Phenolic Profiling and Therapeutic Potential of Certain Isolated Compounds from *Parkia roxburghii* against AChE Activity as well as GABA<sub>A</sub> α5, GSK-3β, and p38α MAP-Kinase Genes

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**ABSTRACT:** *Parkia roxburghii* belongs to the family Mimosaceae; it has been used since ancient times as a cure for different health complications; such as inflammatory and gynecological diseases and hemiplegia. In this investigation, a reversed-phase-high-performance liquid chromatography (RP-HPLC) profile was carried out for *P. roxburghii*; also, the isolated bioactive compounds including quercetin, catechin, and biochaninA were individually and/or in combination investigated for their inhibitory effects on scopolamine-induced memory impairments in mice, implying that they have the ability to reduce the neurodegenerative effects of scopolamine and thus could be employed as a more effective therapeutic agent in the treatment of Alzheimer’s disease (AD) in humans. The possible interactions of *Parkia* flavonoids with acetylcholinesterase (AChE), γ-aminobutyric acid A receptor, alpha5 (GABA<sub>A</sub> α5), glycogen synthase kinase-3 (GSK-3), p38 mitogen-activated protein kinase (p38MAP-kinase), signal-regulated kinase (ERK), and protein-serine/threonine kinase (Akt) were then determined using molecular docking.

**INTRODUCTION**

Alzheimer’s disease (AD) is a type of dementia that affects older persons and is caused by a neurodegenerative illness. Dementia is a condition in which one’s memory, thinking, behavior, and ability to carry out daily tasks deteriorate. Dementia affects over 50 million individuals worldwide, with roughly 10 million new cases diagnosed each year. Dementia is characterized by a psychological shift in the expression of the γ aminobutyric acid (GABA) receptor gene, as well as changes in cholinergic neurons, tau proteins, and amyloid peptide buildup.

GABA is an important inhibitory neurotransmitter in the central nervous system. It regulates neuronal excitability by phasically and tonically activating GABA<sub>A</sub> receptors on neurons; tonic inhibition is mediated by extrasynaptic receptors, whereas phasic inhibition is mediated by synaptic receptors.

Herbal medicines and natural products have a wide variety of chemical structures, which has resulted in the identification of important therapeutic agents and discovery of new drugs.

A wide range of natural products, specially flavonoids, are now known to influence the function of GABA ionotropic receptors, the brain’s primary inhibitory neurotransmitter. *Parkia roxburghii* belongs to the family Mimosaceae, known as tree bean. It grows extensively in Northeast India and South East Asia. It is distributed in Bangladesh, Thailand, Egypt, and the Malaysian regions. It is a huge tree reaching a height of 25–30 m, with pipinnate leaves, flowers that are yellowish, white capitulum, and fruits that are soft, tender, and bright green in color. It is considered a highly important tree, and hence, pods and seeds of this plant are edible; they also are considered a good source of proteins, fats, and vitamins. *P. roxburghii* has a variety of applications, viz. antibacterial, medicines, alleopathy, and as human food. Salam et al. reported that its seed oil has insecticidal properties. Plants are known to contain diverse phytochemicals, and most of these substances are secondary metabolites, which are of great interest as sources of natural products. During the last decade, many studies focused on the use of medicinal plants for the prevention of diseases based on scientific studies. Pharmacological studies have been conducted with different *Parkia* species to determine their cytotoxic, antidiabetic, antioxidant, antiinflammatory, and anti-inflammatory activities. However, there have been no reports about biological effects concerning Alzheimer’s disease and memory deficits. The present study was designed to isolate the main bioactive compounds from *P. roxburghii* to evaluate their neuroprotective activity against Scopolamine hydrobromide-induced dementia in mice.

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**DETERMINATION OF TOTAL PHENOLIC CONTENT**

According to the Folin–Ciocalteau procedure, the total phenolic content was determined for *P. roxburghii* MeOH leaf extract.  

Briefly, the extract (100 μL), was transferred into a test tube and the volume adjusted to 3.5 mL with distilled water and oxidized by addition of 250 μL of Folin–Ciocalteau reagent. After 5 min, the mix was neutralized with 1.25 mL of 20% aqueous sodium carbonate (Na2CO3) solution. The absorbance was measured against the solvent blank at 725 nm after 40 min. The total phenolic level was determined by the gallic acid-prepared calibration curve, expressed as mg/g gallic acid equivalent. Further dilution was performed if the measured absorption value was over the linear distance of the standard curve.

**DETERMINATION OF TOTAL FLAVONOID CONTENT**

Total flavonoid content of *P. roxburghii* MeOH leaf extract was determined using the aluminum chloride (AlCl3) colorimetric assay according to Zilić et al.  

Briefly, 100 μL of the extract was mixed with 300 μL of 5% sodium nitrite (NaNO2). After 6 min, 300 μL of a 10% AlCl3 solution was added and the volume was adjusted to 2.5 mL using distilled water. After 7 min, 1.5 mL of 1 M sodium hydroxide (NaOH) was added and the mix was centrifuged for 10 min at 5000g. At 510 nm, the absorbance of the the supernatant was measured against solvent blank. The total flavonoid content was calibrated by catechin and expressed as milligram of catechin equivalent (mg CE) per gram of sample. A further dilution occurred where the measured absorption value was over the linear range of the standard curve.

**Biological Testing. Animals.** Male albino mice weighing around 30 ± 5 g (42 mice) were obtained from the animal house of the Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. They were housed in plastic cages with stainless steel covers at the National Cancer Institute Animal House. All adopted procedures were approved by the Institutional Research Ethics Committee at the Faculty of Applied Medical Sciences, October 6 University, Egypt (No. 20200512).

**EXPERIMENTAL ANIMAL GROUPS**

The mice were divided into seven groups each containing six mice. Food was withdrawn 24 h and water 2 h before the beginning of experiment. The animals were classified to 7 groups as listed in Table 1.

**PLASMA LIPID PROFILE ESTIMATION**

On the 15th day, blood was collected from each animal into heparinized tubes, centrifuged, and plasma was obtained. Total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) using commercially available kits (Asan and Youngdong Pharmaceutical Co., Korea).

**PREPARATION OF BRAIN SAMPLES**

Cerebral dislocation was used to dissect mice, and brain tissues were quickly extracted. To prepare a 25% W/V homogenate, a portion of each tissue was weighed and homogenized with ice-cold saline using an MPW-309 glass homogenizer (Universal Lab. Aid, Poland). Four aliquots were taken from the homogenate. The first was deproteinized with ice-cold 12% trichloroacetic acid, and the resulting supernatant was used to calculate GSH levels following centrifugation at 1000g.

The supernatant from the second aliquot was centrifuged at 1000g and utilized to determine the levels of thiobarbituric acid-reactive substances (TBARS), acetylcholinesterase (AChE) activity, interleukine-6 (IL-6), transforming growth factor beta1 (TGF-β1), inducible nitric oxide synthase (iNOS), and total protein using immunooassay kits (Immunobiological Laboratories ELISA kit). The clear supernatant from the third aliquot of brain homogenate was centrifuged at 10 500g for 15 min at 4 °C in a cooling ultracentrifuge (Servall comilus T-880, Du Pont), and the superoxide dismutase (SOD) and glutathione reductase (GR) activities were determined. The Christiansen technique  

**Determination of brain γ-aminobutyric acid type A receptor alpha5 subunit (GABRA α5), glycogen synthase kinase-3β (GSK-3β), and signal-regulated kinase 1 (pERK1) genes.** 

mRNA Extraction and cDNA Synthesis, brains of tested mice were homogenized in TRIzol reagent (Gibco BRL) for each treatment group, and ribonucleic acid (RNA) was produced according to Chomczynski and Sacchi.  

A total of 2 μg of total RNA, 110 pmol of oligo dT12-18, 3 mM MgCl2, and 200 units of ImProm-II reverse transcriptase were used in a 20 μL reaction containing 2 g of total RNA, 110 pmol of oligo dT12-18, 3 mM MgCl2, and 200 units of ImProm-II reverse transcriptase (Promega, Madison, WI).

**Primer Design.** Primers were created using genomic and mRNA sequences from gene sequence databases such as the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The positions of each intron and exon within the gene sequences were determined using the mRNA sequence to design primers at exon–exon junctions to avoid false-positive results caused by amplification of potentially contaminating genomic DNA. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ensure that no nonspecific binding sites on the same gene or similar sequence sites in other species were present in the primers. The primer sequences and annealing temperatures are depicted in Table 2.

To determine the optimal annealing temperature for the γ-aminobutyric acid type A receptor alpha5 (GABRA α5), glycogen synthase kinase-3β (GSK-3β), and signal-regulated kinase 1 (pERK1) genes. A 25 μL PCR reaction was as follows: 12.5 ul of 2X Green Master Mix, which contains DNA polymerase. The reverse transcription was performed if the measured absorption value was over the linear distance of the standard curve.

**Table 1. Groups of Animals in the Present Study**

| Groups | treatment description |
|--------|-----------------------|
| I      | Normal Control A      |
| II     | Normal Control B      |
| III    | received 3 mL of distilled water orally, daily for 14 days |
| IV     | received SCO (3 mg/kg b.w. ip) + quercetin (QR) (25 mg/kg b.w.) orally, daily for 14 days |
| V      | received SCO (3 mg/kg b.w. ip) + catechin (CAE) (30 mg/kg b.w.) orally, daily for 7 days |
| VI     | received SCO (3 mg/kg b.w. ip) + biochaninA (BCA) (20 mg/kg b.w.) orally, daily for 14 days |
| VII    | received SCO (3 mg/kg b.w. ip) + (QR) (10 mg/kg b.w.) + CAE (10 mg/kg b.w.) + BCA (10 mg/kg b.w.) orally, daily for 14 days |
polymerase supplied in 2X Green reaction buffer (pH 8.5), 400 M dATP, 400 M dGTP, 400 M dCTP, 400 nM dTTP, and 3 mM MgCl₂; 400 nM 10 µM forward and reverse primers for each gene sequence, 200 ng of cDNA template, and an appropriate volume of nuclease free water was added to 0.2 ml nuclease free PCR tubes and centrifuged for 10 sec.

The thermal cycler was set up as follows: an initial denaturation step at 95 °C for 2 min, a subsequent denaturation step at 95 °C for 30 s, optimization of the annealing conditions by performing the gradient reaction starting approximately 5 °C below the calculated melting temperature of the primers and increasing the temperature in increments of 3 °C to the optimized temperature for 30 s, followed by 72 s of cooling. All PCR reaction preparation steps were carried out on ice.

### WESTERN BLOT ANALYSIS

In the radioimmunoprecipitation assay buffer (RIPA; 10 mM Tris-HCl (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and 0.1% protease inhibitor cocktail), 10% brain homogenate (w/v) was prepared. The protein concentration was determined after centrifugation of the supernatant. On a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel, 50 g of denatured protein mixed with a 2X sample loading buffer was determined after centrifugation of the supernatant. On a 12% SDS-PAGE in 10% gels and transferred to poly(vinylidene difluoride) (PVDF) membranes. The blots were then incubated with antibodies specific for protein-serine/threonine kinase (Akt) (Cell Signaling; dilution 1:1000) and p38-MAPK (Novus Biologicals; diluted 1:500) followed by the corresponding secondary antibodies and finally developed using chemiluminescent reagents (Thermo Scientific).

### MOLECULAR DOCKING

**In silico** molecular docking of the three isolated natural compounds, viz quercetin, catechin, and biochaninA, was performed against different biological targets using the MOE program (Molecular Operating Environment, 2008.10: http://www.chemcomp.com). The computational experiments were performed on a Windows 10 pro with an Intel R CoreTM i5-3210M CPU@4.00GHz processor and 12 GB RAM. The three-dimensional (3D) structures of selected proteins were downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) (Table 7) with a good resolution. The protein structures were prepared for the docking process, and the co-crystalline ligands were redocked into the active pocket for each enzyme to validate the docking protocol with an RMSD value less than 2.5 Å. The water molecules were kept in the active site in case it was involved in binding interaction of the co-crystalline ligands. For the proteins without co-crystalline ligands, the active site was identified using MOE/Site Finder, the first site in the panel was selected, and then dummy atoms were created for each α sphere in the active site and used as superposition targets for docking calculations. The two-dimensional (2D) structures of the isolated compounds were generated using ChemDraw Ultra 12.0 and then converted into 3D structures, and the energy was minimized using the MMFF94x force field (eps = r, cutoff until the root-mean-square (RMS) gradient of 0.1 kcal/molÅ was achieved). MOE-DOCK default was used to find a suitable binding position for the compounds inside the active site (Algorithm). The MOE program utilized the rigid/flexible (receptor/ligand) technique with five energy maps, including H-bond interaction, electrostatic, two Van der Waal parameters, and hydrophobicity.

#### Statistical Analysis

The results were expressed as means ± SD. Comparisons between groups were performed using one-way analysis of variance (ANOVA). Differences between individual treatment groups were compared using Dunnett’s test. Statistical significance was set at P < 0.05 and 0.01, and the statistical analyses were performed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL).

#### RESULTS

**Chemistry. Compound A.** White powder, MP (186–187 °C), gives a single spot in S1 (Rf = 0.75). Electron ionization/mass spectroscopy (EI/MS) showed a molecular ion peak at m/z 426 calculated for the molecular formula C₂₉H₅₀O, in addition to peaks at m/z 411 (M+⁴H₂O) and 409 (M+⁴H₂O). Based on spectral data, m.p., and in comparison with reference compounds, it was identified as α-amyrin.²⁵

**Compound B.** Yellowish powder, MP (140–142), gives a single spot in S2 with Rf = 0.45. The mass spectrum showed the molecular ion peak at m/z 414 calculated for the molecular formula C₃₀H₅₁O₂, in addition to 399 (M+⁴H₂O), 397 (M+⁴H₂O), and 395 (M+⁴H₂O-2H). Based on the spectral data, m.p., and in comparison with reference compounds, it was identified as β-sitosterol.²⁶

**Compound C.** White powder, MP (215–216, EI/MS (M+)) at m/z 285 corresponding to the molecular formula C₁₅H₂₀O₅.¹ The ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 3.74 (3H, s, OCH₃), 6.40 (1H, d, J = 2.0 Hz, H-6), 6.22 (1H, d, J = 2.0 Hz, H-8), 6.82 (2H, d, J = 8 Hz, 3′, 5′), 7.85 (2H,d, J = 8 Hz, H-2′, H-6′), 8.29 (1H, s, H-2).

**13C NMR (DMSO-d₆, 100 MHz) δ**: 55 (OCH₃), 94.0 (C-8), 98.6 (C-6), 104.4 (C-10), 113.5 (C-3′, 5′), 118.3 (C-1), 122 (C-3), 130.3 (C-2′, 6′), 153.4 (C-2), 157.8 (C-9), 161.4 (C-5), 164.1 (C-7), 171.2 (C-4). It was identified as α-amyrin.²⁸

**Compound D.** Yellow powder, MP (315–316 °C). EI-MS m/z 302 corresponding to molecular formula C₁₃H₁₆O₃.¹ The ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 9.59 (1H, s, OH-3), 12.49 (1H, s, OH-5), 6.19 (1H, d, J = 2.0 Hz, H-6), 10.78 (1H, s, OH-7), 6.41 (1H, d, J = 2.0 Hz, H-8), 7.24 (1H, d, J = 2.0 Hz, H-2′), 9.33 (1H, s, OH-3′, 4′), 6.88 (1H, d, J = 8.5 Hz, H-5′), 7.55 (1H, dd, J = 2.0, 8.0 Hz, H-6′).

**13C NMR (DMSO-d₆, 100 MHz) δ**: 147.2 (C-2), 136.1 (C-3), 176.3 (C-4), 161.1 (C-5), 98.6 (C-6), 164.3 (C-7), 93.8 (C-8), 156.5 (C-9), 103.4 (C-10), 122.4 (C-1′), 115.4 (C-2′),

## Table 2. Primer Sequences of Studied Genes

| official name       | forward primer | reverse primer      |
|---------------------|----------------|---------------------|
| β actin             | 5′ CGTGGCGTGACATAAACGAGA 3′   | 5′ CGCTCATGCGAGTATTAC 3′   |
| GABBRα α5           | 5′ AGTGGAGCCGAAACAGTT 3′   | 5′ AAGGGCGGTTGGCTGAT 3′   |
| GSK-3               | 5′ TCAAGGCTCTCCCCATAGA 3′   | 5′ GTGAAAGGGATGAAATGG 3′   |
| signal-regulated kinase (pERK 1) | 5′ TGGGTTCTCTGCCGAATGAT 3′ | 5′ TGGCCAGGTTGCTGCTGTC 3′   |

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145.2 (C-3′), 147.2 (C-4′), 116 (C-5′), 120.4 (C-6′). It was identified as quercetin; NMR data were consistent with those reported.29

**Compound E.** Buff powder, MP (174–176), EI/MS m/z 291 calculated for the molecular formula C_{15}H_{14}O_{6}. 1H NMR (DMSO-d_{6}, 400 MHz): δ_{H} 6.97, 7.56, 7.01 (1H, s, OH−3), 6.92 (1H, d, J = 2 Hz, H-2′), 6.71 (1H, dd, J = 2, 8 Hz, H-6′), 4.67 (1H, d, J = 8 Hz, OH−3), 6.60 (1H, d, J = 9 Hz, H−5′), 5.90 (1H, d, J = 2 Hz, H-8), 5.97 (1H, d, J = 2.5 Hz, H-5), 4.80 (1H, d, J = 8 Hz, H-2), 3.88 (1H, m, H-3), 2.43 (1H, dd, J = 9, 16 Hz, H-4). 13C NMR (DMSO-d_{6}, 100 MHz): δ_{C} 82.7 (C-2), 66.6 (C-3), 28 (C-4), 166.7 (C-5), 96.2 (C-6), 145.7 (C-4′), 120.4 (C-5′), 115.3 (C-2′), 145 (C-3′), 145.7 (C-4′), 115.7 (C-5′), 119.9 (C-6′). It was identified as catechin.30

**TOTAL FLAVONOID AND PHENOIC CONTENT**

The antioxidant effect of the MeOH plant extract is often measured by the determination of the polyphenolic contents present in it. Their importance comes from the fact that these compounds can inhibit lipid peroxidation by their reaction with active oxygen radicals. In our study, RP-HPLC determinations of polyphenolics of MeOH leaf extract allowed the identification of 10 components, with the prevalence of catechin (308.31 µg/g) and quercetin (273.69 µg/g); the results are shown in Table 3.

| Identified Compounds     | Conc. (µg/g) |
|--------------------------|-------------|
| gallic acid              | 214.75      |
| protocatechuic           | ND          |
| ρ-hydroxybenzoic         | ND          |
| gentisic acid            | ND          |
| catechin                 | 308.31      |
| chlorogenic acid         | ND          |
| caffeic acid             | ND          |
| syringic acid            | 48.27       |
| vanillic acid            | 31.68       |
| ferulic acid             | 236.86      |
| sinapic acid             | 301.84      |
| ρ-coumaric acid          | 31.87       |
| rutin                    | ND          |
| apigenin-7-glucoside     | 174.37      |
| rosmarinic acid          | ND          |
| cinnamic acid            | 69.02       |
| quercetin                | 273.69      |
| apigenin                 | ND          |
| kaempferol               | ND          |
| chrysin                  | ND          |

Note: ND, not detected.

Spectrophotometric determination of the total flavonoid of MeOH leaf extract was 78.15 (mg CE/g) and that of total phenolics was 181.66 (mg GAE/g).

**Biological Studies.** Scopolamine (3mg/kg.b.w.) significantly elevated the plasma total cholesterol (TC) and triglycerides (TGs) as well as depletion of cholesterol-high density lipoprotein (HDL-C) and brain phospholipids (PLs) when compared with normal mice (P < 0.05). Also, QUR, CAE, and BCA ameliorate plasma TC, TG, HDL-C, and PL as compared to the scopolamine control groups of mice (P < 0.05). Administration of QUR, CAE, and BCA combination scopolamine-treated mice showed considerably reduced TC and TG as well as increased HDL-C levels when compared to scopolamine-treated mice (Table 4).

On the other hand, scopolamine injection significantly elevated AChE activity, IL-6, TGF-β1, and iNOS in brain tissues when compared with the negative control group of mice (P < 0.05). Treatment with QUR, CAE, and BCA significantly lowered AChE, IL-6, TGF-β1, and iNOS as compared with the scopolamine control groups of mice (P < 0.05) (Table 5).

We can notice that Table 6 shows that the brain TBARS level was significantly higher and glutathione (GSH), superoxide dismutase (SOD), and glutathione reductase (GR) were significantly lower for the scopolamine control groups of mice when compared to the negative control group of mice (P < 0.05). Also, in QUR, CAE, and BCA combination-fed mice, TBARS accumulation was significantly suppressed and brain GSH, SOD, and GR levels were markedly increased when compared to the scopolamine control groups of mice (P < 0.05).

**Changes in Brain GABA_{A} α5, GSK-3β, and pERK1 mRNA Gene Expression.** GABA_{A} α5 and GSK-3β mRNA levels were significantly upregulated, approximately 5.5- and 2.5-fold, respectively, in brain tissues of the scopolamine group when compared with the negative control (P < 0.01). Also, pERK1 mRNA gene expression was significantly downregulated, approximately 0.5-fold, in brain tissues of the scopolamine group when compared with the negative control group of mice (P < 0.01). These data suggested that delivering these compounds in combination may improve their bioavailability compared to supplementation as a single compound.

This up- and downregulation of these genes was ameliorated by QUR, CAE, and BCA when administered to scopolamine-treated mice in both single or in combination formula when compared with the positive control group of mice (Figures 1–3).

**Changes in Brain Protein Levels of Akt and p38α MAP-kinase.** Western blot analysis clearly showed that scopolamine decreased Akt phosphorylation and increased p38α MAP-kinase levels when compared with the negative control (P < 0.05). QUR, CAE, and BCA when administered to scopolamine-treated mice in either single or combination formula significantly reversed the scopolamine-suppressed phosphorylation of Akt as well as recovered the levels of brain p38α MAP-kinase when compared with the positive control group of mice (P < 0.05) (Figures 4 and 5).

**MOLECULAR DOCKING**

The docking technique was conducted to clarify the binding mode of QUR, CAE, and BCA with different biological targets utilizing the MOE program. Table 7 represents the validation result of the docking protocol. All of the redocking ligands showed a similar conformation with the native redocking results of the docking protocol. All of the redocking ligands showed a similar conformation with the native redocking ligands, and the RMSD values were ≤2.0 Å. Tables 7 and 8 summarize the docking study results, presented as binding energy and binding interaction. In general, the presence of hydroxyl and carbonyl groups of the natural isolated compounds played an important role in the ligand-target interaction through hydrogen-bond formation besides hydro-
Table 4. Levels of Plasma Cholesterol, Triglycerides, and Cholesterol-High-Density Lipoprotein (HDL) as well as Brain Phospholipids in Normal and Experimental Groups of Miceα

| groups | treatment description | cholesterol (mg/dL) | triglycerides (mg/dL) | HDL (mg/dL) | phospholipids (mg/g tissue) |
|--------|-----------------------|---------------------|-----------------------|-------------|-----------------------------|
| I      | normal control A (distilled-water-treated) 3 mL/kg | 185.49 ± 6.95a | 80.44 ± 5.73a | 29.30 ± 3.70a | 5.81 ± 0.17a |
| II     | normal control B (Twee 80-treated) 3 mL/kg | 184.98 ± 4.38b | 81.86 ± 6.43b | 30.00 ± 3.62b | 6.04 ± 0.40b |
| III    | scopolanine hydrobromide (SCO) (3 mg/kg b.w.) | 231.43 ± 5.10b | 129.40 ± 7.83b | 14.50 ± 1.36b | 3.54 ± 0.26b |
| IV     | SCO (3 mg/kg b.w.) + quercetin (QUR) (25 mg/kg b.w.) | 195.54 ± 4.89b | 89.44 ± 5.10b | 24.87 ± 2.76b | 4.79 ± 0.53b |
| V      | SCO (3 mg/kg b.w.) + catechin (CAE) (30 mg/kg b.w.) | 204.36 ± 5.66b | 97.85 ± 4.77b | 22.47 ± 3.67b | 4.50 ± 0.44b |
| VI     | SCO (3 mg/kg b.w.) + biochaninA (BCA) (20 mg/kg b.w.) | 206.44 ± 4.37b | 104.80 ± 6.09b | 20.75 ± 1.44b | 4.70 ± 0.35b |
| VII    | SCO (3 mg/kg b.w.) + (QUR) (10 mg/kg b.w.) + CAE (10 mg/kg b.w.) + BCA (10 mg/kg b.w.) | 187.60 ± 3.65b | 83.90 ± 5.87b | 28.70 ± 3.08b | 5.89 ± 0.64b |

Values represent the mean ± SE (n = 6). Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at P ≤ 0.05.

Table 5. Levels of Brain Acetylcholinesterase (AChE) Activity, Interleukine-6 (IL-6), Transforming Growth Factor Beta1 (TGF-β1), and Inducible Nitric Oxide Synthase (iNOS) in Normal and Experimental Groups of Miceα

| groups | treatment description | AChE (U/mg protein) | IL-6 (pg/mg protein) | TGF-β1 (pg/mg protein) | iNOS (mg/g protein) |
|--------|-----------------------|---------------------|----------------------|------------------------|---------------------|
| I      | normal control A (distilled-water-treated) 3 mL/kg | 0.65 ± 0.040a | 10.76 ± 0.54a | 37.65 ± 3.80a | 18.54 ± 1.96a |
| II     | normal control B (Twee 80-treated) 3 mL/kg | 0.62 ± 0.03a | 10.50 ± 1.04a | 38.15 ± 3.69a | 17.66 ± 2.18a |
| III    | scopolanine hydrobromide (SCO) (3 mg/kg b.w.) | 2.65 ± 0.24b | 86.07 ± 4.20b | 14.90 ± 1.77b | 113.19 ± 5.63b |
| IV     | SCO (3 mg/kg b.w.) + quercetin (QUR) (25 mg/kg b.w.) | 1.10 ± 0.08b | 35.66 ± 1.74b | 28.76 ± 2.17b | 46.50 ± 4.31b |
| V      | SCO (3 mg/kg b.w.) + catechin (CAE) (30 mg/kg b.w.) | 1.57 ± 0.05c | 21.80 ± 2.87c | 21.66 ± 3.09c | 57.69 ± 5.40c |
| VI     | SCO (3 mg/kg b.w.) + biochaninA (BCA) (20 mg/kg b.w.) | 1.65 ± 0.21d | 39.90 ± 3.05d | 24.32 ± 1.86d | 41.67 ± 4.50d |
| VII    | SCO (3 mg/kg b.w.) + (QUR) (10 mg/kg b.w.) + CAE (10 mg/kg b.w.) + BCA (10 mg/kg b.w.) | 0.73 ± 0.05d | 16.17 ± 0.83d | 34.08 ± 2.15d | 28.70 ± 3.52d |

Values represent the mean ± SE (n = 6). Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at P ≤ 0.05.

Table 6. Levels of Brain Reduced Glutathione (GSH), Superoxide Dismutase (SOD), Glutathione Reductase (GR), and Thiobarbituric Acid-Reactive Substances (TBARs) in Normal and Experimental Groups of Miceα

| groups | treatment description | GSH (mg/g tissues) | SOD (U/g protein) | GR (nmol H₂O₂/consume mg protein) | TBARs (nmole/mg protein) |
|--------|-----------------------|--------------------|-------------------|-----------------------------------|------------------------|
| I      | normal control A (distilled-water-treated) 3 mL/kg | 13.26 ± 1.05b | 176.40 ± 12.36b | 83.40 ± 4.50b | 9.76 ± 6.66b |
| II     | normal control B (Twee 80-treated) 3 mL/kg | 11.80 ± 0.94c | 174.82 ± 7.54c | 85.13 ± 6.07c | 10.35 ± 0.53c |
| III    | scopolanine hydrobromide (SCO) (3 mg/kg b.w.) | 6.49 ± 0.37b | 69.80 ± 5.01b | 21.60 ± 2.14b | 42.75 ± 3.06 |
| IV     | SCO (3 mg/kg b.w.) + quercetin (QUR) (25 mg/kg b.w.) | 10.86 ± 0.72b | 154.08 ± 8.63b | 73.70 ± 5.06b | 15.33 ± 2.60b |
| V      | SCO (3 mg/kg b.w.) + catechin (CAE) (30 mg/kg b.w.) | 9.90 ± 0.67b | 126.80 ± 10.77b | 65.90 ± 4.97b | 23.05 ± 3.16b |
| VI     | SCO (3 mg/kg b.w.) + biochaninA (BCA) (20 mg/kg b.w.) | 10.90 ± 0.45b | 134.05 ± 11.04b | 70.44 ± 5.22b | 18.64 ± 2.54b |
| VII    | SCO (3 mg/kg b.w.) + (QUR) (10 mg/kg b.w.) + CAE (10 mg/kg b.w.) + BCA (10 mg/kg b.w.) | 12.54 ± 1.11b | 163.37 ± 7.62b | 80.15 ± 4.30b | 11.40 ± 1.06b |

Values represent the mean ± SE (n = 6). Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at P ≤ 0.05.

**Interaction with Anti-Inflammatory Targets.** Inflammatory markers such as IL-6, TGF-β1, and inducible nitric oxide synthase (iNOS) are widely known as major targets in discovering natural anti-inflammatory drugs for the treatment of inflammation-related diseases.31

The result indicated that catechin showed a better docking score of −17.11 kcal/mol in the interaction with IL-6 (PDB ID: 1ALU) than the native ligand (L(+)-tartaric acid) of −14.83 kcal/mol, while both quercetin and biochaninA showed binding energies of −14.20 and −14.51 kcal/mol, respectively, closely similar to L(+)-tartaric acid (Table 7). Additionally, the mentioned isolated compounds exhibited good fitting inside the active site of IL-6 through formation of multiple H-bonds with the amino acid residues ARG 30,
GLN 175, ARG 179, ARG 182, and ASP 34 (Table 8, Figure 7).

However, in the interaction with TGF-β, quercetin exhibited the lowest binding energy of $-28.33$ kcal/mol compared to catechin and biochaninA, with values of 22.74 and $-20.89$ kcal/mol, respectively, comparable to the native ligand of the docking score of $-30.13$ kcal/mol (Table 7). Both quercetin and catechin revealed good binding affinity with the active site via formation of four H-bonds with ILE 211, SER 280, LYS 232, LYS 213, and HIS 283 residues (Table 8, Figure 8). However, biochaninA binds to the target via two H-bonds with HIS 283 and LYS 232 (Table 8, Figure 8).

In the case of iNOS, biochaninA revealed a better binding energy of $-20.53$ kcal/mol compared to quercetin, catechin, and ethylisothiourea (native ligand, ITU) with values of $-15.04$, $-11.19$, and $-9.30$ kcal/mol, respectively (Table 7). The quercetin–protein complex was stabilized by three H-bond donors with the amino acid residue Glu 377, TRP 372, and ASN 370 and two $\pi-\pi$ interactions with TRP 149 and PHE 369 (Table 8, Figure 9). The catechin–protein complex was stabilized by one H-bonding with Glu 377 and two $\pi-\pi$ interactions with TRP 149 and PHE 369. However, biochaninA formed a $\pi-\pi$ interaction with TRP 149 (Table 8, Figure 9).

Interaction with Antioxidant Targets. Various diseases are strongly associated with oxidative stress and the metabolism of free radicals. Antioxidation systems, such as SOD and GR, have great effects on organisms, which are responsible for scavenging free radicals and protecting the cell membrane structure and function.32

The crystal structure of the SOD receptor (PDB ID: 1CBJ) was used for the docking study. The result revealed that quercetin and catechin showed potent activation effect with binding energies of $-17.48$ and $-15.65$ kcal/mol and an excellent interaction model via formation of H-bonds between hydroxy groups and VAL 7, ASP11, CYS 144, VAL 146, and LYS 9 residues (Tables 7 and 8 and Figure 10). BiochaninA revealed a docking energy of $-8.60$ kcal/mol and binds to the active pocket of SOD via two H-bonds with ASN 51 and LYS 9 residues and the $\pi-$cation interaction between the p-methoxy...
phenyl ring and the LYS 9 residue (Tables 7 and 8 and Figure 10).

Moreover, docking of the natural compounds inside the active pocket of the glutathione reductase receptor (PDB ID: 1xan) indicated that quercetin showed the lowest binding energy of $-18.18$ kcal/mol compared to catechin, biochaninA, and 3,6-dihydroxy-xanthene-9-propionic acid (native ligand, HXP) with values of $-12.55$, $-10.75$, and $-17.12$ kcal/mol, respectively (Table 8). The quercetin–protein complex was stabilized with two H-bonds between the hydroxyl group with the TYR 407 residue and hydrophobic interaction between the phenyl ring and HIS82 (Table 8, Figure 11). While catechin formed a H-bond with the HIS82 residue in a similar manner to the native ligand, biochaninA formed a $\pi$–cation interaction with HIS 75 (Table 8, Figure 11).

**Interaction with the Beta3-Alpha5 GABAA Receptor.**

The structure of the beta3-alpha5 GABAA complex with P9N, pregnanolone (PDB ID: 5O8F), was used for this study. Quercetin, catechin, and biochaninA revealed favorable binding energies of $-16.86$, $-16.70$, and $-18.11$ kcal/mol, respectively, in comparison to pregnanolone with a value of $-21.40$ kcal/mol (Table 7). The mode of interaction of quercetin and biochaninA with the binding site of the beta3-alpha5 GABAA receptor was similar to that of the native ligand, which formed a H-bond acceptor with the GLN 245 residue besides a $\pi$–$\pi$ interaction with the TRP 249 residue (Table 8, Figure 12). However, catechin linked to the target active site by $\pi$–$\pi$ interactions between the coumarine moiety and Trp 249 (Table 8, Figure 12).

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**Figure 3.** Level of brain signal-regulated kinase 1 (pERK1) gene expression in normal and experimental groups of mice. Representative bar diagram of three independent experiments is presented.

**Figure 4.** Level of brain Akt in normal and experimental groups of mice. Representative bar diagram of three independent experiments is presented.
Interaction with Glycogen Synthase Kinase-3β (GSK-3β). The docking results revealed that quercetin, catechin, and biochanin A showed docking energies of $-21.07$, $-20.26$, and $-19.37$ kcal/mol, respectively, compared to the native ligand with a value of $-21.98$ kcal/mol, in addition to excellent fitting inside the active site of GSK-3β via formation of H-bonds between the hydroxyl group and VAL 135, ASP 200, ILE 62, PRO 136, and LYS 85 residues (Tables 7 and 8 and Figure 13).

Interaction with Signal-Regulated Kinase (pERK). The docking results indicated that biochanin A and quercetin revealed better docking scores of $-19.49$ and $-19.82$ kcal/mol, respectively, compared to the native ligand with a value of $-26.40$ kcal/mol (Table 7). However, both quercetin and catechin exhibited good fitting inside the active site of pERK than biochanin A through formation of multiple H-bonds with different amino acid residues LEU598, ASP 954, CYS 890, ARG 891, and GLN 888 and $\pi-\pi$ interaction with PHE 943 (Table 8, Figure 14).

Interaction with Akt. The docking results indicated that both quercetin and catechin exhibited similar docking scores of $-19.49$ and $-19.82$ kcal/mol, respectively, which are better than biochanin A with a value of $-15.53$ kcal/mol (Table 7). Also, quercetin and catechin revealed good fitting inside the active site of AKt-1 through formation of multiple H-bonds with SER 7, GLU 234, ARG 4, and LYS 179, better than the native ligand (3MVH) and biochanin A (Table 8, Figure 15). However, biochanin A binds to the target via the H-bond donor with TYR 437 and the $\pi-\pi$-cation interaction between $p$-methoxy phenyl and the ARG 4 residue (Table 8, Figure 15).

Interaction with p38α MAP-Kinase. The docking study was carried out using the protein crystal structure of p38α MAP-kinase in complex with SB0 (trans-4-[4-(4-fluorophenyl)-5-(2-methoxy pyrimidin-4-yl)-1H-imidazol-1-yl]-cyclohexanol) (PDB ID: 4FA2). The docking result indicated that QUR, CAE, and BCA revealed better docking scores of $-23.18$, $-19.45$, and $-20.71$ kcal/mol, respectively, in comparison to the native ligand with a value of $-30.35$ kcal/mol (Table 7). Quercetin and catechin showed good fitting inside the active site through the H-bond interaction with PHE 169, GLY 170, ASP 112, SER 154, PHE 169, and LYS 53, while biochanin A formed a H-bond with the PHE 169 residue (Table 8, Figure 16).

Pharmacokinetic Properties. The pharmacokinetic properties of natural compounds that can be used as drug candidates were assessed using Lipinski’s rule. The molecular weight should be approximately $500$ Da, $\text{LogP}$ (octanol–water division coefficient) should be $5$, and there should be no more than five donor sites and no more than ten acceptor sites of the hydrogen bond. The coefficients are four parameters.

Also, topological polar surface area (TPSA) is used in medicinal chemistry for the optimization of a drug’s ability to penetrate cells. In accordance with Veber’s rule for good oral bioavailability, TPSA values must be $\leq 140$ Å. The drug-likeness parameters were calculated using the MOE program (Table 9). Moreover, the percentage of absorption (ABS) of the three isolated compounds was calculated using the formula of Zhao et al.34

\[
\% \text{ABS} = 109 - (0.3345 \times \text{TPSA})
\]

The result indicated that the three isolated compounds had molecular weights in the range of $284.26–302.23$, with log $P$ of $1.97–2.17$, four to six H-bond donors, and two to six H-bond acceptors and hence follow Lipinski’s rule of five (Table 9). Moreover, the three compounds had TPSA less than $140$ Å and good absorption percentage ranging from $66.36$ to $83.58$ (Table 9).

**DISCUSSION**

Recently, the need for herbal medicine has increased and is considered as a dietary supplement for the maintenance of health and prevention of disease; in Africa, up to 90% of the population depends on traditional herbal medicine for their primary health care.

Five compounds have been isolated from CHCl3 and EtOAc leaf extract; they have been identified by spectral data as α-
Table 7. Docking Score of Cocrystalline Ligands, Quercetin, Catechin, and BiochaninA

| molecular target (ref)       | PDB ID (resolution) | cocrystalline ligand name       | docking score                                      |
|-----------------------------|---------------------|--------------------------------|----------------------------------------------------|
| AChE                         | IACL (2.45)         | DME (decamethonium ion)        | cocrystalline ligand quercetin catechin biochaninA |
|                             |                     |                                | -21.74 (RMSD 1.25) −23.41 −19.84 −21.93           |
| IL-6                         | 1ALU (1.90 Å)       | TLA (l(+)-tartaric acid)       |                                                   |
|                             |                     |                                | -14.83 (RMSD 0.80) −14.20 −17.11 −14.51           |
| TGF-β                       | 5QIK (1.58 Å)       | J2M (N-{4-[3-(6-fluoropyridin-3-yl)-4-oxo-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c] pyridin-2-yl] pyridin-2-yl} acetamide) |                                                   |
|                             |                     |                                | -30.13 (RMSD 0.43) -28.33 -22.74 -20.89           |
| Inos                        | 4NOS(1.51 Å)        | ITU (ethylisothiourea)         |                                                   |
|                             |                     |                                | -9.30 (RMSD 1.51) −15.04 −11.19 −20.53           |
| SOD                         | 1CBJ (1.65 Å)       | HXP 3,6-dihydroxy-xanthene-9-propionic acid |                                                   |
|                             |                     |                                | -17.12 (RMSD 1.57) −18.18 −12.55 −10.75          |
| glutathione reductase        | 1XAN (2.00 Å)       | P9N (pregnanolone)             |                                                   |
|                             |                     |                                | -21.40 (RMSD 0.61) −16.86 −16.70 −18.11          |
| beta3-alpha5 GABAA receptor  | 5O8F (3.20 Å)       |                                |                                                   |
| glycogen synthase kinase-3β | 1QSK (1.94 Å)       | TMU (N-(4-methoxybenzyl)-n’-(5-nitro-1,3-thiazol-2-yl)urea) |                                                   |
| (GSK-3β)                    |                     |                                | -21.98 (RMSD 0.85) −21.07 −20.26 −19.37          |
| signal-regulated kinase 1/2 | 4G31 (2.28 Å)       | OWH (1-[[5-(4-amino-7-methyl-1H-pyrrolo[2,3-d]pyrimidin-5-yl]-2,3-dihydro-1H-indol-1-yl]-2-[3-(trifluoromethyl) phenyl]ethanone) |                                                   |
| (pERK)                      |                     |                                | -26.40 (RMSD 0.63) −21.92 −16.40 −24.66          |
| AKT-1                       | 3MVH (2.01 Å)       | WPE (N’-[[35S]-3-amino-1-(5-ethyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl]pyrrolidin-3-yl)methyl]-2,4-difluorobenamide) |                                                   |
|                             |                     |                                | -28.99 (RMSD 1.42) −19.49 −19.82 −15.53          |
| p38α MAP-kinase             | 4FA2 (2.00 Å)       | SB0 (trans-4-[4-(4-fluorophenyl)-5-(2-methoxypyrimidin-4-yl)-1H-imidazol-1-yl]cyclohexanol) |                                                   |
|                             |                     |                                | -30.35 (RMSD 0.62) −23.18 −19.45 −20.71          |
Table 8. Binding Interaction of the Cocrystalline Ligands, Quercetin, Catechin, and BiochaninA, Indicating the Type of Interaction, Compound Atoms-Amino Acid Involved in the Interaction and Distance of Hydrogen Bonds (Å)

| compounds | enzyme ID | cocrystalline ligand | quercetin | catechin | biochaninA |
|-----------|-----------|----------------------|-----------|----------|------------|
| 1ACL      | arene−cation [NH4−TRP 84, TRP 279] | H-don [OH−TYR 130, 2.74 Å]; H-don [OH−GLU 199, 1.37 Å]; H-acc [OH−TYR 130, 2.74 Å]; arene−arene [phenyl, coumarine moiety−TRP 84, PHE 330] | H-don [OH−GLU 199, 1.43 Å]; H-acc [OH−HIS640 2.86 Å]; arene−arene [phenyl, coumarine moiety−TRP 84, TRP 334] | H-don [OH−GLU 199, 1.36 Å]; H-don [OH−GLU 199, 1.43 Å]; H-acc [OH−HIS440 2.86 Å]; H-acc [OH−ARG 198, 2.55 Å]; arene−arene [phenyl, coumarine moiety−HIS640] |
| 1ALU      | H-don [OH−GLN 175, 1.95 Å]; H-acc [CO−ARG 179, 3.04 Å]; H-acc [O−ARG 179, 2.77 Å]; H-acc [OH−ARG 183, 2.79 Å]; H-acc [O−ARG 182, 2.74 Å] | H-acx [O−ARG 30, 2.90 Å]; H-acc [O−ARG 30, 2.62 Å]; H-acx [O−GLN 175, 2.76 Å]; H-acx [O−ARG 179, 3.10 Å]; H-acc [O−ARG 179, 2.86 Å] | H-acx [O−ARG 30, 2.85 Å]; H-acx [O−GLN 175, 2.73 Å]; H-acx [O−ARG 182, 2.87 Å]; H-acc [O−ARG 182, 2.55 Å] | H-don [H−ASP 34, 1.24 Å]; H-acc [O−ARG 179, 3.02 Å]; H-acc [O−ARG 179, 3.04 Å] |
| 3QIK      | H-don [NH−HIS 283, 2.05 Å]; H-don [NH−ASP 351, 2.03 Å]; H-acc [CO−LYS 232, 2.72 Å]; H-acc [N−HIS 283, 3.01 Å] | H-don [H−ILE 211, 1.63 Å]; H-don [O−SER 280, 2.55 Å]; H-acc [O−LYS 232, 3.00 Å]; H-acc [O−SER 280, 2.55 Å] | H-don [H−LYS 213, 1.55 Å]; H-don [O−SER 280, 2.47 Å]; H-acc [O−LYS 232, 3.04 Å]; H-acc [O−SER 280, 2.47 Å] | H-don [H−HIS 283, 1.49 Å]; H-acc [O−LYS 232, 3.10 Å] |
| 4NOS      | H-don [NH−GLU 377, 2.12 Å]; H-don [NH−GLU 377, 2.21 Å]; H-don [H−GLU 377, 1.82 Å]; H-don [NH−TRP 372, 2.21 Å] | H-don [OH−GLU 377, 1.56 Å]; H-don [O−TRP 372, 1.43 Å]; H-don [OH−ASN 370, 1.67 Å]; arene−arene [phenyl ring−TRP 149 and PHE 369] | H-don [OH−GLU 377, 1.56 Å]; H-don [O−TRP 372, 1.43 Å]; H-don [OH−ASN 370, 1.67 Å]; arene−arene [phenyl ring−TRP 149 and PHE 369] | arene−arene [phenyl ring of the coumarine moiety−Trp 149] |
| 1CBJ      | H-don [OH−VAL 7, 3.66 Å]; H-don [OH−ASP 11, 1.47 Å]; H-don [OH−CYS 144, 1.56 Å]; H-acc [O−VAL 146, 2.88 Å] | H-don [OH−VAL 7, 3.73 Å]; H-don [OH−VAL 7, 2.07 Å]; H-acc [O−LYS 9, 2.49 Å]; H-acc [O−LYS 9, 2.49 Å]; H-acc [O−VAL 146, 2.83 Å] | H-don [OH−VAL 7, 3.73 Å]; H-don [OH−VAL 7, 2.07 Å]; H-acc [O−LYS 9, 2.49 Å]; H-acc [O−LYS 9, 2.49 Å]; H-acc [O−VAL 146, 2.83 Å] | arene−cation [p−methoxy phenyl ring−LYS 9] |
| 1XAN      | H-acx [O−HIS 75, 2.32 Å]; H-acc [O−HIS82, 2.42 Å] | H-don [OH−TYR 407, 2.78 Å]; H-acx [OH−TYR 407, 2.78 Å]; arene−cation [phenyl ring−HIS82] | H-don [OH−TYR 407, 2.78 Å]; H-acx [OH−TYR 407, 2.78 Å]; arene−cation [phenyl ring−HIS82] | arene−arene [phenol of the coumarine moiety−Trp 249] |
| 3O8F      | H-acc [OH−GLN 245, 2.67 Å] | H-acc [OH−GLN 245, 2.94 Å]; arene−arene [coumarine moiety−TRP 249] | H-acc [OH−GLN 245, 2.94 Å]; arene−arene [coumarine moiety−TRP 249] | arene−arene [phenol of the coumarine moiety−Trp 249] |
| 1QSK      | H-don [NH−VAL 135, 1.59 Å]; H-don [NH−ASP 201, 1.39 Å]; H-acc [N−VAL 135, 2.99 Å]; arene−cation [p−methoxy phenyl−ARG 141] | H-don [OH−VAL 135, 1.54 Å]; H-don [OH−ASP 201, 1.39 Å] | H-don [OH−VAL 135, 1.54 Å]; H-don [OH−ASP 201, 1.39 Å] | H-don [OH−VAL 135, 1.47 Å]; H-don [OH−PRO 136, 1.69 Å]; H-acc [O−LYS 85, 3.30 Å] |
| 4G31      | H-don [NH−GLU 888, 1.89 Å]; H-acc [N−CYS 890, 2.79 Å]; arene−arene [pyrano pyrazole−PHE 943] | H-don [OH−LEUS98, 1.80 Å]; H-don [OH−ASP 954, 1.34 Å]; H-acc [CO−LYS 890, 2.57 Å]; arene−arene [coumarine moiety−PHE 943] | H-don [OH−LEUS98, 1.47 Å]; H-don [OH−GLU 888, 2.58 Å]; H-acc [OH−ARG 893, 2.01 Å]; H-acx [OH−CYS 890, 2.87 Å] | H-don [OH−ARG 891, 1.81 Å]; arene−arene [coumarine moiety−PHE 943] |
| 3MVH      | H-don [NH−GLU 228, 2.07 Å]; H-don [NH−GLU 234, 1.56 Å]; H-don [NH−ASP 292, 2.33 Å]; H-acc [NH−ALA 230, 3.18 Å] | H-don [OH−GLU 234, 1.36 Å]; H-acc [OH−ARG 4, 2.79 Å]; two H-acc [OH−ARG 7, 2.66 Å] and 3.05 Å]; H-acc [OH−LYS 179, 2.87 Å] | H-don [OH−GLU 234, 1.36 Å]; H-acc [OH−SER 7, 2.82 Å]; two H-acc [OH−SER 7, 2.82 Å]; H-acc [OH−LYS 179, 2.81 Å] | H-don [OH−TYR 437, 1.68 Å]; arene−cation [p−methoxy phenyl−ARG 4] |
| 4FA2      | H-don [OH−ASP 112, 2.18 Å]; H-acc [N−MET 109, 3.00 Å] | H-acc [H−PHE 169, 1.50 Å]; H-acc [CO−GLY 170, 2.61 Å] | H-acc [H−PHE 169, 1.50 Å]; H-acc [CO−GLY 170, 2.61 Å] | H-don [OH−PHE 169, 1.48 Å] |
amyrin, β-sitosterol, biochaninA, quercetin, and catechin. Biological results revealed that three bioactive compounds isolated from the EtOAc extract of *P. roxburghii* inhibited AChE, GABA<sub>α</sub>5, GSK-3β, and p38α MAP-kinase activities and suggested that they have the potential to suppress the neurodegeneration effect of scopolamine.

**BIOLOGICAL STUDIES**

The druglike property predictions showed that the three isolated compounds followed Lipinski’s rule of five with TPSA less than 140 Å and a good absorption percentage (Table 9). Hyperlipidemia has been recognized as the leading cause of scopolamine injection in mice for 14 days.

Quercetin at a dose of 25 mg/kg reduced the plasma cholesterol and triacylglycerol levels in experimental hyperlipidemia scopolamine-treated mice, which was a more potent effect than that of catechin (30 mg/kg b.w.) and biochaninA (20 mg/kg b.w.). Also, delivering these compounds in combination ameliorates plasma HDL-cholesterol (HDL-C) levels and improved plasma phospholipid levels in experimental hyperlipidemia mice, which was comparable to that of scopolamine-treated mice. The present results were confirmed by reported data, which showed the neurodegeneration effect of scopolamine in animals and humans.

![Figure 6](image6.png)

**Figure 6.** Three-dimensional docked conformations of the native ligand DME (green), QUR (red), CAE (yellow), and BCA (blue) in the active site of the AChE receptor (PDB ID: 1ACL); H-bonds (purple lines).

![Figure 7](image7.png)

**Figure 7.** Three-dimensional docked conformations of the native ligand TLA (green), QUR (red), CAE (yellow), and BCA (blue) in the active site of the IL-6 receptor (PDB ID: 1ALU); H-bonds (purple lines).
Figure 8. Three-dimensional docked conformations of the native ligand J2M (green), QUR (red), CAE (yellow), and BCA (blue) in the active site of the TGF-β receptor (PDB ID: 5QIK); H-bonds (purple lines).

Figure 9. Three-dimensional docked conformations of the native ligand ITU (green), QUR (red), CAE (yellow), and BCA (blue) in the active site of the iNOS receptor (PDB ID: 4NOS); H-bonds (purple lines).
Figure 10. Three-dimensional docked conformations of QUR (red), CAE (yellow), and BCA (blue) in the active site of the SOD receptor (PDB ID: 1CBJ); H-bonds (purple lines).

Figure 11. Three-dimensional docked conformations of the native ligand HXP (green), QUR (red), CAE (yellow), and BCA (blue) in the active site of the GR receptor (PDB ID: 1XAN); H-bonds (purple lines).
Furthermore, hyperlipidemia is also closely related to atherosclerosis and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease. Also, quercetin, catechin, and biochaninA have a significant hypolipidemic effect. Phospholipids are structurally and biologically important molecules, which form cellular membranes and are involved in the behavior of membrane proteins, receptors, enzymes, and ion channels intracellularly or at the cell surface. Since the brain is one of the richest organs in lipid content, changes in the brain phospholipid levels could lead to different pathogenic processes.

Decreased brain phospholipid levels in scopolamine-treated mice were found when compared to the control group of mice via an acceleration of the neurodegeneration rate, leading to Alzheimer’s disease. In parallel, a decrease in brain phospholipids has been reported. The isolated compounds have been reported to possess neuroprotective effects.
Hyperactivity of AChE as well as dysfunction of inflammatory mediators (IL-6, TGF-β1, and iNOS) in the brain tissue was reported among aged people with cognitive impairments. Also, scopolamine caused severe deficits in cholinergic neurons and increased the activity of brain AChE, resulting in neurodegeneration elevation. Scopolamine injection also increased the IL-6 as well as decreased TGF-β1 levels, leading to neuroinflammation. Moreover, scopolamine administration can increase the iNOS through the activation of NF-κB. iNOS are major regulators in the progression of the proinflammatory signaling pathways, which further release proinflammatory mediators such as IL-1β, IL-2, IL-6, and TNF-α. Furthermore, pretreatment with quercetin, catechin, and biochaninA reduced AChE activity and cholinergic activity loss in brain tissues, as well as modulated cytokine production and proinflammatory enzyme expression.

To the best of our knowledge, the present study is the first report of the inhibition of AChE activity and levels of IL-6 and iNOS as well as elevation of TGF-β1 by P. roxburghii bioactive compounds (quercetin, catechin, and biochaninA) evaluated...
biochemically and computationally. We used an MOE program and different AChE, IL-6, iNOS, and TGF-β1 crystal structures. Our results found that quercetin, catechin, and biochanin A revealed docking scores with the AChE receptor (PDB ID: 1ACL) that were better than or closely similar to the native ligand DME and had excellent binding affinity with the AChE active site via formation of three to four hydrogen bonds with the amine acid TYR 130, GLU 199, HIS440, and SER 200 residues besides π−π and π−ion interaction with TRP 84, PHE 330, TYR 334, and HIS440, which helped in stabilization of the ligand–protein complex (Tables 7 and 8, Figure 6).

The model interaction of QUR, CAE, and BCA with the IL-6 binding site was similar to the model interaction of the native ligand, L(+)-tartaric acid, due to the formation of H-bonds with GLN 175, ARG 179, and ARG 182 residues, with additional H-bonds formed between hydroxyl groups and ARG 30 and ASP 34 residues (Table 8, Figure 7). Furthermore, QUR, CAE, and BCA had binding energies of −14.20, −17.11, and −14.51 kcal/mol, respectively, which were lower than or comparable to the native ligand’s binding energy of −14.83 kcal/mol (Table 7).

Furthermore, QUR, CAE, and BCA had higher binding energies than the native ligand (J2M) in their interactions with TGF1, and the protein–ligand complexes were stabilized by H-bonds established with the same amino acid residues (LYS 232 and HIS 283) as the original ligand, as well as H-bonds produced with other amino acid residues, such as ILE 211 and SER 280 (Tables 7 and 8, and Figure 8).

Quercetin, on the other hand, demonstrated excellent binding affinity to the active site of the iNOS receptor (PDB ID: 4NOS) in the same way as the native ligand (ITU), via H-bond donors formed between the hydroxyl group and Glu 377, TRP 372 in addition to H-bond donors with ASN 370, and hydrophobic interactions with the amino acids TRP 149 and PHE 369. The CAE–protein complex was stabilized by a H-bond donor with Glu 377 and two interactions between the phenyl ring and TRP and PHE 369 residues (Table 8, Figure 9). On the other hand, BCA revealed excellent binding energy compared to QUR, CAE, and the native ligand but showed a poor binding affinity to the target via formation of only one π−π interaction between the phenyl ring of the coumarin moiety and the Trp 149 residue (Table 8, Figure 9).

Furthermore, scopolamine generates reactive oxygen species (ROS) from mitochondria and also cell mediators from monocytes and macrophages, which are implicated in brain damage due to oxidative stress. In agreement with these findings, the group that was injected with scopolamine (3 mg/kg b.w, daily dose for 14 days) demonstrated clear oxidative stress, as manifested by lowered levels of GSH, SOD, and GR and upsurged lipid peroxidation, as illustrated by the increased level of TBARS in the brain hippocampus and increased nitric oxide levels.

To prevent or slow down the progression of free-radical-mediated oxidative stress, brain antioxidant defense enzymes such as GR and SOD play a vital role in protecting tissues against oxidative damage. Antioxidant mRNA alteration caused by scopolamine injection was significantly ameliorated for GSH, SOD, and GR and upsurgence of lipid peroxidation, as illustrated by the increased level of TBARS in the brain hippocampus and increased nitric oxide levels.

In our study, quercetin and catechin showed excellent binding affinity to the SOD active site by H-bonds between...
hydroxyl groups and VAL 7, ASP11, CYS 144, VAL 146, and LYS 9 residues, which stabilized the protein–ligand complexes. Also, the biochaninA–protein complex was stabilized by hydrogen bonding with the amino acid residues ASN 51 and LYS 9 (Tables 7 and 8, Figure 10).

Also, quercetin helped stabilize itself within the GR active site by H-bonding with the amino acid residue TYR 407 and π–cation interaction between the phenyl ring and HIS82. Also, the catechin–protein complex stabilized by H-bonding with the His82, while biochaninA stabilized itself by the π–cation interaction with HIS 75 (Table 8, Figure 11).

Our findings demonstrate that scopolamine therapy increased GABA<sub>α5</sub> gene expression levels in the brain of mice. Pretreatment with isolated compounds individually and/or in combination has been found to have a modulatory effect on the expression of the γ-aminobutyric acid A alpha5 subunit (GABA<sub>α</sub>5) gene. The current findings are consistent with those of Moghbelinejad et al.,<sup>51</sup> who found that quercetin has a modulatory effect on the expression of the GABA<sub>α</sub>5 receptor genes in a epileptic model.

Flavonoids, on the other hand, have been demonstrated to inhibit GABA<sub>α</sub>5 by acting as positive, modulatory receptors.<sup>52</sup> Isolated compounds administered individually and/or in combination may inhibit GABA<sub>α</sub>5π via reducing the level of expression of α1β1γ2 GABA<sub>α</sub> and p1 GABAc receptors in a neurodegeneration mice model.<sup>53</sup>

Our docking study on the beta3-alpha5 GABA<sub>α</sub> receptor indicated that biochaninA exhibited the best docking score of −18.11 kcal/mol compared to quercetin and catechin with values of −16.86 and −16.70 kcal/mol, respectively, compared to pregnanolone with a value of −21.40 kcal/mol (Table 7). Also, quercetin and biochaninA showed powerful binding interaction with the target active site by H-bonding with the same amino acid GLN 245 residue as the native ligand, in addition to π–π interaction with the Trp 249 residue. However, the catechin–protein complex was stabilized by π–π interaction between the phenyl of the coumarine moiety and Trp 249 (Table 8, Figure 12).

Previous studies indicated that GSK-3β is the key enzyme that leads to tau hyperphosphorylation and increased extracellular β-amyloid (Aβ) aggregation in AD. The dysfunctionality of GSK-3β is involved in AD. Our study showed a significant elevation of the expression of GSK-3β in scopolamine mice compared to normal mice. Our results were in agreement with those of Das et al.,<sup>56</sup> who found that scopolamine administration elevated the GSK-3β gene expression in a mice AD model. Also, the isolated compounds inhibit GSK-3β induced by scopolamine in mice. In addition, the downregulation effect of these compounds for GSK-3β gene expression was in agreement with the results of Johnson Jodee et al.<sup>57</sup>

Our study demonstrated that the lowered levels of GSK-3β in scopolamine-treated mice could potentially caused by P. roxburghii bioactive compounds in AD mice, resulting in reduced tau phosphorylation and Aβ aggregation in the brain. The present results were in agreement with the results of Das et al.<sup>56</sup>

Our docking results found that quercetin showed an excellent binding energy of −21.07 kcal/mol, closely similar to the native ligand (TMU) of −21.98 kcal/mol and a good interaction model with the active site by the H-bond formed with the same amino acid VAL 135 residue as the native ligand, besides the H-bond with ASP 200. Also, catechin and biochaninA revealed a similar interaction model as the native ligand by H-bonds formed between the hydroxyl group and VAL 135 and PRO 136 residues, in addition to H-bonds with ILE 62 and LYS 85 residues, respectively (Tables 7 and 8 and Figure 13).

Ueda et al.<sup>58</sup> determined that flavonoids and phenolics with hydroxyl groups present on QUR, CAE, and BCA had the highest inhibitory effect on tumor necrosis factor production in vitro. Several studies have also demonstrated that the total number and location of hydroxyl groups on flavonoids greatly influence their impact on several mechanisms of antioxidant activity.<sup>59</sup>

This study showed that scopolamine injection decreased pERK1 gene expression as well as Akt levels in the brain tissue compared to the negative control group of mice. Our results are in agreement with the results of Samrat et al.,<sup>60</sup> who reported decreased pERK gene expression as well as Akt levels in the group of mice treated with scopolamine.

However, ERK make induction of translation through phosphorylation of translation factors eIF4E and 4EBP1 and the ribosomal protein S6 in the late LTP phase.<sup>61</sup> The potentiated action of acetylcholine through the injection of AChE inhibitors was reported to increase acute Akt phosphorylation in brain tissues.<sup>62</sup> Accordingly, flavonoid-induced activation of brain Akt and pERK could be one of the molecular mechanisms underlying the memory-enhancing effect.<sup>63</sup> Also, quercetin showed a similar interaction model to the native ligand of the pERK1 receptor (PDB ID: 4G31) by a hydrophobic interaction formed between the coumarin moiety and PHE 943 and the H-bond acceptor with the CYS 890 residue. Also, quercetin formed two H-bond donors with LEU598 and ASP 954 residues. In addition, catechin exhibited excellent binding affinity by formation of two H-bonds with GLN 888 and CYS 890 similar to the native ligand and two H-bonds with LEU598 and ARG 891 residues. The biochaninA–protein complex was stabilized by the π–π interaction with PHE 943 and H-bonding with the ARG 891 residue (Table 8, Figure 14).

In the present study, treatment with P. roxburghii bioactive compounds attenuated the pERK1 signaling pathway, increased Akt protein levels, and further enhanced the scopolamine-injured neurons.

On the other hand, either ligand and/or quercetin helped stabilize themselves within the Akt protein active site by hydrogen bonding with the amino acid residues GLU 228, GLU 234, ASP 292, and ALA 230 as well as ARG 4, SER 7, and LYS 179. Also, catechin stabilized itself within the Akt protein active site by H-bonding with the SER 7, GLU 234, ARG 4, and LYS 179 residues. On the other hand, biochaninA stabilized itself within the target by H-bonding with Tyr 437 and π–cation interaction with Arg4 (Table 8, Figure 15).

Aldo et al.<sup>64</sup> demonstrated how the cognitive function of scopolamine-treated mice with quercetin, catechin, and biochaninA improves their neuroprotective action by inhibiting oxidative stress.

In our study, scopolamine injection also increased the AChE and IL-6 levels, leading to neuroinflammation. Also, scopolamine administration can increase the iNOS level through the activation of NF-κB, iNOS, and TGF-β1, which are major regulators in progression of the proinflammatory signaling pathways, which further release proinflammatory mediators...
such as IL-6 and TGF-β1. Furthermore, MAPKs play crucial roles in modulating cytokine production and the expression of proinflammatory enzymes. MAPKs are compelling molecules in the inflammatory process and crucial targets for therapy. P. roxburghii-isolated compounds significantly inhibited the p38α-MAPK protein in scopolamine-treated mice. These data suggested that the administration of these compounds individually and/or in combination regulated inflammatory reactions by inhibiting the p38-MAPK signaling pathway. As previously reported by Xiao et al., neuroprotective effects of flavonoids against H2O2 and Aβ-induced toxicity in human neuroblastoma SH-SY5Y cells were due to enhancement of the MAPK pathway. Yoon et al. also reported that NF-κB and MAPK are major elements in the inflammatory process and are compelling targets for anti-inflammatory molecules.

However, either ligand and/or quercetin–protein complexes were stabilized within the active site of the p38α-MAPK receptor by hydrogen bonding with the amino acid residues ASP 112 and MET 109 as well as PHE 169 and GLY 170. Also, catechin linked to the target active site by a H-bond formed between the hydroxyl group and ASP 112, SER 154, PHE 169, and LYS 53 residues. On the other hand, biochaninA revealed poor binding affinity to the target by formation of only one H-bond with PHE 169 (Table 8, Figure 16).

## MATERIALS AND METHODS

### Plant Collection

Leaves of the plant were collected from El Zohria Garden, Giza, Egypt, in January 2020. The identification of the plant was confirmed by Dr. Ahmed Wahba, Executive Manager of the garden. A voucher specimen was deposited in the herbarium of the Faculty of Pharmacy, Pharmacognosy Department, October 6 University.

### EQUIPMENT AND MATERIAL

The melting point was determined using Gallenkamp apparatus for all isolated compounds. Nuclear magnetic resonance (NMR) analysis was performed for determination of proton-NMR (1H NMR) (400 MHz) and carbon 13 NMR (13C NMR) (100 MHz), measured on a Bruker high-performance digital FT-NMR spectrometer Avance III 400 MHz. The NMR spectra were recorded in deuterated dimethyl sulfoxide (DMSO-d6) and deuterated methanol (CD3OD). Also, mass spectrometry: electron ionization/mass spectrometry (EI/MS) spectra were obtained on Thermo Scientific. The following chemicals were used of high analytical grade: Scophedex LH-20 (Pharmacia), silica gel (E. Merck), and precoated thin-layer chromatography (TLC) plate silica gel 60 GF254 (E. Merck), (20 × 20 cm²). Scopolamine bromide and Tween 80 were obtained from Merck Ltd., Germany.

### PLANT EXTRACT PREPARATION

Leaves of the plant were dried in shade (1 kg). The plant leaves were comminuted then macerated with 4 L of methanol (MeOH). The solvent was evaporated using a rotary evaporator, leaving a brown sticky residue (190 g). The residue was then suspended in 500 mL of water and successively partitioned with 2 L of chloroform and 2 L of ethyl acetate; solvents were then evaporated to produce fractions of 20 g and 24 g under reduced pressure.

### EXTRACTION AND ISOLATION

- The chloroform fraction was subjected to column chromatography (CC) using a silica gel column and then subjected to Sephadex LH-20 with MeOH to yield three subfractions; silica gel columns were eluted with hexane/ethyl acetate (EtOAc) (90:10 and 80:20) S1 and S2, which afforded compounds A (15 mg) and B (10 mg), respectively.
- The EtOAc fraction was applied to the silica gel column and eluted with methylene chloride/methanol (CH2Cl2-MeOH) (from 100:0 to 60:40) to give two main fractions, which were separately subjected to several runs on Si gel column chromatography using different concentrations of CH2Cl2-MeOH to give C (20 mg), D (30 mg), and E (15 mg).

### FLAVONOID AND PHENOLIC PROFILE

Agilent Technologies 1100 series liquid chromatography fitted with an autosampler and a diode-array detector was used to perform reversed-phase-high-performance liquid chromatography (RP-HPLC) analysis of MeOH leaf extract. An analytical column XDB-C18 (150 × 4.6 mm²; 5 μm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was held constant at 0.8 mL/min for a total run length of 70 min, and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min, and 0% B to 100% B in 5 min. The injection volume was 50 μL, and the peaks were monitored simultaneously at 280 and 320 nm. Before injection, all samples were filtered via a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI). Congruent retention durations and UV spectra were used to identify the peaks, which were then compared to the standards.

### CONCLUSIONS

P. roxburghii bioactive compounds (quercetin, catechin, and biochaninA) have beneficial properties against general mechanisms of AD etiology. The tested compounds protected neuronal cells by attenuating oxidative stress and neuro-inflammation. Our study demonstrated that a variety of P. roxburghii bioactive compounds can inhibit AChE, GABA<sub>A</sub>, α<sub>5</sub>, GSK-3β, and p38α MAP-kinase as well as increase SOD and GR activities directly by binding in the active site of these enzymes when administrated to scopolamine-treated mice individually and/or in combination. The inhibitory effect of the isolated compounds was potentiated by the presence of hydroxyl side groups, which were available for hydrogen bonding with the amino acid residues in the enzyme. The inhibitory effects of quercetin, catechin, and biochaninA used in combination were more pronounced than when administrated individually. Hence, the study showed stimulating results; P. roxburghii could be used as an anti-inflammatory agent as well as for preventing memory impairments in Alzheimer’s disease. To the best of our knowledge, this is the first report of its kind on P. roxburghii concerning complications of Alzheimer’s disease and phenolic profiling.

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Notes

The authors declare no competing financial interest.

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