Anti-Parkinson’s Activity of *Tribulus terrestris* via Modulation of AChE, α-Synuclein, TNF-α, and IL-1β

Uzma Saleem,* Zunera Chauhdary, Zohaib Raza, Shahid Shah, Mahmood-ur Rahman, Parwasha Zaib, and Bashir Ahmad

**ABSTRACT:** *Tribulus terrestris* (T.T.) is a rich source of flavonoids and saponins, which have been reported to have neuroprotective and antioxidant potential. The current study was planned to investigate the anti-Parkinson’s activity of *T. terrestris* methanol extract (TTME). It was hypothesized that TTME possessed antioxidant potential and can ameliorate Parkinson’s disease (PD) via modulation of α-synuclein, acetylcholinesterase (AChE), TNF-α, and IL-1β. To test this hypothesis, in silico and in vivo studies were performed. The PD model in rats was prepared by giving haloperidol, 1 mg/kg, i.p. Rats were divided into six groups: control, disease control, standard, and treatment groups receiving TTME orally at 100, 300, and 1000 mg/kg dose levels for 21 days. Behavioral observations and biochemical analyses were done. The TTME modulatory effect on mRNA expression of α-synuclein, AChE, TNF-α, and interleukins in the brain homogenate was estimated by RT-PCR. Compounds detected in HPLC analysis disrupted the catalytic triad of AChE in in silico studies. Behavioral observations showed significant (*p* < 0.05) improvement in a reversal of catatonia, muscular strength, locomotor functions, stride length, and exploration in a dose-dependent manner (1000 > 300 > 100 mg/kg) of PD rats. Endogenous antioxidant enzyme levels CAT, SOD, GSH, and GPx were significantly restored at a high dose (*p* < 0.05) with a notable (*p* < 0.05) decrease in the MDA level in TTME-treated groups. TTME at a high dose significantly (*p* < 0.05) decreased the level of acetylcholinesterase. RT-PCR results are showing down-regulation in the mRNA expression levels of IL-1β, α-synuclein, TNF-α, and AChE in TTME-treated groups compared to the disease control group, indicating neuroprotection. It is concluded that TTME has potential to ameliorate the symptoms of Parkinson’s disease.

1. **INTRODUCTION**

Parkinson’s disease (PD) is an idiopathic chronic neurodegenerative disorder manifested by cardinal motor dysfunctions such as bradykinesia, muscle stiffness, rigidity, resting tremors, and postural instability. PD, a hypokinetic rigid syndrome, involves slowly progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Pathogenesis of PD involves various environmental and genetic factors, causing inhibition of mitochondrial complex-I, inflammation, apoptosis, protein aggregation, and excitotoxicity.1 The reduced level of dopamine in the caudate and basal ganglia triggers the emergence of pathological conditions such as the presence of intracellular Lewy body inclusions, neuronal damage, and depigmentation. Oxidative stress has a principal role in the pathogenesis and advancement of PD by disrupting cellular proteins and lipids in the membrane. The prevention of redox potential is required for neuronal endure, and its interruption interferes with the biological processes inside the cells causing cell death.2 In PD, dopaminergic neuronal loss corresponds to oxidative stress, mitochondrial dysfunction, microglial activation, neuroinflammation, and generation of Lewy bodies.3 Available neuroprotective agents such as levodopa, carbidopa, selegiline, amantadine, pergola, and orphenadrine effectively reverse the symptoms of PD by activation of central dopaminergic receptors. These therapeutic agents are associated with many adverse events such as depression, hypertension, ulcer, and toxicity. Therefore, the effective complementary herbal curative agent is an ultimate clinical need of today.

A butyrophenone derivative, haloperidol is a neuroleptic drug used for the treatment of schizophrenia, mania, and Tourette syndrome. It has the activity to block the dopamine receptors (D2) in the basal ganglia and causes a reduction in
Tribulus terrestris L. belongs to the family of Zygophyllaceae, generally known as puncture vine, caltrop, goats head, and yellow vine in different cultures. It is widely distributed in tropical and mild temperate locations. *T. terrestris* is proficiently used as a remedial herb by phytologists and Ayurvedic practitioners. Phytochemical analysis indicated its medicinal importance against neurodegeneration due to the existence of steroidal saponins, alkaloids, flavonoids, and phytosterol. It has traditionally been used as a restorative agent in abdominal distention, sexual dysfunction, leucorrhoea, eye trouble, asthma, hyperlipidemia, hypertension, microbial infection, and veiling. Recent reports showed that saponins and flavonoids have remarkable neuroprotective activity in Alzheimer’s disease, stroke, and PD. Keeping in view of the chemical composition of *T. terrestris* in the literature, the current study was designed to evaluate its potential using the co-crystallized native ligand (Figure 2).

### 2. RESULTS

#### 2.1. HPLC Analysis

Quercetin, gallic acid, caffeic acid, vanillic acid, benzoic acid, syringic acid, cinnamic acid, sinapic acid, and kaempferol were identified by HPLC analysis (Figure 1). The quantity of these compounds in descending order is as follows: kaempferol, 32.98 ppm; quercetin, 26.82 ppm; sinapic acid, 24.81 ppm; gallic acid, 22.40 ppm; cinnamic acid, 14.97 ppm; syringic acid, 13.50 ppm; benzoic acid, 12.41 ppm; caffeic acid, 12.30 ppm; and vanillic acid, 11.23 ppm (Table 1).

#### 2.2. In Silico Modeling: Induced-Fit Molecular Docking

Molecular docking was performed on identified compounds from HPLC mentioned in Table 2 to delineate the potential mechanism for the inhibition of AChE. Re-docking of the cognate ligand generated the pose with 0.173 Å root mean square deviation (RMSD) concerning the apo conformation of the co-crystallized native ligand (Figure 2).

![Table 1. HPLC Analysis of a Methanolic Extract of T. terrestris](https://dx.doi.org/10.1021/acsomega.0c03375)

| serial no | compound     | retention time | area (mV s) | area (%) | amount in ppm |
|-----------|--------------|----------------|-------------|----------|---------------|
| 1         | quercetin    | 2.813          | 50.074      | 2.4      | 2.65          |
| 2         | gallic acid  | 4.840          | 222.419     | 10.8     | 7.99          |
| 3         | caffeic acid | 12.407         | 12.519      | 0.6      | 0.57          |
| 4         | vanillic acid| 13.220         | 24.918      | 1.2      | 1.54          |
| 5         | benzoic acid | 14.947         | 59.501      | 2.9      | 6.37          |
| 6         | syringic acid| 16.507         | 127.503     | 6.2      | 3.71          |
| 7         | cinnamic acid| 24.813         | 153.591     | 7.4      | 4.74          |
| 8         | sinapic acid | 26.820         | 105.455     | 5.1      | 1.37          |
| 9         | kaempferol   | 1.860          | 106.438     | 31.2     | 32.98         |

All the compounds were found to dock with negative binding energy ($\Delta G$), which highlighted their potential binding affinity for the AChE active site. Rivastigmine complexation was stabilized with $−6.22 \Delta G$ (Kcal/mol) and served as the standard threshold. Interestingly, sinapic acid, quercetin, and kaempferol bind the AChE with comparable $\Delta G$ as that of rivastigmine — which implied that the related inhibitory potential was noteworthy, that all the phytochemicals penetrated the active site, established the vital interactions, and emerged as important bioactive compounds with AChE inhibitory potential (Table 2). However, the spatial conformational analysis revealed that sinapic acid, quercetin, and kaempferol were penetrated, through the narrow active-site gorge, in a similar fashion to rivastigmine (Figure 3).

These phytochemicals orient themselves into the spatial configuration, which allows the diverse interactions with vital residues of the active site. Moreover, the spatial configurations of phytochemicals also allowed their functional groups to disrupt the catalytic triad (HIS–SER–GLU) of AChE. The active-site gorge is lined by residues, with aromatic side chains, which has low affinity for acetylcholine and mediates its diffusion into a catalytic triad of the active site — i.e., “aromatic guidance”. Moreover, these aromatic residues exhibit greater flexibility, and renders the narrower entrance of the active-site gorge that offers selectivity, and governs the entrance of small molecules with suitable size. However, similar to rivastigmine,

Figure 1. HPLC chromatogram of TTME.
Table 2. Parameters for Induced-Fit Docking of Compounds at the AChE Active Site

| compound    | binding score (S) (Kcal/mol) | interacting residues | interaction type                        |
|-------------|------------------------------|----------------------|----------------------------------------|
| rivastigmine| -6.22                        | HIS477, GLU202, TYR133, TRP86, TYR337, PHE297, PHE338, GLY122 | van der Waals, π–alkyl, H-bonding     |
| sinapic acid| -6.17                        | SER203, GLY122, GLY121, TYR124, TRY337, TYR133 | van der Waals, H-bonding, amide–π stacking |
| quercetin   | -6.16                        | GLU202, SER203, PHE297, GLY122, GLY121, TYR124, TRY337, TRP86, TYR133 | van der Waals, H-bonding, π–π stacking |
| kaempferol  | -6.02                        | GLU202, TRP86, PHE297, GLY121, TYR124, TYR133 | H-bonding, π–π stacking |
| syringic acid| -5.89                        | GLY122, TRP86, TYR337, HIS447 | H-bonding, π–π stacking, T-shaped     |
| caffeic acid| -5.32                        | SER203, TYR337, GLY122 | H-bonding, π–π stacking |
| vanillic acid| -5.24                        | HIS447, GLU202, TYR337, TYR124, TRP86, GLY448 | π–π stacking |
| cinnamic acid| -4.99                        | TRP86               | π–π stacking |
| gallic acid | -4.91                        | HIS447, GLY448, GLU202, TYR337, TRP86 | H-bonding, π–π stacking, π–lone pair |
| benzoic acid| -4.85                        | TRP86, TYR337       | H – bonding, π–π stacking |

sinapic acid, quercetin, and kaempferol blocked the aromatic guidance phenomenon of the active-site gorge and efficiently penetrated its bottom, which further disrupted the catalytic triad of AChE.

Rivastigmine absorbed into the AChE binding pocket by π–alkyl interaction with PHE338, PHE297, and TRP86, thus blocking the aromatic lining of the active-site gorge (Figure 4). Rivastigmine also hinders the closure of the active-site swinging gate (i.e., TYR337) by forming a H-bond with its corresponding TYR133 residue. Rivastigmine complexed with the AChE catalytic triad by π–alkyl and H-bond to HIS447 and GLU202, respectively. The sinapic acid complex was stabilized by the H-bond with TYR124, TYR133, and TRY337, thus blocking the absorption, aromatic guidance, and penetration of acetylcholine into the AChE active site. Besides, sinapic acid also established a H-bond with SER203 at the catalytic triad, and its further amide–π stacking with GLY121 blocked the oxyanion hole of the active site, which ultimately blocks the catalysis. The quercetin complex was stabilized by strong and diverse interactions along the path of its penetration into the AChE active-site gorge. In addition to its steric effect on the swinging gate, it blocked the entrance by forming H-bonds with TYR124, TYR133, and TRP86 and π–π stacking with PHE297. Interestingly, it also blocked the AChE catalytic triad by strong H-bonds with SER203 and GLU202 and further hindered the bridging of SER–GLY at the oxyanion hole. Kaempferol revealed a similar binding pattern to quercetin, with the exception that it lacked the H-bonding with SER at the catalytic triad that may justify the moderation of its affinity. We also noted an interesting binding behavior of gallic acid. Despite the lower ΔG, gallic acid blocked the closure of the swinging gate by interacting with TYR337 and TRP86 and penetrated deep into a catalytic triad, where it established strong H-bonds with GLU202 and HIS447.

2.3. In Vivo Anti-Parkinson’s Activity. 2.3.1. Behavioral Observations: Tests to Observe the Effects on Motor Function. 2.3.1.1. Catalepsy Test. In the present study, haloperidol (1 mg/kg i.p.) induced significant (P < 0.001) catalepsy in the disease control group after 30 min and maximum catalepsy after 120 min compared to the control group (Table 3). Catatonia induced by haloperidol was significantly reversed in TTME-treated groups in dose-dependent mode after 60 min, indicating its neuroprotective potential by preserving dopaminergic neurotransmission in the striatum.

2.3.1.2. Hang Test. In the current study, the wire hanging test indicated that fall-off time was significantly decreased (P < 0.05) in the disease control group compared to the control group and significantly improved hanging time (greater than 60 min) was observed in TTME-treated groups (Figure 5). The neuromuscular strength was significantly reduced (P < 0.05) by haloperidol due to blockage of descending commands initiated by basal and cortical motor pathways. This study indicated that TTME has a protective role in neuromuscular coordination by improving hanging time.

2.3.1.3. Grip Walk Test. The ladder climbing test indicated that grip walk strength was significantly reduced (P < 0.05) by haloperidol in the disease control group and improved in TTME-treated groups dose-dependently, manifesting decreased foot slips and that lesser time is taken by animals to reach the platform (Figure 6).

2.3.1.4. Open-Field Test. An open-field test was used to investigate the protective effect of TTME on exploration, locomotion, and anxiety. In the open-field task, haloperidol significantly worsen (P < 0.001) the locomotion and exploratory behavior in disease control, standard, 100 mg/kg, and 300 mg/kg TTME-treated groups (Table 4). TTME (1000 mg/kg) has significantly improved (P < 0.001) total number of lines crossed and locomotion. Freezing time was significantly increased in disease control and improved in the
1000 mg/kg TTME-treated group. Grooming and rearing frequencies were significantly reduced \((P < 0.001)\) in the disease control group and significantly recovered in TTME-treated groups and standard groups.

2.3.1.5. Footprinting Test. Stride length was estimated by the footprinting test, which indicated that haloperidol induces significant reduction \((P < 0.05)\) in motor dysfunction and freezing behavior due to depletion of the dopamine level in the substantia nigra in the disease control group compared to the control group. L-Dopa and carbidopa, standard drugs of PD, and TTME at high dose significantly reversed motor dysfunctions and akinesia by improving stride length (Figure 7).

2.3.2. Test to Observe the Effects on Nonmotor Function.

2.3.2.1. Hole-Board Test. The frequency of head dipping was significantly decreased \((P < 0.05)\) in the disease control group compared to the control group in the hole board apparatus (Figure 8). Haloperidol induced depression and impaired the exploratory and locomotor abilities of animals in the disease control group. In TTME-treated groups, head dipping was significantly improved in a dose-dependent way indicating its protective propensity against dopaminergic depletion.

2.3.2.2. Biochemical Estimation. In this study, haloperidol provoked significant reduction \((P < 0.05)\) in the levels of CAT, SOD, GSH, and GPx in the disease control group compared to the control group (Table 6). The level of MDA indicating lipid peroxidation was significantly increased \((P < 0.05)\) in the disease control group compared to the control group. TTME (300 and 1000 mg/kg) and standard drug (l-Dopa + carbidopa) significantly increased the levels of antioxidant enzymes CAT, SOD, GSH, and GPx and decreased the level of MDA (Table 5).

Dopamine and acetylcholine are the main neuromodulators in the basal ganglia regulating motor and cognitive abilities. The acetylcholine level is depleted due to the degeneration of choline acetyltransferases with cognitive disabilities in PD. In this study, TTME has a protective propensity on the level of acetylcholine by its inhibitory action on acetylcholinesterase, which causes the breakdown of acetylcholine. The level of acetylcholinesterase was significantly increased \((P < 0.05)\) in the disease control group compared to the control group. TTME at high dose significantly decreased the level of acetylcholinesterase compared to standard, 100 mg/kg, and 300 mg/kg (Figure 9).

2.4. RT-PCR Analysis. The mRNA expression levels of PD indicators named acetylcholinesterase (AChE) \((2.26 \pm 0.13\) fold), \(\alpha\)-synuclein \((2.76 \pm 0.152\) fold), interleukin-1\(\beta\) (IL-1\(\beta\)) \((3.82 \pm 0.058\) fold), and tumor necrosis factor-alpha (TNF-\(\alpha\)) \((2.43 \pm 0.08\) fold) were raised significantly \((p<0.05)\) in the disease control group as compared to control group values. TTME showed a decline in these PD indicators dose-dependently indicating that treatment decreases the severity of Parkinson’s disease in rats (Figure 10).

3. DISCUSSION

In dopaminergic neurons of the substantia nigra, oxidative stress is one of the foremost factors leading to neurodegenerative PD disorder. Some medicinal plants embody appreciative quantities of flavonoids, glycosaponins, alkaloids, and polyphenolic compounds that are considered to be effective against oxidative stress-induced neurotoxicity. T. terrestris is enriched with alkaloids, flavonoids, and steroidal saponins, which are helpful against neurodegeneration and mental disorders.

Our previous findings of GCMS were in corroborate with Jung Choi et al. They isolated benzene dicarboxylic acid dinonyl ester from the ethanol extract of Rosa laevigata, and they estimated its neuroprotective potential by improving...
Figure 4. Rivastigmine (A), sinapic acid (B), quercetin (C), and kaempferol (D) interaction patterns with the residues of the AChE active site; the two-dimensional (2D) perspective of the compound’s interactions with key binding pocket residues illustrated as balls and sticks colored by type of interaction.

Table 3. Effect of TTME on Catalepsy in the Haloperidol-Induced Parkinson’s Model

| group               | 30 min | 60 min | 90 min | 120 min | 150 min | 180 min |
|---------------------|--------|--------|--------|---------|---------|---------|
| control             | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| disease control     | 0.6 ± 0.16** | 0.83 ± 0.16* | 1.00 ± 0.5** | 1.16 ± 0.16*** | 1.66 ± 0.16*** | 1.83 ± 0.16*** |
| standard            | 0.5 ± 0.00** | 0.66 ± 0.16** | 1.00 ± 0.00** | 0.83 ± 0.44* | 0.83 ± 0.16** | 0.5 ± 0.28** |
| 100 mg/kg           | 0.3 ± 0.16** | 1.33 ± 0.16*** | 1.16 ± 0.16*** | 1.16 ± 0.16*** | 0.83 ± 0.16** | 0.83 ± 0.16** |
| 300 mg/kg           | 0.6 ± 0.16** | 1.00 ± 0.00** | 1.16 ± 0.16*** | 1.33 ± 0.16*** | 0.83 ± 0.16** | 0.83 ± 0.16** |
| 1000 mg/kg          | 0.5 ± 0.00** | 0.80 ± 0.15* | 1.00 ± 0.28** | 0.60 ± 0.33** | 0.66 ± 0.44** | 0.33 ± 0.16** |

*Values are expressed as mean ± SEM (n = 6). **p < 0.001, *p < 0.01, *p < 0.05, ns: nonsignificant as compared to control.

Figure 5. Effect of TTME on a wire hanging test in the haloperidol-induced Parkinson’s disease model. HT: hanging time, values are expressed as mean ± SEM (n = 6). **p < 0.001, *p < 0.01, *p < 0.05, ns: nonsignificant as compared to control.

Figure 6. Effect of TTME on the ladder climbing test in haloperidol-induced Parkinson’s disease model. Values are expressed as mean ± SEM (n = 6). *p < 0.05, ns: nonsignificant as compared to control.
Molecular docking is a reliable approach that simulates the binding mode, and mechanism of inhibition. Silico modeled to address their AChE inhibitory potential, the phytochemicals were further in silico modeled to address their AChE inhibitory potential, simulate the binding mode, and mechanism of inhibition. Molecular docking is a reliable approach that simulates the complexity of compounds within the active site and delineates the binding mechanism. Induced-fit docking is a vital extension to address the conformational flexibility of the binding pocket upon ligand binding while accurately scoring the ligands. Herein, we showed that sinapic acid, quercetin, and kaempferol efficiently penetrated the binding pocket of AChE and established the important interactions such as in the case of gallic acid. However, the binding mode analysis provided the mechanistic insights to predict the modulation of AChE catalysis by these phytochemicals. Interestingly, sinapic acid, quercetin, and kaempferol efficiently blocked the aromatic guidance for acetylcholine at the active-site gorge. Moreover, they also rendered the retraction of the swinging gate, out of the active site, and diffused toward catalytic triad, where they further blocked the catalysis of acetylcholine at the site. Therefore, these insights indicated these phytochemicals as potential lead compounds that may not just competitively block the acetylcholine entrance into an active-site gorge of AChE but also competitively inhibit the catalysis of acetylcholine at the active site — thereby supporting the inhibition of AChE and amelioration of experimental cholinergic deficits. Certain neurosteroids such as allopregnanolone depletion or excessive metabolism by their metabolizing enzymes are associated with the pathogenesis of PD. TTME as previously reported has pregnane-11-20-dione with a percentage peak area of 7% indicating its adjunctive role in neuroprotection. The existence of benzene acetic acid with maximum peak area in TTME indicates its vigorous neuroprotective role in the PD model in corroboration with a previous study by Yun et al. in which they evaluated the neuroprotective role of 4-hydroxy-

Table 4. Effect of TTME on the Open-Field Test in the Haloperidol-Induced Parkinson’s Model

| group            | number of lines crossed | freezing (s) | rearing/10 min | grooming/10 min |
|------------------|-------------------------|--------------|----------------|-----------------|
| control          | 25.00 ± 0.3             | 0.00 ± 0.0   | 6.00 ± 0.1     | 2.99 ± 0.3      |
| disease control  | 8.0 ± 0.0***            | 76.00 ± 0.5**| 0.00 ± 0.0***  | 0.00 ± 0.0***   |
| standard         | 24.99 ± 0.6***          | 11.03 ± 0.8***| 6.00 ± 1.3**   | 3.54 ± 0.76**   |
| 100 mg/kg        | 18.99 ± 1.8***          | 31.05 ± 1.1**| 4.76 ± 1.1**   | 6.00 ± 0.45**   |
| 300 mg/kg        | 22.75 ± 0.5***          | 18.09 ± 0.5**| 3.55 ± 0.42**  | 2.34 ± 0.2**    |
| 1000 mg/kg       | 22.02 ± 0.5**           | 0.54 ± 0.0** | 4.8 ± 0.45**   | 3.90 ± 0.34**   |

Values are expressed as mean ± SEM (n = 6). ***p < 0.001, **p < 0.01, *p < 0.05, ns: nonsignificant as compared to control.

Figure 7. Effect of TTME on stride length in the footprinting test in the haloperidol-induced Parkinson’s disease. SL: stride length, values are expressed as mean ± SEM (n = 6). ***p < 0.001, *p < 0.05, ns: nonsignificant as compared to control.

Figure 8. Effect of TTME on the hole-board test in the haloperidol-induced Parkinson’s disease model. HD: head dipping, values are expressed as mean ± SEM (n = 6). ***p < 0.001, *p < 0.05, ns: nonsignificant as compared to control.

Table 5. Effect of TTME on First-Line Antioxidant Enzymes in the Haloperidol-Induced Parkinson’s Model

| group            | CAT (IU/μL) | SOD (IU/μL) | MDA (TBA mg/mL) | GPs (μg/mg protein) | GSH (μg/mg protein) |
|------------------|-------------|-------------|-----------------|---------------------|---------------------|
| control          | 0.88 ± 0.17 | 0.20 ± 0.9  | 2.73 ± 0.3      | 6.94 ± 0.1          | 0.51 ± 0.6          |
| disease control  | 0.73 ± 0.02*** | 0.07 ± 0.1*** | 4.50 ± 0.3***   | 5.14 ± 0.01***     | 0.22 ± 0.12***     |
| standard         | 0.787 ± 0.8** | 0.17 ± 0.6* | 2.80 ± 0.1 **  | 6.33 ± 0.17*       | 0.53 ± 0.12**      |
| 100 mg/kg        | 0.70 ± 0.60*** | 0.15 ± 0.6 *** | 3.08 ± 0.4 ***  | 5.42 ± 0.9 ***     | 0.46 ± 0.6***      |
| 300 mg/kg        | 0.72 ± 0.60*** | 0.18 ± 0.6 *** | 2.83 ± 0.18***  | 6.30 ± 0.17***     | 0.49 ± 0.6***      |
| 1000 mg/kg       | 0.86 ± 0.02**  | 0.22 ± 0.6* | 2.75 ± 0.6*     | 6.90 ± 0.5*        | 0.56 ± 0.05*       |

Values are expressed as mean ± SEM (n = 6). ***p < 0.001, **p < 0.01, *p < 0.05, ns: nonsignificant as compared to control.

Figure 9. Effect of TTME on acetylcholinesterase in the haloperidol-induced Parkinson’s disease model. Values are expressed as mean ± SEM (n = 6). ***p < 0.05, ns: nonsignificant as compared to control.

antioxidant enzymes, memory, and cognitive disabilities, and level of acetylcholine. The phytochemicals were further in silico modeled to address their AChE inhibitory potential, simulate the binding mode, and mechanism of inhibition. Molecular docking is a reliable approach that simulates the complexity of compounds within the active site and delineates the binding mechanism. Induced-fit docking is a vital extension to address the conformational flexibility of the binding pocket upon ligand binding while accurately scoring the ligands. Herein, we showed that sinapic acid, quercetin, and kaempferol have a superior binding affinity, which was comparable to rivastigmine. Although the rest of the phytochemicals exhibited lower binding affinity, they also sufficiently penetrated the binding pocket of AChE and established the important interactions such as in the case of gallic acid. However, the binding mode analysis provided the mechanistic insights to predict the modulation of AChE catalysis by these phytochemicals. Interestingly, sinapic acid, quercetin, and kaempferol efficiently blocked the aromatic guidance for acetylcholine at the active-site gorge. Moreover, they also rendered the retraction of the swinging gate, out of the active site, and diffused toward catalytic triad, where they further blocked the catalysis of acetylcholine at the site. Therefore, these insights indicated these phytochemicals as potential lead compounds that may not just competitively block the acetylcholine entrance into an active-site gorge of AChE but also competitively inhibit the catalysis of acetylcholine at the active site — thereby supporting the inhibition of AChE and amelioration of experimental cholinergic deficits.

https://dx.doi.org/10.1021/acsomega.0c03375

ACS Omega 2020, 5, 25216−25227

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benzeneacetic acid and 4-methoxybenzeneacetic acid isolated from Dianthus superbus L. in glutamate-induced cell death in HT22 cells. Haloperidol induces depression and impairs the exploratory and locomotor abilities of animals in the disease control group. This study indicated that TTME has a protective role in neuromuscular coordination by improving hanging time. Depression is the most recurrent psychiatric issue in the prognosis of PD along with motor deficit. Our findings from the wire hanging test and hole-board test were in corroboration with a previous study by Bagewadi and Khan, in which Aloe vera at doses of 200 mg/kg and 400 mg/kg significantly improved hanging time, many heads dip, and decreased oxidative stress in the haloperidol-induced PD model. TTME effects on footprinting tests were harmonious to Withania somnifera extract effects, which conclusively showed significant improvement in physiological abnormalities such as improved stability in walking, stride length, and muscular coordination.

In this study, our findings from the ladder-climbing test and open-field test were in agreement with a previously reported study by Srimathi Priyanga and Vijayalakshmi, showing that administration of quercetin and hesperidin in the rotenone-induced PD model restored neuromuscular strength, cognition, and biochemical parameters due to their strong antioxidant activity. Haloperidol-induced neurotoxicity by blocking dopamine receptors results in the production of free radicals and lipid peroxidation. Evidence suggested that drugs that ameliorated the extrapyramidal side effects induced by neuroleptic drugs can considerably decrease oxidative stress. L-Dopa is effective in the symptomatic treatment of PD, but its long-term use increases oxidative stress. Antioxidant agents are an unorthodox robust therapeutic approach to intercept expeditious degenerative phenomena, i.e., trafficking in the central nervous system and free radical mediated tissue demobilization ascribing to neurodegeneration. In PD, the modulatory role of antioxidant agents indicates that antioxidant therapy is an attractive approach.

Neuroleptic agents such as haloperidol convulse the catecholaminergic system and induce reversible akenisia by blocking D_{2} receptors. In this study, oxidative stress was measured by estimating the levels of CAT, SOD, MDA, GSH, and GPx. The first line of defense, antioxidant enzymes such as catalase neutralize noxious execution of hydrogen peroxide by transforming hydrogen peroxide to water and nonreactive oxygen. Haloperidol inoculation induced oxidative stress resulting in a decreased level of catalases. SOD enzyme is involved as a catalyst in the dismutation of superoxide into water and nonreactive oxygen and halting oxidative stress by counteracting the harmful action of free radicals. Lipid peroxidation is a sensitive marker of oxidative insult, which is measured by estimating the level of TBA. Lipid peroxidation occurs due to the attack of free radicals on arachidonic acid and on the double bond of unsaturated fatty acids, which produce lipid peroxide radicals, starting a chain reaction of further attack. GSH is a predominant enzyme in the pathogenesis of PD, and depletion of glutathione levels in the substantia nigra is attributed to the neuronal loss in PD. A positive interdependence subsists among neuronal loss and glutathione depletion. The diminished level of GSH impairs the capability of neurons to detoxify hydrogen peroxide, which consequently causes the generation of free radicals and lipid peroxidation. A raised level of lipid peroxidation was observed in the substantia nigra of PD brain, consistent with our current findings of MDA. Dopamine and acetylcholine are the main neuromodulators in the basal ganglia regulating motor and cognitive abilities. The acetylcholine level is depleted due to the degeneration of choline acetyltransferases with cognitive disabilities in PD. In this study, TTME has a protective propensity on the level of acetylcholine by its inhibitory action on acetylcholinesterase, which causes breakdown of acetylcholine.

Our findings of TTME consequences on behavioral and biochemical parameters were in agreement with a previous study where Cannabis sativa showed antiparkinsonian propensity by reducing catalepsy duration and improving oxidative stress by reducing lipid peroxidation in the haloperidol-induced experimental model. TTME expressions on neurochemical parameters like GSH, GPX, SOD, CAT, and MDA were inconsistent with the effect of the Ficus religiosa petroleum ether extract in haloperidol- and 6-OHDA-induced PD animal models, in which this plant improved behavioral parameters like catalepsy, muscular rigidity, locomotion, and attenuated oxidative stress.

Overexpression of α-synuclein protein due to a mutation in the SNCA gene has a vital role in pathogenetic dysfunctions of PD. This protein is considered as a possible early diagnostic biomarker and therapeutic target for PD in current scientific approaches. It is reported in prior studies that cinnamon extract in vitro and in vivo suppressed α-synuclein aggregation and precipitated oligomer stabilization in A53T- and α-synuclein-induced fly model of PD. The study results by Siddique et al. are in unison with our findings that Eucalyptus citriodora ameliorated climbing abilities and reduced oxidative stress in the drosophila PD model. Compatible to the...
abovementioned studies, TTME ameliorates mRNA expression of α-synuclein in treatment groups compared to the model group due to exhibiting multiple secondary metabolites, alkaloids, glycosides, flavonoids, and steroidal saponins.

The principal role of acetylcholinesterase (AChE) is the cessation of cholinergic neurotransmission at synapses by hydrolysis of acetylcholine. In prior literature, it is well reported that increased expression of a synaptic isoform of mRNA of AChE is associated with induction of apoptosis in numerous cell lines. The AChE level and its mRNA expression were elevated by the neurotoxic action of haloperidol in the model group of PD. TTME induces inhibition of AChE expression in the treatment group compared to the model group indicating its therapeutic potential against apoptotic dopaminergic neuronal cell death, compatible with previous findings.21

It is evident from the previous literature that PD etiopathogenesis is strongly associated with raised expression of proinflammatory cytokines such as TNF-α and IL-1β by activation of microglial cells in the striatum and CSF.22,23 It is reported that the sustained raised level of proinflammatory cytokines IL-1β is responsible for dopaminergic neuronal cell death in PD brain.24 It is reported that raised levels of TNF-α cause forelimb akinesia and neurodegeneration.25 TTME ameliorated the raised level of proinflammatory cytokines TNF-α and IL-1β in treatment groups compared to a model group via inhibition of neuroinflammation and oxidative stress, in agreement with a previous study.26

The microglia cells, resident macrophages of the brain, activated in response to neurotoxic substances like haloperidol, MPTP, 6-OHDA, etc. These cells ubiquitously activated in the substantia nigra in PD and induced activation of inflammatory cytokines and neuroinflammation. TNF-α and IL-1β are potent mediators of microglial functions. These two mediators are principally involved in dopaminergic neuronal cell death in the background of PD pathogenesis. The higher mRNA expression level of TNF-α at the site of neuronal damage suggested that it is a feasible therapeutic target in the treatment of the neurological disorder. It is evident from the current study that TTME at high dose remarkably attenuated the mRNA expression of TNF-α.27

In recent advance studies, it is reported that neurodegenerative disorders can be treated and their progression is delayed by anti-inflammatory agents also comprising medicinal plants via hampering microglial activation. TTME modulations of inflammatory cytokines were similar to the effect of the F. religiosa leaf methanol extract, which attenuated the mRNA expression of TNF-α, IL-1β, and IL-6 in mice in a dose-dependent manner. IL-1β modulated the mechanism of neuroinflammation through the up-regulation of MAPK pathways and repressed the level of NF-Kb.28,29

Molecular docking was performed using a computer-aided diagnostic tool capable of mimicking human reasoning of PD pathogenesis. This application is helpful in the identification of novel therapeutic strategies for pharmacological interventions. In this study, computational figures were drawn to identify binding and interaction of phytochemicals existing in TTME (identified in HPLC analysis) with target enzyme acetylcholinesterases.29,30 Current study findings manifested that the acetylcholinesterase active sites were complementary to these phytochemicals in terms of binding parameters.30

Most of the phytoconstituents identified regulated cell-stress reaction pathways resulting in neuroprotection.31 The findings of in silico studies revealed that anti-Parkinson’s potential of TTME phytochemicals is harmonious to the previous computational literature in which quercetin and kaempferol modulated the enzyme monoamine oxidase through the interaction of Parkinson’s target amino acid residues: I1LE51,32 ALA68, GLN443, GLN66, GLN443, MET445, TYR69, and ASN181.33

Therefore, we concluded that TTME has modulatory action against a behavioral and biochemical alteration in haloperidol neurotoxicity, and it will be considered as a lead compound for promising future neuroprotective agents.

4. CONCLUSIONS

It is concluded from behavioral, biochemical, RT-PCR, and in silico induced-fit molecular docking that T. terrestris possessed the potential to ameliorate Parkinson’s disease. It increased the levels of endogenous antioxidant enzymes and decreased the levels of AChE, α-synuclein, IL-1β, and TNF-α. Hence, T. terrestris should be considered as a potent reservoir of lead compounds for the development of neuroprotective agents.

5. MATERIALS AND METHODS

5.1. Chemicals. Methanol (Sigma-Aldrich, St. Louis, MO, USA), haloperidol (Searle Pakistan Pvt. Ltd.), levodopa, and carbidopa (Platinum Pharmaceuticals (Pvt.) Ltd.), carboxymethylcellulose, triazole (Invitrogen), cDNA kit (Thermo Scientific), cyber green (SYBR Green master mix of Bio-Rad), and primers (Thermo Fischer Scientific) were the chemicals used.

5.2. Plant Collection, Authentication, and Extract Preparation. T. terrestris fruits were collected from West Canal Office, Irrigation Department, Faisalabad, Pakistan in June 2017. These were identified and authenticated by a taxonomist “Dr. Mansoor” from the University of Agriculture Faisalabad, Pakistan, and a voucher specimen (No: 625-1-2018) was kept in their herbarium for reference. The fruits were dried under a shade for 2 months, and after coarse grinding, the fine powder was obtained by sieving. The T. terrestris methanol extract (TTME) was prepared by macerating 2 kg of T. terrestris powder in 5 L of methanol for 7 days with occasional shaking. The extract was filtered and passed through a rotary evaporator at 40 °C to get the semisolid extract.34,35 The percentage yield was calculated by the following formula

\[
\text{extract yield percentage (\%) = \left( \frac{\text{weight of pure extract}}{\text{weight of powder macerated}} \right) \times 100}
\]

5.3. HPLC Analysis. HPLC was performed with a Shimadzu autosampler equipped with a pump LC-10AT and UV–visible detector SPD-10AV. A column of Shim-pack CLC-ODS (C-18), 25 cm × 4.6 mm, 5 μm diameter particles packed and volume injection 40 μL was used. The mobile phase consisted of water and acetic acid at 94:6 as solvent A and acetonitrile 100% as solvent B. The flow rate of the mobile phase was 1 mL/min. The UV absorption spectrum was recorded at a wavelength of 280 nm for both standard and sample. The mobile phase used for kaempferol was acetonitrile, dichloromethane, and methanol in a ratio of 60:20:20. The absorption spectrum was recorded at wavelength 248 nm for kaempferol. The stock solution of the standard was prepared in...
methanol of HPLC grade in a concentration of 1 mg/mL. The mobile phase and stock solution of both sample and standard were degassed by placing them in a sonicator and filtered through a membrane filter of size 0.45 μm (Millipore). HPLC analysis was carried out at room temperature and in triplicate. The identification of compounds was done by comparing retention times and UV absorption spectra of standards and samples.36,37

5.4. In Silico Modeling: Induced-Fit Molecular Docking. The identified phytochemicals were computationally modeled to complement the insights into acetylcholinesterase (AChE) inhibition. These phytochemicals were computationally docked into the active site of AChE by induced-fit docking protocol in Molecular Operating Environment (MOE) 2015.10. The three-dimensional (3D) X-ray crystallized structure of AChE (4EY6) was retrieved from RSCB Protein Data Bank (http://www.rcsb.org). The 3D conformers of Rivastigmine (CID: 77991), benzoic acid (CID: 243), caffeic acid (CID: 689043), cinnamic acid (CID: 444539), gallic acid (CID: 370), sinapic acid (CID: 637775), syringic acid (CID: 10742), vanillic acid (CID: 8468), kaempferol (CID: 5280863), and quercetin (CID: 5280343) were fetched from the PubChem Database. The macromolecule was prepared and optimized in the Structure Preparation panel to correct the structural errors—such as H-Count, Termini Capping, and Alternates. The Protonate 3D application was used to optimize the H-bonds and potential energy to withstand the molecular mechanics refinement of docked poses. The molecular system was energy-minimized with Amber10: EHT force field to add tether restraints. The unbound water molecules and extraneous co-factors were removed. The binding site was identified by specifying the contact-residues of the co-crystallized ligand of a macromolecule. The test ligands were docked into the binding pocket by the Triangular Matcher Placement Method with London dG scoring, and the induced-fit method was specified to refine the generated poses, which were further scored with the GBV1/WSA dG scoring function. The pose with the lowest conformational energy or ΔG (i.e., S) was used to analyze the ligand—receptor complex orientation and interactions in Discovery Studio Visualizer v17.2. The apo conformation of the co-crystallized ligand was docked, and RMSD of the docked pose was computed as a measure to validate the docking protocol.36-40

5.5. Animals. Healthy adult male Wistar rats (weight range 180–220 g) were obtained from the animal house of the University of Agriculture Faisalabad, Pakistan, and housed in properly aerated polypropylene cages under standard conditions (temperature 25 ± 2 °C, 12 h light and dark cycle, and 55–60% humidity), and provided with standard commercial pelleted rat feed and water ad libitum in the animal house of Government College University Faisalabad, Pakistan. After acclimatization for 1 week, animals were divided randomly into six groups (n = 6).

5.6. Ethical Approval. The study design was approved by the Institutional Review Board of Government College University Faisalabad with reference no. GCUF/ERC/1980.

5.7. In-Vivo Anti-Parkinson’s Activity. 5.7.1. Haloperidol-Induced Parkinson Disease Model. All the rats except the control group were given haloperidol 1 mg/kg once daily by intraperitoneal (i.p.) route 1 h before the administration of respective treatments throughout the 21 day study.

5.7.2. Experimental Design. Thirty-six rats were divided into six groups (n = 6).

(1) Group I: control group received vehicle (carboxymethylcellulose 1% (w/v), 10 mL/kg, p.o.)

(2) Group II: disease control received haloperidol (1 mg/kg, i.p.)

(3) Group III: standard, receiving l-dopa (100 mg /kg, orally) and carbidopa (25 mg/kg, orally)

(4) Groups IV–VI: treatment groups administered with 100, 300, and 1000 mg/kg TTME orally, respectively

All the doses were given for 21 days. Weight and behavioral observations were recorded before and after the study. On the 22nd day, rats were sacrificed under anesthesia by cervical dislocation. Brain from all groups was removed, washed with ice-cold phosphate buffer (pH 7.4), and preserved for biochemical estimations.

5.7.3. Behavioral Observations. 5.7.3.1. Tests to Observe the Effects on Motor Functions. 5.7.3.2. Catalepsy Test. Catalepsy is the inability of animals such as rodents to correct externally imposed position or posture. The previously elucidated method41 was followed for the determination of catatonia induced by haloperidol (1 mg/kg i.p.). Cataleptic behavior was observed for 30 to 120 min after the administration of haloperidol by the standard bar test (with 3 and 9 cm elevated bars). Catalepsy was scored zero when a rat was able to move freely on a table, 0.5 when a rat moved on touch or pushed, 1 when a rat was unable to remove its front paws set on a 3 cm high bar (0.5 for each paw), and 2 when a rat was unable to correct its forepaws set on a 9 cm high bar (1 for each paw).

5.7.3.3. Hang Test. The wire hanging test was performed to assess neuromuscular strength by following the method previously elucidated. The grip walk test was performed by placing a rat on a wooden ladder inclined at 45° with 2 cm apart consecutive steps of the ladder. The muscular activity was observed and scored according to the previously adopted method.41

5.7.3.4. Grip Walk Test. This task was designed to investigate neuromuscular coordination, locomotor functions, and forelimb/hind limb deficit in PD animals. This test is usually adopted due to its consistent, unambiguous, and reliable results between observers as compared to the open-field test.42 A wooden ladder was designed with 45 steps along with a small box on the top of the apparatus, which was considered as a platform. A small wooden box was constructed on the top to provide a secure and hidden place for animals. The grip walk test was performed by placing a rat on the wooden ladder inclined at 45° with 2 cm apart consecutive steps of a ladder. The muscular activity was observed and scored according to a previously adopted method.42 Foot slips and arrival time to the platform were noticed.

5.7.3.5. Open-Field Test. The task was used for concurrent investigation of exploration, anxiety, and locomotion in animals. It was constructed of a plywood material of 72 × 72 cm dimensions with walls of height 36 cm like a hollow square chamber painted with white resins. One wall was made of Plexiglas material to visualize rat movement inside the apparatus. The floor of this chamber was divided into 16 equal squares (18 × 18 cm) with black lines. The central square was drawn of 18 × 18 cm dimension with red lines to distinguish the central area from other locations in its surroundings. Ethanol (70%) was used to wipe the apparatus after each
animal trial. The rat was handled by its tail gently and placed at one corner of this apparatus, and the total distance traveled and total lines crossed by its four paws were observed for 10 min. The time spent in the central area, stretches attend, postures, grooming, defecation, rearing, and freezing were observed.

5.7.3.6. Footprinting Test. This test was performed by following Yadav et al.’s method in which the animals were inculcated to walk on a white sheet. Forepaws were placed in black ink, and stride length was measured.

5.7.3.7. Test to Observe the Effects on Nonmotor Function. 5.7.3.8. Hole-Board Test. Anxiety and exploratory behavior of rats were observed by head dipping in the hole-board apparatus. This apparatus was constructed of Plexiglas material of dimensions (25 cm × 25 cm and 30 cm wall height), and the floor was divided into 16 equally divided holes and 1.5 m high from the ground. The animal was leniently left on the floor and permitted to explore for 8 min. The distance traveled in the periphery of the apparatus and central portion and the number of head dipping in holes were recorded. Head dipping was scored only when both eyes of animals were disappeared in the hole.

5.8. Biochemical Estimations. 5.8.1. Preparation of the Tissue Homogenate. Brain tissues were homogenized with 10% w/v in Tris–HCl 30 Mm, pH 7.4 in a tissue homogenizer, and centrifuged at 3000 rpm for 10 min. These brain homogenates were used for the estimation of first-line defense endogenous antioxidant enzymes.

5.8.2. Determination of Catalases (CAT) Activity. For this assay, the reaction mixture was composed of 50 μL of brain homogenate, 1.95 mL of phosphate buffer (pH 7.4, 50 mM), and H2O2 (1 mL, 30 mM). Optical density was noted at 240 nm. The following formula was used to estimate the catalases activity.

\[
\text{CAT activity} = \frac{\delta \text{O.D.}}{E \times \text{vol. of sample (mL)} \times \text{mg of protein}}
\]

Here, \( E \) is 0.071 mmol cm\(^{-1}\) (the extinction coefficient of hydrogen peroxide) and \( \delta \text{O.D.} \) is the absorbance shift per minute.

5.8.3. Determination of Superoxide Dismutase (SOD) Activity. The reaction mixture was prepared by mixing sodium phosphate buffer (pH 8.3, 0.052 M) 1.2 mL with 100 μL of tissue homogenate, 100 μL of phenazine methosulfate (186 μM), 300 μL nitro blue tetrazolium (300 μM), and 200 μL of Triton X. It was incubated for 95 s at 30 °C. Glacial acetic acid was incorporated to terminate a chemical reaction. It was stirred vigorously upon the addition of 4 mL of \( n \)-butanol, allowing it to settle down for 10 min and to get a separate layer of \( n \)-butanol centrifuged at 1000 rpm. \( n \)-Butanol served as a blank against which color intensity of chromogen was recorded at 560 nm and compared with the known SOD standard curve, expressed as unit/mL.

5.8.4. Determination of the Malondialdehyde (MDA) Level. The MDA level indicates the level of lipid peroxidation. For this assay, in a falcon tube, 200 μL of brain homogenate, sodium dodecyl sulfate (200 μL, 8.1%), 20% acetic acid, and thiobarbituric acid (TBA) 0.8% (1.5 mL of each) were mixed and poured into 4 mL of distilled water. The mixture was heated for 1 h at 90 °C in a water bath and cooled. The solution was further blended with \( n \)-butanol (5 mL) and distilled water (1 mL) and centrifuged at 4000 rpm for 10 min after vigorous shaking. The overlying top butanol surface was separated, and at 532 nm, absorbance was recorded. Different thiobarbituric acid (TBA) concentrations (10–100 μL) were prepared to plot the standard curve. The following regression equation was used to calculate the MDA level.

\[
Y = 0.0278x - 0.2485
\]

5.8.5. Determination of Glutathione Peroxidase (GPx). The assay mixture was composed of brain homogenate (0.1 mL), sodium azide (0.2 mL), 0.2 mL of each EDTA, and distilled H2O2. Trichloracetic acid (TCA) was added to stop the reaction and centrifuged to get the supernatant at 2000 rpm for 10 min. The separated overlying top layer coalesced with disodium hydrogen phosphate (4 mL) and of 5,5′-dithiobisnitrobenzoic acid (DTNB: 0.5 mL). At 420 nm, absorbance was recorded. Glutathione peroxidase activity was expressed as μmol of glutathione oxidized/minute/mg protein.

5.8.6. Determination of Reduced Glutathione (GSH) Activity. In this assay, 1 mL of potassium chloride and 1 mL of tissue homogenate were mixed in 4 mL of cold distilled water. Trichloracetic acid (1 mL) was added to the mixture and centrifuged for 30 min at 3000 rpm at 4 °C. Tris-buffer (0.4 M, 4 mL) and 0.001 M DTNB (0.1 mL) were incorporated in 2 mL of supernatant. The absorbance of sample and blank (composed of all reagents excluding the brain homogenate) was recorded at 412 nm. The concentration of GSH was calculated by using the following formula.

\[
\text{GSH} (\mu \text{mol/mL}) = \frac{(A_s - A_b)}{(C_b - C_s)} \times C_b
\]

5.8.7. Estimation of Acetylcholinesterase Activity. For the estimation of acetylcholinesterase activity, the brain homogenate (0.4 mL) was prepared in a cuvette containing 2.6 mL of 0.1 M phosphate buffer (pH 8.0) followed by 100 μL of 2,4-dinitrobenzene reagent (DTNBcd) and 20 μL of acetylthiocholine iodide. The yellow color was produced due to the reaction between dithiobisnitrobenzoic acid (DTNB) and

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**Table 6. List of Primers Used in the qRT-PCR Analysis of Haloperidol-Induced Parkinson’s Disease Rat Model**

| primer               | sequence                      | expected product size (bp) | accession no. |
|----------------------|-------------------------------|----------------------------|---------------|
| interleukin 1 beta   | forward GACTTCTACCATGGAACCGGT | 104                        | NM_031512.2   |
| acetylcholinesterase | forward GGAGAGATGCCATCTCAGAC | 200                        | NM_172009.1   |
| tumor necrosis factor | forward GGAGGGGAAGAAGCAACTCC | 168                        | NM_012675.3   |
| α-synuclein          | forward TCTGAGAAGCCTGGATCCATC | 156                        | XM_017592500.1|
| glyceraldehyde 3-phosphate | forward GGAGTCCATCACCACAACCA | 173                        | XM_017592351.1|

https://dx.doi.org/10.1021/acsomega.0c03375
ACS Omega 2020, 5, 25216–25227
thiocholine, which was measured spectrophotometrically at 412 nm.

The following formula was used for the estimation of enzyme activity:

\[ R = 5.74 \times 10^{-4} \times \frac{A}{CO} \]

In this equation, \( R \) is the rate in moles of substrate hydrolyzed per minute per gram of tissue, \( CO \) is the original concentration of tissue (mg/mL), and \( A \) is the change in absorbance/minute.\(^{50}\)

5.9. RT-PCR Analysis. RNA was extracted from rat brain hemispheres. The brain tissue was homogenized using a Polytron (VWR) device and then treated with TriZol (Life Technologies, Carlsbad, CA, USA). RNA samples were transcribed to cDNA in a 20 μL volume using the QuantiTect reverse transcription kit (Qiagen).

The thermal cycling comprised the real-time PCR as per the following conditions: 95 °C for 5 min followed by 40 cycles (denaturation for 15 s at 95 °C, annealing for 20 s at 60 °C, and extension for 20 s at 72 °C). The primer sequences and PCR product size for the target and reference genes are listed in Table 6.

mRNA expression levels of different markers were detected by real-time PCR with GADPH as an internal reference, using a Mesa Blue qPCR Master Mix Plus for the SYBR assay (Eurogentec) on the Master cycler Realplex2 (Eppendorf).

Relative quantitation was calculated by the comparative threshold cycle (CT) method with realplex software. Mean CT of triplicate measurements was used to calculate \( \Delta CT \) as the difference in CT for target and internal reference (GADPH) genes. The difference between the CT of the control experiment and the CT of each sample was calculated to give \( \Delta \Delta CT \). Fold increase in mRNA was calculated by \( 2^{-\Delta \Delta CT} \).

The PCR products of tissue samples after real-time PCR were electrophoresed by E-Gel Precast Agarose Electrophoresis System.

5.10. Statistical Analysis. All values were expressed as mean ± SEM. One and two-way ANOVA followed by Bonferroni post-test were applied for statistical analysis using GraphPad Prism version 5. \( P < 0.05 \) was set as a statistically significant value.

■ AUTHOR INFORMATION

Corresponding Author

Uzma Saleem — Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University, Faisalabad 38000, Pakistan; orcid.org/0000-0002-1541-4236; Phone: +92-333 4904928; Email: uzma95@gmail.com

Authors

Zunera Chauhdary — Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University, Faisalabad 38000, Pakistan

Zohair Raza — Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University, Faisalabad 38000, Pakistan

Shahid Shah — Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Government College University, Faisalabad 38000, Pakistan

Mahmood-ur Rahman — Department of Bioinformatics and Biotechnology, Government College University, Faisalabad 38000, Pakistan

Parwasha Zaib — Department of Bioinformatics and Biotechnology, Government College University, Faisalabad 38000, Pakistan

Bashir Ahmad — Riphah Institute of Pharmaceutical Sciences, Riphah International University, Lahore 54000, Pakistan

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c03375

Notes
The authors declare no competing financial interest.

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