBioSource, Inc., San Diego, USA, Product Number MBS7251608), according to the manufacturer’s instructions.

Histopathological Examination of Different Brain Regions

Brain tissue samples from all groups were quickly excised, cut into small pieces, and fixed in 10% buffered formalin for 24 hours. Then tissue pieces were washed, alcohol-dehydrated, xylene-cleaned, and paraffin-embedded in a hot-air oven for 24 hours at 56°C. For histopathological analysis, serial sections of about 4 μm thick were cut and stained with hematoxylin and eosin (Bancroft & Gamble, 2008). To prevent bias, all histopathologic processing and evaluation was done by a professional blinded observer.

Statistical Analysis

Data were expressed as the mean ± SEM and statistical analysis was carried out by one way ANOVA followed by Tukey multiple comparisons test to calculate the significance of the difference between treatments. Values of $p < .05$ were considered significant. All statistical analyses were performed and graphs were sketched using GraphPad Prism (ISI, USA) software (version 5) computer program.

Results

In the present work, the concurrent administration of L-dopa alone or with all POM combinations could reverse the deficits of rotenone in the rat animal model of parkinsonism. This observation was confirmed via conducting three different types of experiments: behavioral, biochemical and histopathological investigations.

The Behavioral Efficacies of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa against the Development of Parkinsonism in a Rat Animal Model

To investigate the neuroprotective potential of POM and other compounds employed in this study, we conducted several behavioral analyses such as catalepsy, swimming, Y-maze, and open field tests.
The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Change in Catalepsy in the Grid Test

It is illustrated in Figure 1 (A), administration of rotenone significantly prolonged moving latency by 277.8% as compared to the corresponding control group. However, the treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly reduced moving latency by (70%, 36.7%, 42.7%, 42.3%, and 41.7%) respectively, as compared to the RT-treated group. Therefore, the combination of POM with each of VIN, propolis, cocoa or L-dopa significantly reduced moving latency as compared to the RT, and RT + L-dopa groups, but they showed no significant difference from each other.
The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Changes in the Swimming Time and the Swimming Direction Score

As illustrated in Figure 1 (B,C), administration of rotenone significantly elevated the swimming time by 185.04% and significantly reduced the direction score by 50% as compared to the corresponding control group. However, the treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly decreased the swimming time by (74.1%, 56.5%, 71.7%, 68.6%, and 71.0%), and significantly elevated the direction score by (181.8%, 190.9%, 172.94%, 181.8% and 200.05%), respectively, as compared to the RT-treated group. Therefore, the combination of POM with each of propolis, cocoa, or L-dopa significantly improved the swimming time and score as compared to the RT-treated group and they showed no significant difference from the RT + L-dopa group. However, RT + POM + VIN group significantly improved the swimming time as compared to RT + L-dopa group.

The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Change in Spatial Working Memory in the Y Maze Test

It is illustrated in Figure 1 (D), administration of rotenone caused a significant impairment in spatial working memory, as evidenced by a significant reduction in the percentage of spontaneous alternations by 74.32% as compared to the corresponding control group. However, treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly improved the spatial working memory, as evidenced by a significant increase in the percentage of spontaneous alternations by (123%, 123.5%, 121.5%, 121.3%, and 135.1%) respectively, as compared to the RT-treated group. Therefore, the combination of POM with each of VIN, propolis, cocoa, or L-dopa significantly improved spatial working memory as compared to RT-treated group and they showed no significant difference from the RT + L-dopa group. However, RT + POM + L-dopa group significantly improved spatial working memory as compared to RT + L-dopa group.

The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Changes in the Open-field Test

As illustrated in Figure 2 (A, B, C, D), administration of rotenone significantly reduced the locomotor activity of rats by increasing the latency time to move from the center by 506.4%, significantly decreased ambulation frequency, rearing frequency, and grooming time by (30.3%, 22.4%, and 23.1%) respectively, as compared to the control group. However, the treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly decreased the latency time by (64.8%, 38.23%, 50.1%, 48.7%, and 50.1%), significantly elevated the ambulation frequency by (245.1%, 265.5%, 267.11%, 287.8% and 289.6%), the rearing frequency by (230.9%, 284.76%, 357.97%, 369.51%, and 361.9%), and finally the grooming time by (288.2%, 334.5%, 394.5%, 3.94.5%, and 403.1%), respectively, as compared to the RT-treated group. Therefore, the combination of POM with each of VIN, propolis, cocoa, or L-dopa significantly enhanced
the motor activity of the rats as compared to the RT-treated group but, they showed no significant difference in the ambulation frequency as compared to RT + L-dopa group. However, RT + POM + VIN group significantly improved the latency time as compared to RT + L-dopa group. Finally, all groups except RT + POM + VIN significantly improved rearing frequency, and grooming time as compared to RT + L-dopa group.

The Biochemical Efficacies of Pomegranate with Different Drugs

The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Changes on the Levels of Different Brain Neurotransmitters in Rats

As shown in Figure 3 (A, B, C, D, E), administration of rotenone significantly reduced brain striatal neurotransmitters levels of DA, NE, 5-HT, and glutamate by (23.1%, 33.6%, 29.64%, and 23.95%) respectively, but significantly elevated the brain striatal GABA by 838.8% as compared to the corresponding control group. However, the treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly increased brain striatal DA by (190.72%, 270.6%, 206.1%, 223.53%, and 371.4%), NE by (193.2%, 206.74%, 186.1%, 202.13%, and 246.7%), 5-HT by (148.6%,
224.4%, 189.8%, 189.8% and 316.3%), and glutamate by (162.32%, 194.64%, 155.35%, 157.41%, and 266.7%) respectively, but significantly decreased the brain striatal GABA by (51.7%, 54.06%, 58.2%, 59.3%, and 44.3%) respectively, as compared to the RT-treated group. Therefore, the combination of POM with each of VIN, propolis, cocoa, or L-dopa significantly improved brain striatal neurotransmitters levels as compared to the RT-treated group but, they showed no significant difference in GABA level as compared to L-dopa group. However, RT + POM + L-dopa significantly improved NE level as compared to RT + L-dopa group. Additionally, RT + POM + VIN and RT + POM + L-dopa significantly improved DA, 5-HT levels as compared to RT + L-dopa group. Finally, RT + POM + VIN; RT + POM + propolis and RT + POM + L-dopa significantly improved glutamate as compared to RT + L-dopa group.

The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Changes on the Brain Oxidative Stress (MDA, SOD, TAC) Markers in Rats

As shown in Figure 4 (A, B, C), administration of rotenone significantly elevated brain striatal MDA, a biomarker for lipid peroxidation, by 863.7%, and a significant reduction in
the antioxidant enzyme levels of SOD and TAC activity by about 11.1%, and 23.9% as compared to the corresponding control group, indicating that rotenone increased brain striatal oxidative stress. However, the treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly decreased MDA by (35.2%, 33.2%, 40.8%, 48.1%, and 22.8%), significantly increased SOD by (293.2%, 670.3%, 379.73%, 379.73%, and 381.9%), and TAC by (162.5%, 193.84%, 155.4%, 155.4%, and 267%) respectively, as compared to the RT-treated group, indicating that the treatment with the combination of POM with each of VIN, propolis, cocoa or L-dopa can reduce brain striatal oxidative stress as compared to the RT-treated group. However, RT + POM + VIN showed the best improvement regarding the SOD level as compared to RT + L-dopa group. However, RT + POM + VIN and RT + POM + L-dopa showed the best improvement in TAC level as compared to RT + L-dopa group. Finally, RT + POM + propolis and RT + POM + L-dopa significantly improved MDA as compared to RT + L-dopa group.

**The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa or L-dopa on Rotenone-induced Changes on the Brain Inflammatory Mediators (TNF-α, IL1β, iNOS) Markers in Rats**

As shown in Figure 5 (A, B, C), administration of rotenone significantly elevated brain striatal TNF-α, IL1β, and iNOS contents by about 501.04%, 680%, and 2822.6%, as compared to the corresponding control group, indicating that rotenone increased brain striatal inflammatory mediators. However, treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly decreased brain striatal TNF-α by (35.2%, 33.2%, 40.8%, 48.1%, and 22.8%), IL1β by (293.2%, 670.3%, 379.73%, 379.73%, and 381.9%), and iNOS by (293.2%, 670.3%, 379.73%, 379.73%, and 381.9%) respectively, as compared to the RT-treated group, indicating that the treatment with the combination of POM with each of VIN, propolis, cocoa or L-dopa can reduce brain striatal inflammatory mediators as compared to the RT-treated group. However, RT + POM + VIN; RT + POM + propolis and RT + POM + L-dopa showed the best improvement regarding the

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**Figure 4.** (A-C): The neuroprotective effect of pomegranate with each of vinpocetine, propolis, cocoa, or L-dopa on the brain oxidative stress (MDA, SOD, TAC) markers in rotenone-induced parkinsonism in rats. Data expressed as mean ± SEM. (n = 8). a: Significantly different from the control group at p < .05. b: Significantly different from the rotenone group at p < .05. c: Significantly different from the rotenone/L-dopa group at p < .05.
**Figure 5.** (A-C): The neuroprotective effect of pomegranate with each of vinpocetine, propolis, cocoa, or L-dopa on the brain inflammatory mediators (TNF-α, IL1β, iNOS) markers in rotenone-induced parkinsonism in rats. Data expressed as mean ± SEM. (n = 8). a: Significantly different from the control group at p < .05. b: Significantly different from the rotenone group at p < .05. c: Significantly different from the rotenone/L-dopa group at p < .05.

striatal IL1β, and iNOS contents as compared to RT + L-dopa group, but they showed no significant difference regarding striatal TNF-α as compared to RT + L-dopa group.

**The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa, or L-dopa on Rotenone-induced Change on the Gene Expression of Brain Striatal Apoptotic Marker (Caspase-3) in Rats**

As shown in Figure 6, administration of rotenone significantly elevated brain striatal caspase-3 gene expression by 1041.2%, as compared to the corresponding control group. However, the treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly decreased striatal caspase-3 gene expression by (47.86%, 48.2%, 61.1%, 58.5%, and 34.2%), as compared to the RT-treated group. However, RT + POM + VIN; RT + POM + propolis and RT + POM + L-dopa showed no significant difference as compared to RT + L-dopa group.

**The Effects of Pomegranate with Each of Vinpocetine, Propolis, Cocoa or L-dopa on Rotenone-induced Change on the Levels of ACHE, GSK-3β, and BDNF in Rats**

As shown in Figure 7 (A-C), administration of rotenone significantly elevated brain striatal ACHE and GSK-3β levels by 513.6%, and 592.7% respectively, and significantly reduced brain striatal BDNF by 37.8% as compared to the corresponding control group. However, treatment with RT + L-dopa; RT + POM + VIN; RT + POM + cocoa; RT + POM + propolis and RT + POM + L-dopa significantly decreased brain striatal ACHE by (32.14%, 40.5%, 57.1%, 49.4%, and 26.02%), GSK-3β by (58.7%, 63.1%, 69%, 67.5%, and 44.3%) respectively, and significantly elevated brain striatal BDNF by (184%, 185.2%, 182.6%, 183%, and 214.2%) as compared to the RT-treated group. However, RT + POM + L-dopa showed the best improvement regarding the striatal GSK-3β, and BDNF as compared to RT + L-dopa group, but RT + POM + propolis and RT + POM + cocoa groups significantly improved ACHE as compared to RT + L-dopa group.
Histopathological Alterations of Pomegranate with Different Drugs in the Brain Tissues

Histopathological Alterations in the Brain Tissues

Histopathological alterations in brain specimens from different treated groups are shown in Figure 8 (A1-G4). It showed marked degeneration for the rotenone group, whereas the rest of the treated groups improved this degeneration. Microscopic examination of brain tissues from
the control group showed normal histological structure and no histopathological alterations in the cerebral cortex, hippocampus (subiculum, fascia dentate and hilus), and striatum Figure 8 (A1-A4). On the other hand, brain specimens of rats treated with rotenone showed nuclear pyknosis and degeneration in most neurons of the cerebral cortex and striatum also nuclear pyknosis in some neurons of the fascia dentate and hilus with multiple focal eosinophilic plagues, but it showed no histopathological alterations in the neurons of the subiculum of the hippocampus Figure 8 (B1-B4). Moreover, brain tissue sections from the RT + L-dopa treated group showed mild congestion in the blood vessels of the striatum and hemorrhage in the meninges and the cerebellum fig. 9 (C1-C2). While RT + POM + VIN treated group showed intact histological structure and no alteration in the neurons of the cerebral cortex, fascia dentate and hilus as well as in the striatum, but it showed nuclear pyknosis and degeneration in the subiculum of hippocampus Figure 8 (D1-D4). Additionally, RT + POM + Cocoa treated group showed nuclear pyknosis and degeneration in the cerebral cortex and no histopathological alterations in the hippocampus (subiculum & fascia dentate), but it showed multiple focal eosinophilic plagues formation with nuclear pyknosis and degenerations in some neurons of the striatum Figure 8 (E1-E4). Furthermore, RT + POM + Propolis treated group showed nuclear pyknosis and degeneration in the cerebral cortex and in some neurons of the striatum with multiple focal eosinophilic plagues formation, while it showed few nuclear pyknosis and degeneration in the subiculum of the hippocampus Figure 8 (F1-F3). Finally, brain specimens

**Figure 8.** (A1-G4): Representative photomicrographs of brain tissue sections of rats stained by Hematoxylin and Eosin: Sections were taken from the brain of all groups, respectively, showed different histopathological alteration in the cerebral cortex, hippocampus (subiculum, fascia dentate and hilus), and striatum.
from the RT + POM + L-dopa treated group showed normal histological structure and no histopathological alterations in the cerebral cortex, subiculum and fascia dentate of the hippocampus, while few neurons in striatum showed nuclear pyknosis and degeneration Figure 8 (G1-G4).

Discussion

Parkinsonism is a geriatric disorder in which age, genetic and environmental factors are considered as predisposing factors for parkinsonism. Several lines of evidence indicate oxidative stress and inflammation as important pathogenic factors in this disease. Besides, it was recorded previously that parkinsonism is associated with decreased striatal dopamine levels. Unfortunately, the details of the neurodegenerative cascade of parkinsonism disease remain unclear. However, it was proposed previously that oxidative damage plays a crucial role in the neuropathology of parkinsonism. Moreover, Complex I was reported to be impaired in parkinsonism which leads to NADH accumulation resulting in an excessive ROS production. In agreement with this, recent studies demonstrated an accumulation of MDA in patients with parkinsonism. Increased generation of ROS leads to elevation of the lipid peroxidation level yielding MDA that can be considered as a biomarker for oxidative stress. Also, other factors observed in parkinsonism pathology are GSH depletion, enhanced superoxide activity, and cellular apoptosis (Silva et al., 2019; Wichmann, 2019).

L-dopa was always considered as the best treatment for parkinsonism despite its long-term treatment side effects which may include further addition to the free radical oxidative load produced via normal metabolism processes. This may lead to the progression of the disease. Therefore, L-dopa is only limited to the relief of parkinsonism symptoms. Therefore, developing new agents that decrease the rate of parkinsonism progression is crucial (Kelly et al., 2019; Malek et al., 2019). Since the key factor to relief the oxidative stress seen in parkinsonism is to repair the damage due to free radicals via antioxidants that might have a preventive action against free radical-mediated tissue destruction, a combination of pomegranate, propolis, vinpocetine and cocoa was investigated in the current study.

Rotenone is a pesticide of lipophilic nature and hence can cross biological membranes. RT is known to mimic the neurochemical and behavioral features of parkinsonism in rats. Indeed, RT impaired behavioral and biochemical profile of rats under investigation in the current study. Examples for such features are reduced mobility and flexed posture (Rekha & Inmozhi Sivakamasundari, 2018). It was proposed previously that this is because RT damages neurons via oxidative mechanisms and hence increases lipid peroxidation. RT-treated rats have previously demonstrated elevated levels of TNF-α. The released TNF-α may be responsible for neuronal degeneration in parkinsonism disease.

Pomegranate with different combinations significantly reversed the deficits observed with RT. Pomegranate possesses a well-known health-promoting effect. Pomegranate fruits are rich in polyphenols. Indeed, 23 phenolic compounds were identified including cyanidin-3,5-O-diglucoside and pelargonidin-3,5-O-diglucoside. It was previously indicated that polyphenols attenuate neuronal death in animal models of neurodegeneration. This can be explained based on their possession to antioxidant and anti-inflammatory actions (Mazumder, Choudhury, & Borah, 2019; Tapias, Cannon, & Greenamyre, 2014).

Vinpocetine is a broad-spectrum antioxidant and neuroprotective agent. It was reported previously to have a therapeutic effect in the treatment of some cerebrovascular diseases. It
was previously demonstrated that VIN inhibited oxidative stress, memory impairment, and neuroinflammation via enhancement of the antioxidant defense system and inhibition of neuroinflammatory cytokines. The neuroprotective effect of VIN may be due to modulation of monoamines, antioxidant, anti-inflammatory, and anti-apoptotic activities (Nadeem, Ahmed, & El-Sayeh, 2018). In addition to that, alteration of the rheological properties of red blood cells is a unique mechanism of VIN. This unique mechanism enables the penetration of the small vessels of the cerebro-microvasculature by blood cells to deliver nutrients improving neurocognitive function. Findings from a previous study demonstrated that VIN improved the symptoms of parkinsonism via improvement of the antioxidant defense system and inhibition of neuroinflammatory mediators. Moreover, VIN significantly reduced MDA and increased GSH levels as compared to the control-positive group (Kujawska et al., 2019; Zaitone et al., 2019).

It was reported previously that propolis with the caffeic acid phenethyl ester, as the main active component, attenuated dopaminergic neurodegeneration in a mouse model of parkinsonism disease (Cova, Leta, Mariani, Pantonì, & Pomati, 2019). Propolis and cocoa were shown previously to possess anti-inflammatory as well as antioxidant properties (Costa et al., 2019; Cova et al., 2019).

In the current study, different levels of biochemical markers were assessed in addition to histopathological examinations of different brain regions were also determined.

Brain-derived neurotrophic factor is secreted in the mammalian central nervous system. Diminished BDNF expression level in the substantia nigra in the animal can mimic the symptoms of parkinsonism. In the present study, POM with other combinations elevated the decreased level of BDNF following the injection of RT (Petit-Paitel, 2010). In addition to that, the elevated GSK-3β level, which was implicated in the pathogenesis of several neurodegenerative diseases such as AD, was reported to decrease following administration of POM with other combinations [50].

The authors of this study were able to show that combinations of POM together with each of VIN, propolis, or cocoa have beneficial effects against the development of parkinsonism in rats even without being given as an adjuvant to L-dopa.

**Disclosure statement**

The authors declare no conflict of interest.

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**Ethical approval**

Animal experiments followed the national institute of health guidelines for the care and use of laboratory animals (NIH publications No. 8023, revised 1978). Animal experiments were usually carried out at a fixed time around 9 am-2 pm. All experimental procedures of the study were approved by the animal care and use committee of the Faculty of Pharmacy, Al-Azhar University (ethical approval number 212).
References

Alam, M., Mayerhofer, A., & Schmidt, W. (2004). The neurobehavioral changes induced by bilateral rotenone lesion in medial forebrain bundle of rats are reversed by L-DOPA. Behavioural Brain Research, 151(1–2), 117–124. doi:10.1016/j.bbr.2003.08.014

Alder, S., & Zbinden, G. (1983). Neurobehavioral tests in single- and repeated-dose toxicity studies in small rodents. Archives of Toxicology, 54, 1–23. doi:10.1007/BF00277811

Ali, A., Hamed, M., & El-Sayed, M. (1992). Effect of protein malnutrition on postnatal neurobehavioural response to drugs. M Sc. Thesis, Pharmacology, Faculty of Pharmacy, Cairo University. pp 59–160.

Ballance, W. C., Qin, E. C., Chung, H. J., Gillette, M. U., & Kong, H. (2019). Reactive oxygen species-responsive drug delivery systems for the treatment of neurodegenerative diseases. Biomaterials, 217, 119292. doi:10.1016/j.biomaterials.2019.119292

Bancroft, J. D., & Gamble, M. (2008). Theory and practice of histological techniques. 6th Edition, Churchill Livingstone, Elsevier, China.

Ben-Ari, Y., Gaiarsa, J. L., Tzyio, R., & Khazipov, R. (2007 Oct). GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiological Reviews, 87(4), 1215–1284. doi:10.1152/physrev.00017.2006. PMID: 17928584.

Bhide, N., Lindenbach, D., Surrena, M. A., Goldenberg, A. A., Bishop, C., Berger, S. P., Paquette, M. A. (2013). The effects of BMY-14802 against L-DOPA- and dopamine agonist-induced dyskinesia in the hemiparkinsonian rat. Psychopharmacology (Berl), 227, 533–544. doi:10.1007/s00213-013-3001-4

Botsford, E., George, J., & Buckley, E. E. (2018). Parkinson’s disease and metal storage disorders: A systematic review. Brain Sci, 8. doi:10.3390/brainsci8110194

Costa, C. M., de Oliveira, G. L., Fonseca, A. C. S., Lana, R. D. C., Polese, J. C., Pernambuco, A. P. (2019). Levels of cortisol and neurotrophic factor brain-derived in parkinson’s disease. Neurosci. Lett, 708, 134359. doi:10.1016/j.neulet.2019.134359

Cova, I., Leta, V., Mariani, C., Panti, L., & Pomati, S. (2019). Exploring cocoa properties: Is theobromine a cognitive modulator?. Psychopharmacology (Berl), 236(2), 561–572. doi:10.1007/s00213-019-5172-0

Cunha, J. M., & Masur, J. (1978). Evaluation of psychotropic drugs with a modified open field test. Pharmacol, 16, 259–267. doi:10.1159/000136777

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7(2), 88. IN191–9095. doi:10.1016/0006-2952(61)90145-9

Ginsberg, Y., Khatib, N., Saadi, N., Ross, M. G., Weiner, Z., Beloosesky, R. (2018). Maternal pomegranate juice attenuates maternal inflammation-induced fetal brain injury by inhibition of apoptosis, neuronal nitric oxide synthase, and NF-κB in a rat model. Am. J. Obstet. Gynecol, 219, 113.e1–113.e9. doi:10.1016/j.ajog.2018.04.040

Hamed, M., & El-Sayed, M. (1991). Influence of protein malnutrition on behavioral response to drugs in rats. JOURNAL OF DRUG RESEARCH-CAIRO, 20, 241.

Hamed, M. R., Khayyal, M. T. E., Mansour, H. A. F., & Al-Ansary, D. M. (2006). Immunomodulatory effects of dimethyl- 4, 4- Dimethoxy-5,5,5,6- Dimethylene Dioxy-Biphenyl- Dicarboxylate (D.D. B). J Drug Res Egypt, 27(1–2), 32–43.

Hassanzadeh, K., Rahimmi, A., & Hassanzadeh, K. (2014). Effect of N-acetylcysteine on TNF-α level of substantia nigra and striatum in rat model of parkinson’s disease. J. Mazandaran Univ. Med. Sci, 24(118), 40–48.

Hirsch, E. C., & Hunot, S. (2009). Neuroinflammation in parkinson’s disease: A target for neuroprotection?. Lancet Neurology, 8(4), 382–397. doi:10.1016/S1474-4422(09)70062-6

Hirsch, E. C., Hunot, S., & Hartmann, A. (2005). Neuroinflammatory processes in parkinson’s disease. Parkinson. Relat. Disord, 11(Suppl. 1), S9–S15. doi:10.1016/j.parkreldis.2004.10.013

Ishola, I. O., Akinyede, A. A., Adeluwa, T. P., & Micah, C. (2018). Novel action of vinpocetine in the prevention of paraquat-induced parkinsonism in mice: Involvement of oxidative stress and neuroinflammation. Metab. Brain Dis, 33, 1493–1500. doi:10.1007/s11011-018-0256-9
Kaur, G., Jabbar, Z., Athar, M., & Alam, M. S. (2006). Punica granatum (pomegranate) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice. *Food Chem. Toxicol.*, 44, 984–993. doi:10.1016/j.fct.2005.12.001

Kelly, M. J., Lawton, M. A., Baig, F., Ruffmann, C., Barber, T. R., Lo, C., Klein, J. C., Ben-Shlomo, Y., & Hu, M. T. (2019). Predictors of motor complications in early parkinson’s disease: A prospective cohort study. *Mov. Disorder*. doi:10.1002/mds.27783

Kilari, E. K., Rao, L. S. N., Sreemanthula, S., & Kola, P. K. (2015). Anti-stress and nootropic activity of aqueous extract of piper longum fruit, estimated by noninvasive biomarkers and Y-maze test in rodents. *Environ Exp Biol*, 13, 25–31.

Kovalchuk, Y., Hanse, E., Kafitz, K. W., & Konnerth, A. (2002). Postsynaptic induction of BDNF-mediated long-term potentiation. Science, 295, 1729–1734.

Kujawska, M., Jourdes, M., & Kurzik, M. (2019). Neuroprotective effects of pomegranate juice against parkinson’s disease and presence of ellagitannins-derived metabolite-Urolithin A in the brain. *International Journal of Molecular Sciences*, 21(1), Published 2019 Dec 27, 202. doi:10.3390/ijms21010202

Liu-T, H., Luo-C, L., Huang, B., & Lu-T, S. (2018). Fu-Y-S 2018: the caffeine effects on rotenone-induced parkinson’s disease model in vitro and in vivo. *Experimental Biology*, 32(S1), 740.2.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, 25, 402–408. doi:10.1006/meth.2001.1262

Malek, N., Kanavou, S., Lawton, M. A., Pitz, V., Grosset, K. A., Bajaj, N., Barker, R. A., Ben Shlomo, Y., Burn, D. J., Foltynie, T., Hardy, J., Williams, N. M., Wood, N., Morris, H. R., Grosset, D. G. (2019). L-dopa responsiveness in early parkinson’s disease is associated with the rate of motor progression. *Parkinsonism Relat. Disord*. doi:10.1016/j.parkreldis.2019.05.022

Mazumder, M. K., Choudhury, S., & Borah, A. (2019). An in silico investigation on the inhibitory potential of the constituents of pomegranate juice on antioxidant defense mechanism: relevance to neurodegenerative diseases. *IBRO Reports*, 6, 153–159. doi:10.1016/j.ibro.2019.05.003

Medina, A. E. (2010). Vinpocetine as a potent antiinflammatory agent. *PNAS*, 107(22), 9921–9922. doi:10.1073/pnas.1005138107

Messaoudi, M., Bisson, J.F., Nejdi, A., Rozan, P., & Javelot, H. (2008). Antidepressant-like effects of a cocoa polyphenolic extract in wistar-unilever rats. *Nutr. Neurosci*, 11, 269–276. doi:10.1179/147683008X344165

Nadeem, R. I., Ahmed, H. I., & El-Sayeh, B. M. (2018). Protective effect of vinpocetine against neurotoxicity of manganese in adult male rats. *Naunyn. Schmiedebergs. Arch. Pharmacol.*, 3(91), 729–742. doi:10.1007/s00210-018-1498-0

Petit-Paitel, A. (2010). [GSK-3beta: A central kinase for neurodegenerative diseases?]. *Med. Sci. (Paris)*, 26, 516–521. doi:10.1051/medsci/2010265516

Pfafll, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. 29, e45–e45.

Rekha, K. R., & Inmozhi Sivakamasundari, R. (2018). Geraniol protects against the Protein and Oxidative stress induced by rotenone in an in vitro model of parkinson’s disease. *Neurochem. Res.*, 43, 1947–1962. doi:10.1007/s11064-018-2617-5

Safari, M., Sameni, H. R., Badban, L., Bandegi, A. R., Vafaei, A. A., Pour, A. R., Ghahari, L., & (2015). Protective effects of water extract of propolis on dopaminergic neurons, brain derived neurotrophic factor and stress oxidative factors in the rat model of parkinson’s disease. *International Journal of Pharmacology*, 11(4), 300–308. doi:10.3923/ijp.2015.300.308

Sanberg, P. R. (1988). The catalepsy test: Its ups and downs. *Behavioral Neuroscience*, 102(5), 748. doi:10.1037/0735-7044.102.5.748

Sarter, M., Schneider, H. H., & Stephens, D. N. (1988). Treatment strategies for senile dementia: Antagonist β-carbolines. *Trends in Neurosciences*, 11, 13–17. doi:10.1016/0166-2236(88)90042-2

Silva, J., Alves, C., Freitas, R., Martins, A., Pinteu, S., Ribeiro, J., Gaspar, H., Alfonso, A., Pedrosa, R. (2019). Antioxidant and neuroprotective potential of the brown seaweed bifurcaria bifurcata in an in vitro parkinson’s disease model. *Mar. Drugs*, 17. doi:10.3390/md17020085
Sokolova, A. N., Pavlova, M. A., Klosterhalfena, S., & Enck, P. (2013). Chocolate and the brain: neurobiological impact of cocoa flavanols on cognition and behaviour. Neuroscience and Biobehavioral Reviews, 37(10), 2445–2453. doi:10.1016/j.neubiorev.2013.06.013

Tapia, V., Cannon, J. R., & Greenamyre, J. T. (2014). Pomegranate juice exacerbates oxidative stress and nigrostriatal degeneration in Parkinson’s disease. Neurobiology of Aging, 35, 1162–1176. doi:10.1016/j.neurobiolaging.2013.10.077

Teixeira, M. D., Souza, C. M., Menezes, A. P., Carmo, M. R., & Fonteles, A. A. (2013). Catechin attenuates behavioral neurotoxicity induced by 6-OHDA in rats. Pharmacology, Biochemistry, and Behavior, 110, 1–7. doi:10.1016/j.pbb.2013.05.012

Usman, U. Z., Bakar, A. B. A., & Mohamed, M. (2018). Propolis improves pregnancy outcomes and placental oxidative stress status in streptozotocin-induced diabetic rats. BMC Complement. Altern. Med, 18, 324. doi:10.1186/s12906-018-2391-6

Van den Buuse, M., & De jong, W. (1989). Differential effects of dopaminergic drugs on open-field behavior of spontaneously hypertensive rats and normotensive wistar –kyoto rats. The Journal of Pharmacology and Experimental Therapeutics, 248(3), 1189–1196.

Vorhees, C. V. (1974). Some behavioral effects of maternal hypervitaminosis A in rats. Teratology, 10, 269–273. doi:10.1002/tera.1420100309

Vorhees, C. V., Klein, K. L., & Scott, W. J. (1982). Aspirin-induced psychotereatogenesis in rats as a function of embryonic age. Teratogenesis, Carcinogenesis, and Mutagenesis. 2, 77–84. doi:10.1002/1520-6866(1990)2:1<77::AID-TCM1770020108>3.0.CO;2-C

Wichmann, T. (2019). Changing views of the pathophysiology of parkinsonism. Mov. Disord. doi:10.1002/mds.27741

Yu, A. J., & Dayan, P. (2005). Uncertainty, neuromodulation, and attention. Neuron, 46(4), 681–692. doi:10.1016/j.neuron.2005.04.026

Zaitone, S. A., Ahmed, E., Elsherbiny, N. M., Mehanna, E. T., El-Kherbetawy, M. K., ElSayed, M. H., Alshareef, D. M., Moustafa, Y. M. (2019). Caffeic acid improves locomotor activity and lessens inflammatory burden in a mouse model of rotenone-induced nigral neurodegeneration: relevance to parkinson’s disease therapy. Pharmacol. Rep, 71, 32–41. doi:10.1016/j.pharep.2018.08.004