Novel Diels–Alder Type Adducts from *Morus alba* Root Bark Targeting Human Monoamine Oxidase and Dopaminergic Receptors for the Management of Neurodegenerative Diseases

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**Abstract:** In this study, we delineate the human monoamine oxidase (*hMAO*) inhibitory potential of natural Diels–Alder type adducts, mulberrofuran G (1), kuwanon G (2), and albanol B (3), from *Morus alba* root bark to characterize their role in Parkinson’s disease (PD) and depression, focusing on their ability to modulate dopaminergic receptors (D_{1R}, D_{2L R}, D_{3R}, and D_{4R}). In *hMAO*-A inhibition, 1–3 showed mild effects (50% inhibitory concentration (IC_{50}): 54–114 μM). However, 1 displayed moderate inhibition of the *hMAO*-B isozyme (IC_{50}: 18.14 ± 1.06 μM) following by mild inhibition by 2 (IC_{50}: 57.71 ± 2.12 μM) and 3 (IC_{50}: 90.59 ± 1.72 μM). Our kinetic study characterized the inhibition mode, and in silico docking predicted that the moderate inhibitor 1 would have the lowest binding energy. Similarly, cell-based G protein-coupled receptors (GPCR) functional assays in vector-transfected cells expressing dopamine (DA) receptors characterized 1–3 as D_{1R}/D_{2L R} antagonists and D_{3R}/D_{4R} agonists. The half-maximum effective concentration (EC_{50}) of 1–3 on DA D_{3R}/D_{4R} R was 15.13/17.19, 20.18/21.05, and 12.63/- μM, respectively. Similarly, 1–3 inhibited 50% of the DA response on D_{3R}/D_{4R} R by 6.13/2.41, 16.48/31.22, and 7.16/18.42 μM, respectively. A computational study revealed low binding energy for the test ligands. Interactions with residues Asp110, Val111, Tyr365, and Phe345 at the D_{1R} receptor and Asp115 and His414 at the D_{3R} receptor explain the high agonist effect. Likewise, Asp187 at D_{1R} and Asp114 at D_{2L R} play a crucial role in the antagonist effects of the ligand binding. Our overall results depict 1–3 from *M. alba* root bark as good inhibitors of *hMAO* and potent modulators of DA function as D_{1R}/D_{2L R} antagonists and D_{3R}/D_{4R} agonists. These active constituents in *M. alba* deserve in-depth study for their potential to manage neurodegenerative disorders (NDs), particularly PD and psychosis.

**Keywords:** dopamine; GPCRs; human monoamine oxidase; *Morus alba* L.; Parkinson’s disease

1. Introduction

Monoamine oxidase (MAO) is a flavoenzyme in the outer mitochondrial membrane of neuronal and non-neuronal cells that has a vital role in the etiology of age-regulated neurodegenerative disorders (NDs). MAO catalyzes the oxidative deamination of monoamine neurotransmitters, dietary amines, and xenobiotics and regulates their levels and functions in the brain. During oxidative deamination, MAO liberates hydrogen peroxide, the reactive oxygen species (ROS) most potent in causing oxidative stress and mitochondrial dysfunction [1]. Though the etiology of NDs remains unclear, apoptosis,
oxidative stress, mitochondrial dysfunction, inflammation, an impaired ubiquitin-proteasome system, and excitotoxicity are common disease-modifying factors [2]. Two isoforms (MAO-A and MAO-B) with specific functions have been identified in different brain regions and cell types [3].

MAO-A displays a higher affinity for serotonin (5HT) and norepinephrine, whereas MAO-B prefers phenylethylamine. Dopamine (DA) and tyramine are common substrates for both isozymes [4]. MAO-A is associated with the onset of psychiatric disorders (Figure 1), including depression, and antisocial aggressive impulsive behaviors through its ability to decrease neurotransmitter levels (DA and serotonin) [5,6]. During a normal physiological state, DA levels in substantia nigra pars compacta (SNpc) are regulated as an equilibrium between synthesis, synaptic vesicle loading, uptake, and catabolism. MAO enzyme mediates oxidative deamination of DA to DOPAL along with H$_2$O$_2$ generation, leading DA deficit and oxidative stress state. And MAO-A inhibition prevents the deamination of neurotransmitters, reduces oxidative stress, and increases the availability of neurotransmitters within noradrenergic and serotonergic neurons of the CNS to regulate neuron signaling via their respective receptors [4,7]. Similarly, MAO-B metabolizes DA to DOPAC and catechol-O-methyltransferase (COMT) degrades it to homovanillic acid (HVA) in astrocyte [8,9]. Therefore, MAO inhibitors function as neuroprotective agents against age-related NDs.

![Figure 1. Activity of monoamine oxidase (MAO) enzyme in neuronal cells.](image-url)

The concept of precision medicine relies on protein targeting, and G protein-coupled receptors (GPCRs) are the largest family of target receptors and membrane proteins. At present, 34% of FDA-approved drugs target GPCRs [10]. GPCRs are widely expressed and activated by a broad range of ligands, including neurotransmitters, hormones, and ions, as well as sensory signals [11]. Neurotransmitters bind to their specific receptors at the postsynaptic cleft and trigger or inhibit neuronal functions and signals by regulating the activity of ion channels. In NDs, especially Parkinson’s disease (PD), the selective loss of dopaminergic neurons in the SNpc produces DA deficiency, which triggers cell-specific alterations in intrinsic excitability and synaptic plasticity [12]. Therefore, regulating DA levels or DA receptor signaling is a standard approach to PD treatment. Numerous neurotransmitters and their analogs have therapeutic properties, serve as medicaments for various diseases, and have been the subject of extensive pharmacological studies [13]. In this study, we discuss the critical physicochemical interactions between our test ligands and different residue side chains and the adjacent amino acids.

*Morus alba* Linn, commonly known as mulberry, is a perennial woody plant of the family Moraceae that is widely cultivated in tropical, subtropical, and temperate zones in Asia, Europe, and North and South America. The leaves of this plant are used as feed for animals and sericulture, the fruit
is used as food, and the wood as timber. Furthermore, in traditional Chinese medicine, the leaves, twigs, fruit, and root bark are used as antioxidant, anti-inflammatory, anti-hypertensive, hypoglycemic, immunomodulatory, hypolipidemic, antibacterial, and anti-tumor agents [14]. The plant thus has unique medicinal and ethnic values. It is rich in flavonoids, alkaloids, steroids, and coumarins. Diels–Alder-type adducts are prototypical metabolites in the root bark [15]. In a previous study, mulberry fruit extract protected dopaminergic neurons in in vitro and in vivo PD models by regulating ROS generation through its antioxidant and anti-apoptotic effects [16]. A crude water extract of *M. alba* leaf ameliorated alterations in the retinal neurotransmitters adrenaline, DA, gamma-aminobutyric acid, histamine, noradrenaline, and serotonin in the pups of diabetic and hypercholesterolemic mother rats [17] and ameliorated kidney damage in diabetic rats by suppressing inflammation and fibrosis via peroxisome proliferator-activated receptor γ (PPARγ) modulation [18]. Similarly, a leaf-ethanol extract possessed anxiolytic and muscle-relaxant activities, probably via a γ-aminobutyric acid A-benzodiazepine (GABA_A-BZD) mechanism [19]. No previous reports have considered the root bark of *M. alba*. In our recent work, we reported the antidiabetic [20,21], anti-Alzheimer's disease activity [22,23], and antioxidant and anti-browning property [24] of Diels–Alder-type adducts and arylbenzofurans from *M. alba* root bark. More recently, kuwanon G and albanin G from the root bark were hypothesized as the components responsible for the appetite suppression activity of root-bark extract via cannabinoid (CB1) receptor antagonism [25]. In the present study, we characterize the multi-target effects of Diels–Alder-type adducts, mulberrofuran G (1), kuwanon G (2), and albanol B (3) (Figure 2), via human monoamine oxidase (*hMAO*) inhibition and the modulation of dopaminergic receptors (D_1R, D_2R, D_3R, and D_4R), and we use a molecular simulation to explore the action mechanism of the ligand–receptor interaction.

![Figure 2. Structures of compounds isolated from *Morus alba*.](image)

### 2. Results

#### 2.1. *In Vitro hMAO Inhibition and Enzyme Kinetics*

The *in vitro* *hMAO* inhibition potentials of 1–3 and the reference compound selegiline was evaluated via a chemiluminescent assay in a white, opaque, 96-well plate using the MAO-Glo kit (Promega, Madison, WI). At first, 1–3 were screened for *hMAO* activity at 100 µg/mL and the % inhibition was 93.87%, 99.05%, and 74.85%, respectively. Then the compounds were retested at different micromolar concentrations in triplicates and the 50% inhibitory concentration (IC_{50}) values obtained from the log-dose inhibition curve are tabulated in Table 1.
Table 1. Human monoamine oxidase (hMAO) inhibitory potential of compounds from Morus alba.

| Compounds | Human Monoamine Oxidase A (hMAO-A) | Human Monoamine Oxidase B (hMAO-B) |
|-----------|-----------------------------------|-----------------------------------|
|           | IC₅₀ (µM, Mean ± SD) a             | Kᵢ Value b                        | Inhibition Type c |
| 1         | 54.79 ± 0.03                       | 26.96 ± 3.98                      | Competitive       |
| 2         | 70.16 ± 2.60                       | 28.29 ± 2.02                      | Competitive       |
| 3         | 114.31 ± 2.30                      | 46.93 ± 4.12                      | Competitive       |
| Selegiline d | 12.51 ± 1.11                  | NT                               | NT                |
| Harmine d, e | 0.006 [26]                     | NT                               | NT                |

|                      |                                    |                                    |                    |
| 1         | 18.14 ± 1.06                       | 17.01 ± 3.31                      | Noncompetitive    |
| 2         | 57.71 ± 2.12                       | 52.09 ± 5.56                      | Noncompetitive    |
| 3         | 90.59 ± 1.72                       | 55.19 ± 7.79                     | Mixed             |
| Selegiline d | 0.30 ± 0.01                     | NT                               | NT                |
| Safinamide d,e | 0.00512 [27]                    | NT                               | NT                |

NT: Not tested. a The 50% inhibitory concentration (IC₅₀) values (µM) were calculated from a log dose-inhibition curve and expressed as the mean ± SD of triplicate experiments. b The hMAO inhibition constant (Kᵢ) was determined using a Dixon plot. c The hMAO inhibition type was determined using Lineweaver–Burk plots and Dixon plots. d Reference inhibitor. e Values extracted from the literature. f g Kᵢ and Kᵢ values, respectively.

As shown there, 1–3 displayed mild inhibition of hMAO-A activity. Among the test compounds, 1 showed the best inhibition, with an IC₅₀ value of 54.79 ± 0.03 µM, followed by 2 (IC₅₀: 70.16 ± 2.60 µM) and 3 (IC₅₀: 114.31 ± 2.30 µM). The inhibition potentials of 1–3 were better against hMAO-B, though the pattern of inhibition was similar: 1 showed moderate inhibition effect, with an IC₅₀ value of 18.14 ± 1.06 µM, and compounds 2 and 3 mildly inhibited hMAO-B, with IC₅₀ values of 57.71 ± 2.12 and 90.59 ± 1.72 µM, respectively. The reference inhibitor selegiline inhibited the activity of isozymes -A and -B at IC₅₀ values of 12.51 ± 1.11 and 0.30 ± 0.01 µM, respectively. However, compared to the reference reversible hMAO-A inhibitor (harmine, IC₅₀: 0.006 µM) [26] and reversible hMAO-B inhibitor (safinamide, IC₅₀: 0.00512 µM) [27], the potency of 1-3 is significantly weaker.

The enzyme inhibition patterns of compounds at different substrate concentrations in the kinetic study are tabulated in Table 1 and represented in Figures 3 and 4. Compounds 1–3 competitively inhibited hMAO-A isozyme activity with Kᵢ values of 26.96 ± 3.98, 28.29 ± 2.02, and 46.93 ± 4.12 µM, respectively (Table 1 and Figure 3a–c). The Lineweaver–Burk plots (1/V vs. 1/[S]) for hMAO-A isozyme activity (Figure 3d–f) reveal an increase in Kₘ with an increase in the concentrations of 1–3, whereas 1/V max remained constant. Meanwhile, 1 and 2 were noncompetitive inhibitors (V max value decreased in a concentration-dependent manner without changing the Kₘ value), and 3 was a mixed type inhibitor (increase in inhibitor concentration increased the Kₘ value but decreased the V max value) of the hMAO-B isozyme (Figure 4c, f). From a Secondary plot (plot not shown here), the binding constants of 3 with a free enzyme (K ic) and with enzyme-substrate complex (K icu) identified were 55.19 ± 7.97 and 186.2 ± 10.26 µM, respectively. Likewise, the Kᵢ value of 1 and 2 for hMAO-B inhibition was 17.01 ± 3.31 and 52.09 ± 5.56 µM, respectively.
2.2. In Silico Docking Simulation of hMAO

Computational modeling was performed to obtain insights into the binding affinity between ligands and the enzyme using AutoDock 4.2. To validate the docking result, the reference inhibitor selegiline as well as reversible inhibitor harmine (for hMAO-A) and safinamide (for hMAO-B) were docked into the active site cavity of the hMAO enzyme, and the ligands were re-docked. The results of the simulation study are tabulated in Tables 2 and 3 and represented in Figures 5–7.
Table 2. Binding site residues and docking scores of 1–3 and reference inhibitors in human monoamine oxidase A (hMAO-A) (2BXR) obtained using Autodock 4.2.

| Compound   | Binding Energy (kcal/mol) | H-bond Interacting Residues | Hydrophobic Interacting Residues | Electrostatic Interacting Residues |
|------------|--------------------------|------------------------------|----------------------------------|-----------------------------------|
| 1          | −9.54                    | Gly110, Thr336, Ile207, Gly214, Ser209 | Val210 (Pr-Sigma, Pr-Alkyl), Ile325 (Pr-Sigma), Phe208 (Pr-Pi Stacked, Pr-Pi T-Shaped), Ile358 (Alkyl), Leu337 (Alkyl), Ile335 (Alkyl), Met350 (Alkyl), Val93 (Pr-Alkyl), | - |
| 2          | −6.74                    | Met300, Leu298, Asp359, Gly404, Cys398, Trp397, Glu400 | Ala302 (Pr-Alkyl, Alkyl) | - |
| 3          | −8.62                    | Gln296, Ile295, Gly404, Tyr410, Met300, Thr183, Ser184 | Pro299, Ala279, Ala302 (Pr-Alkyl) | Gla188 (Pr-Anion) |

Selegiline | −6.54 | Ile335 (Pr-Sigma), Leu337 (Pr-Alkyl), FAD600 (Pr-Alkyl), Tyr407 (Pr-Alkyl), Tyr444 (Pr-Alkyl) | - |

HRM (Harmine) | −6.46 | FAD600 | Ty444 (Pr-Sigma), FAD600 (Pr-Sigma, Pi-Pi T-shaped, Pi-Alkyl), Tyr444 (Pr-Pi Stacked), Phe352 (Pr-Pi T-shaped), Tyr407 (Pr-Alkyl), Ile335 (Pr-Alkyl) | - |

* Estimated binding free energy of the ligand–receptor complex.  
* The number of hydrogen bonds and all amino acid residues from the enzyme–inhibitor complex were determined with the AutoDock 4.2 program.  
7-Methoxy-1-methyl-9H-pyrido [3,4-b]indole.

Table 3. Binding site residues and docking scores of 1–3 and reference inhibitors in human monoamine oxidase B (hMAO-B) (2BYB) obtained using Autodock 4.2.

| Compound | Binding Energy (kcal/mol) | H-bond Interacting Residues | Hydrophobic Interacting Residues | Electrostatic Interacting Residues |
|----------|--------------------------|------------------------------|----------------------------------|-----------------------------------|
| 1        | −11.09                   | His115, Pro476, Glu483       | Phe103 (Pr-Pi Stacked, Pr-Pi T-shaped, Pr-Alkyl), Val106 (Pr-Alkyl), Ile477 (Pr-Alkyl) | Glu188 (Pr-Anion) |
| 2        | −12.65                   | Pro104, Asn116, Glu483, Phe103, Thr478 | Tyr112 (Pr-Sigma), Phe103 (Pr-Pi Stacked), Val106 (Alkyl, Pr-Alkyl), Pro102 (Alkyl, Pr-Alkyl), Tyr112 (Pr-Alkyl), Trp119 (Pr-Alkyl), Pro104 (Pr-Alkyl), Leu164 (Pr-Alkyl) | Glu188 (Pr-Anion) |
| 3        | −10.05                   | Thr195, Pro104, Asn116, Thr478, Gly193 | Ile477 (Pr-Sigma), Trp119 (Pr-Pi Stacked), Phe103 (Pr-Pi Stacked), Val106 (Alkyl, Pr-Alkyl), | Asp123 (Pr-Anion), Glu188 (Pr-Anion) |

Selegiline | −7.06 | Ile198 | Tyr398 (Pr-Pi Stacked), Tyr435 (Pr-Pi Stacked), FAD600 (Pr-Pi T-shaped), Leu171 (Alkyl), Cys172 (Alkyl), Phe188 (Pr-Alkyl) | - |

Safinamide | −9.86 | Cys172, Ile199, Tyr326, Thr201 | Tyr398 (Pr-Pi Stacked), Tyr326 (Pr-Pi T-shaped), Ile199 (Pr-Alkyl) | - |

* Estimated binding free energy of the ligand–receptor complex.  
* The number of hydrogen bonds and all amino acid residues from the enzyme–inhibitor complex were determined with the AutoDock 4.2 program.  
* Reference inhibitors.
**Figure 5.** (a) *h*MAO-A inhibition mode of 1–3 and selegiline. (b–d) 2D ligand interaction diagram of *h*MAO-A inhibition by 1–3, respectively.
Figure 6. (a) hMAO-B inhibition mode of 1–3 and selegiline. (b–d) 2D ligand interaction diagram of hMAO-B inhibition by 1–3, respectively.
Figure 7. (a) hMAO-A and (b) hMAO-B inhibition mode of selegiline with flavin adenine dinucleotide (FAD). (c,d) 2D ligand interaction diagram of hMAO-A and hMAO-B inhibition by selegiline.

As shown in Table 2, the test ligand (1–3)–hMAO-A complexes showed lower binding energies (−6.74 to −9.54 kcal/mol) than the reference ligand selegiline (−6.54 kcal/mol) and harmine (−6.46 kcal/mol). Ligand 1 posed in the active site by interacting with Gly110, Thr336, Ile207, Gly214, and Ser209 via a hydrogen bond (Figure 5). Meanwhile, ligands 2 and 3 shared Met300 and Gly404 as common H-bond interacting residues. Reversible inhibitor harmine interacted with flavin adenine dinucleotide (FAD)600, Ile335, and Tyr444 residues at the active site cavity, which were not observed for test ligand-binding. In the case of hMAO-B, ligands 1–3 showed high affinity with binding energies (−11.09, −12.65, and −10.05 kcal/mol, respectively) by forming three and five H-bond interactions, respectively (Figure 6). With the lowest binding energy, ligand 1 stably positioned in the hMAO-B active site by interacting with His115, Pro476, and Glu483 via H-bonds. Moreover, 1 interacted with peripheral residues, including Phe103, Val106, and Ile477. Interacting residues Val106 and Phe103 were shared by all three ligands as a noncompetitive inhibitor. Ligand 2 shared the most abundant H-bond interaction residues: Pro103, Asn116, Glu483, Phe103, and Thr478. Ligand 3 also showed high affinity via H-bond interactions with Thr195, Pro104, Asn116, Thr478, and Gly193. Selegiline interacted with Ile198 and safinamide with Ile199, Cys172, Tyr326, and Thr201 via H-bonds in the active site of hMAO-B (Figure 7).

2.3. Cell-Based Functional GPCR Assays

To characterize the possible role of compounds 1–3 in neuronal diseases, we first screened their functional activity at 100 µM on DA (D₁, D₂, D₃, D₄) receptors by measuring their effects on secondary messengers (cAMP modulation or Ca²⁺ ion mobilization) in transfected cell lines expressing human cloned receptors of interest. The data in Table 4 represent the agonist/antagonist effects of 1–3 at 100 µM on the various receptors.
Table 4. Efficacy values (% stimulation and % inhibition) of Diels–Alder type adducts (1–3) from *M. alba* on DA (D₁, D₂L, D₃, and D₄) receptors.

| Receptors | % Stimulation a (% Inhibition b) | % Stimulation a (% Inhibition b) | % Stimulation a (% Inhibition b) | Reference Drugs |
|-----------|---------------------------------|---------------------------------|---------------------------------|-----------------|
| D₁ (h)    | 17.2 ± 8.4 (87.65 ± 1.19)       | 0.85 ± 0.24 (67.80 ± 9.05)     | INTER                          | 28              |
| D₂L (h)   | 7.10 ± 1.47 (99.15 ± 0.77)      | 4.10 ± 1.06 (78.55 ± 3.61)     |                   | 12              |
| D₃ (h)    | 119.9 ± 2.44 (124.3 ± 0.76)     | 102.8 ± 1.36 (102.8 ± 1.36)    |                   | 4.1             |
| D₄ (h)    | 86.30 ± 0.99 (90.45 ± 0.14)     | 46.10 ± 1.76 (26.9 ± 5.09)     |                   | (150)           |

a, b % Stimulation and % inhibition, respectively, of control agonist response at 100 µM of test compounds. c EC₅₀ (nM) values of standard agonist DA. d IC₅₀ (nM) values of standard antagonists (D₁: SCH-23390, D₂L: butaclamol, D₃: (+)-butaclamol, D₄: clozapine. INTER: Test compound interfered with the assay detection method. NSI: Test compound interfered nonspecifically in the assay.

As shown in the table, 1–3 exhibited a full antagonist effect on the D₁/D₂ receptors and a full agonist effect on the D₃/D₄ receptors. The agonist effects of 1–3 at 100 µM on D₃R/D₄R were 119.9/86.30, 124.3/90.45, and 102.8/46.10%, respectively. Similarly, at 100 µM, 1–3 inhibited the DA response on D₁R/D₂L R by 87.65/101.30, 98.85/99.15, and 67.80/78.55%, respectively. Figure 8 shows the concentration-dependent functional effect of 1–3 on the DA receptor subtypes with corresponding EC₅₀/IC₅₀ values.

![Figure 8](image-url)

**Figure 8.** Concentration-dependent % of control agonist response on human dopamine D₃ receptor (hD₃R) (a) and human dopamine D₄ receptor (hD₄R) (b), and % inhibition of control agonist response on human dopamine D₁ receptor (hD₁R) (c) and hD₂L R (d) of test compounds 1–3.
As shown there, all compounds showed promising antagonist effects on D₁R/D₂L⁺R, with IC₅₀ values in the range of 2.41-31.22 µM (Figure 8c,d). The rank order for the antagonist effect was D₂L⁺R > D₁R for 1 and D₁R > D₂L⁺R for 2 and 3. Compared to 1 and 2, the dose–response curve for 3 (Figure 8c) looks unusual due to relatively higher standard deviation in response at 50 µM concentration. Similarly, 1-3 showed an agonist effect on D₃R/D₄⁺R, with EC₅₀ values in the range of 12.63-21.05 µM (Figure 8a,b), and the rank order for the agonist effect was D₃⁺R = D₄⁺R for 1 and 2 and D₃⁺R > D₄⁺R for 3. These results indicate that compounds 1-3 mediate the DA function by acting as D₁R/D₂L⁺R antagonists and D₃⁺R/D₄⁺R agonists.

2.4. In Silico Docking Simulation of Dopamine Receptors

To validate the results of the functional assays and investigate and identify the ligand–receptor interactions for novel lead discovery, we carried out a computational docking simulation using AutoDock 4.2 (Figures 9-12). Since the effect of 1-3 on DA receptors was promising, a simulation study was carried out on DA receptors. The binding affinities of reference ligands for each receptor were also evaluated to better understand and validate the docking results. The homology model of DA receptor subtype D₁R was based on the structure of the β₂-adrenergic receptor because it has a higher similarity to the DA D₁ receptor in the binding site region and sequence identity [28]. Subtypes D₂L⁺R, D₃⁺R, and D₄⁺R were obtained from the protein data bank (PDB) IDs for 6CM4, 3PBL, and 5WIV, respectively. The results of the docking simulation are tabulated in Tables 5-8 and represented in Figures 9-12. The dotted colored lines in Figures 9-12 represent specific interactions (green line: H-bond; purple line: π-sigma; pale pink: π-alkyl, alkyl; pink: π-π T-shaped, π-π stacked; orange: π-anion).

**Table 5. Binding sites and docking scores of compounds on hD₁R.**

| Target Compounds | Binding Energy (kcal/mol) | H-bond Interaction Residues | Hydrophobic Interacting Residues | Electrostatic Interacting Residues |
|------------------|---------------------------|----------------------------|----------------------------------|----------------------------------|
| Dopamine a (agonist) | −5.59                     | Asp103 (Salt bridge), Ser202, Asn292, Ser199 | Phe289 (Pi-Pi T-shaped), Ile104 (Pi-Alkyl) | Phe288 (Pi-Cation) |
| SCH23390 a (antagonist) | −6.94                     | Asp103 (Salt bridge), Ser199, Ser202 | Leu190 (Pi-sigma), Phe288 (Pi-Pi T-shaped), Ile104 (Pi-Alkyl), Ala195 (Pi-Alkyl) | - |
| 1 D₁R | −9.22 | Lys81, Leu291, Asp314, Ser188 | Leu295 (Pi-sigma), Phe313 (Pi-Pi Stacked), Phe306 (Pi-Pi T-shaped), Ser188 (Amide-Pi Stacked), Leu295 (Pi-Alkyl), Leu291 (Pi-Alkyl) | Lys81 (Pi-Cation), Asp314 (Pi-Anion) |
| 2 | −7.1 | Lys81, Ser107, Ser202, Asp187, Asp103, Ser198 | Val100 (Pi-sigma), Val317 (Pi-Sigma, Pi-Alkyl), Phe313 (Pi-Pi T-shaped), Leu190 (Alkyl), Cys186 (Alkyl), Phe288 (Pi-Alkyl), Ile104 (Pi-Alkyl) | Asp187 (Pi-Anion) |
| 3 | −9.2 | Asp187, Ser188 | Asp187 (Pi-Sigma), Leu295 (Pi-Sigma, Pi-Alkyl), Phe306 (Pi-Pi T-shaped), Pro171 (Pi-Alkyl), Arg192 (Pi-Alkyl), Ala195 (Pi-Alkyl) | - |

a Reference ligand for hD₁R.
Table 6. Binding sites and docking scores of compounds on hD2L.

| Target | Compounds       | Binding Energy (kcal/mol) | H-bond Interaction Residues | Hydrophobic Interacting Residues | Electrostatic Interacting Residues |
|--------|-----------------|---------------------------|-----------------------------|----------------------------------|-----------------------------------|
|        | Dopamine a (agonist) | -6.98                     | Asp114 (Salt bridge), Tyr416, Thr119 | Trp386 (Ps-Pi T-shaped), Val115 (P-Alkyl) | -                                 |
|        | Risperidone a (agonist) | -12.7                     | Asp114 (salt bridge), Thr119 | Trp100 (Ps-Pi T-shaped, P-Alkyl), Trp386 (Ps-Pi T-shaped), Val101 (Alkyl), Leu89 (Alkyl), Val86 (Alkyl), Tyr359 (P-Alkyl), Cys114 (P-Alkyl), Ala122 (P-Alkyl) | -                                 |
|        | Butaclamol a (antagonist) | -6.9                      | Asp114 (Salt bridge), Ser193 | Phe389 (P-Pi Stacked, P-Pi T-shaped, P-Alkyl), Tyr416 (P-Pi Stacked), Cys118 (Alkyl), Phe198 (P-Alkyl), Trp386 (P-Alkyl), Phe390 (P-Alkyl) | -                                 |

Table 7. Binding sites and docking scores of compounds on hD3R.

| Target | Compounds       | Binding Energy (kcal/mol) | H-bond Interaction Residues | Hydrophobic Interacting Residues | Electrostatic Interacting Residues |
|--------|-----------------|---------------------------|-----------------------------|----------------------------------|-----------------------------------|
|        | Dopamine a (agonist) | -5.72                     | Asp110 (Salt bridge), Tyr373, Val111, Thr115, Ser196 | Val111 (P-Alkyl), Cys114 (P-Alkyl) |                                  |
|        | Etiopropide a (antagonist) | -9.22                     | Asp110 (Salt bridge), Tyr373 | Phe345 (P-Pi T-shaped), Ile83 (Alkyl), Phe189 (Alkyl), Val111 (P-Alkyl) |                                  |
|        | (+)-butaclamol a (antagonist) | -10.69                   | Asp110(Salt bridge) | Val111 (Alkyl), Cys114 (Alkyl), Trp342 (P-Alkyl), Phe345 (P-Alkyl), Phe346 (P-Alkyl), Val86 (P-Alkyl) |                                  |
|        | (+)-butaclamol a (antagonist) | -10.69                   | Asp110(Salt bridge) | Val111 (Alkyl), Cys114 (Alkyl), Trp342 (P-Alkyl), Phe345 (P-Alkyl), Phe346 (P-Alkyl), Val86 (P-Alkyl) |                                  |

a Reference ligand for hD2L/R.

a Reference ligand for hD3R.
Table 8. Binding sites and docking scores of compounds on hD4R.

| Target       | Compounds          | Binding Energy (kcal/mol) | H-bond Interaction Residues | Hydrophobic Interacting Residues | Electrostatic Interacting Residues |
|--------------|--------------------|---------------------------|----------------------------|----------------------------------|-----------------------------------|
| Dopamine a   | (agonist)          | −6.1                      | Asp115 (Salt bridge), Thr120, Ser196, Tyr438 | Cys119 (Pi-Alkyl), Val116 (Pi-Alkyl), Phe411 (Pi-Pi T-shaped) |                                   |
| Nemonapride a| (agonist)          | −13.08                    | Asp115 (Salt bridge), Tyr438, Ser196 | Val116 (Pi-Sigma), Phe91 (Pi-Pi T-shaped), Phe410 (Pi-Pi T-shaped), Leu90 (Amide-Pi Stacked), Val193 (Alkyl), Leu111 (Pi-Alkyl) |                                   |
| Clozapine a  | (antagonist)       | −10.14                    | Asp115 (Salt bridge)        | Leu187 (Pi-Sigma), Phe410 (Pi-Pi T-shaped), His414 (Pi-Pi T-shaped), Val116 (Alkyl), Pi-Alkyl, Val193 (Pi-Alkyl) |                                   |

$hD_4R$

1 | −9.67                      | Ser196, Leu187, Val430, Thr434 | Val116 (Pi-Sigma, Pi-Alkyl), Leu187 (Pi-Sigma), Thr434 (Pi-Sigma), Phe411 (Pi-Pi T-shaped), His414 (Pi-Pi T-shaped), Phe410 (Pi-Pi T-shaped), Met112 (Alkyl), Cys185 (Alkyl), Cys119 (Alkyl), Pi-Alkyl, Arg186 (Pi-Alkyl) | Asp115 (Pi-Anion) |

2 | −10.34                    | Ser197, Thr434, Asp115, Tyr438 | Val193 (Pi-Sigma), His414 (Pi-Stacked, Pi-Pi T-shaped), Met112 (Alkyl), Leu187 (Alkyl), Pi-Alkyl, Phe91 (Pi-Alkyl), Arg186 (Pi-Alkyl), Val116 (Pi-Alkyl) | Asp115 (Pi-Anion) |

3 | −12.42                    | Leu187, Asp115, Ser196        | Leu187 (Pi-Sigma, Alkyl, Pi-Alkyl), Phe410 (Pi-Pi T-shaped), His414 (Pi-Pi T-shaped), Val116 (Alkyl, Pi-Alkyl), Val193 (Pi-Alkyl) | Asp115 (Pi-Anion) |

a Reference ligand for hD4R.
Figure 9. (a–c) Molecular docking simulation of 1–3 with human dopamine D₁ receptor (hD₁R). (d–f) 2D diagram of the ligand binding sites.
Figure 10. (a–c) Molecular docking simulation of 1–3 with human dopamine D$_{2L}$ receptor (hD$_{2L}$R). (d–f) 2D diagram of the ligand-binding sites.
Figure 11. (a–c) Molecular docking simulation of 1–3 with human dopamine D₃ receptor (hD₃R). (d–f) 2D diagram of the ligand-binding sites.
Figure 12. (a–c) Molecular docking simulation of 1–3 with human dopamine D_4 receptor (hD_4R). (d–f) 2D diagram of the ligand-binding sites.
As shown in Table 5, the 1-D₁R complex exhibited four strong H-bond interactions with Lys81, Leu291, Ser188, and Asp314 with low binding energy (−9.22 kcal/mol). The ligand 2-D₁R complex (−7.1 kcal/mol) interacted with Ser202 and Asp103 in H-bonds, similar to the reference antagonist SCH-23390 and agonist DA. Ligand 3, with a binding energy of −9.2 kcal/mol, shared Ser188 and Asp187 with D₁R via H-bonds.

Furthermore, Asp187, Leu295, Phe306, Pro171, Ala192, and Ala195 were revealed as hydrophobic residues in the ligand 3-D₁R complex (Figure 9). Figure 10 provides a close-up view of ligands 1–3 binding at the active site of D₂L-R. As shown in Table 6, ligands 1–3 bound strongly to the active site of D₂L-R with low binding energies (−8.11 to −10.45 kcal/mol). Risperidone and butaclamol are D₂L-R agonist and antagonist and they bound to the active site of the receptor with binding energies −12.7 and −6.9 kcal/mol, respectively, by forming salt-bridge with Asp114.

Though ligands 1–3 did not form a salt-bridge with Asp114, they showed H-bond and π-anion interactions with the residue. Furthermore, the number of H-bond interactions was higher for test ligands compared to risperidone and butaclamol.

Specifically, Asp114 was an H-bond and electrostatic residue for both ligands 1 and 3, indicating high affinity with the receptor, whereas Ile184 was a crucially active residue in the second extracellular loop of D₂L-R, forming a π-alkyl interaction with 2. Phe189 and Val190 are necessary key residues in antagonist-ligand binding, and they were well observed in the 2-D₂L-R and 3-D₂L-R complexes.

Among the test ligands, 3 showed the highest affinity (−10.41 kcal/mol) for ligand-D₂L-R interactions (Table 7). Ligand 1 had a slightly higher binding energy than 3 but was comparable to that of reference agonist DA (−6.9 kcal/mol).

The key conserved interacting residue, Asp110 in the transmembrane III of D₁R, could be seen in all three ligands-D₂L-R complexes via electrostatic interaction (Figure 11). Other important residues, such as Phe345 and Tyr365, fit tightly into ligands 1–3 via π-π hydrophobic bonds. Val111 at helix III was also observed forming a π-alkyl interaction with both ligands 1 and 3. Ligands 1–3 also interacted with neighboring residues, including His349, Ile183, Thr369, Val86, and Cys114. Similarly, at D₁R, all the test ligands showed strong interactions with lower binding energies (−12.42 to −9.67 kcal/mol) than the reference drugs (Table 8).

DA, nemonapride, and clozapine were used as the reference agonist and antagonist and had binding energies of −6.1, −13.08, and −10.14 kcal/mol, respectively.

One of the most crucial residues in stimulating D₄R, Asp115 interacted with all three ligands in π-anion form at helix III, whereas Ser197 interacted only with 1 and 3 on in helix V (Figure 12). Ligand 3 showed an H-bond interaction with Asp115 and Ser196, which is probably why it had the lowest binding energy (−12.42 kcal/mol) among the three ligands. Similarly, Val116, His344, and Leu187 were common interaction sites for three ligands at D₄R. Other surrounding residues, including Met112, Thr434, Arg186, Phe410, Cys119, and Val193, were involved in hydrophobic interactions with the ligands.

3. Discussion

In the present study, we tested Diels–Alder type adducts 1–3 from the root bark of M. alba and found that they exhibit a mild-to-moderate hMAO inhibition effect. The inhibition effect was slightly higher on the hMAO-B isozyme (IC₅₀: 18.14 to 90.59 µM) than on hMAO-A (IC₅₀: 54.79 to 114.31 µM). In particular, ligand 1 demonstrated a moderate inhibition effect on the hMAO-B isozyme, with an IC₅₀ value of 18.14 ± 1.06 µM. Among test compounds 1–3, 1 and 3 are fused benzofurans, and 2 is a mono-isoprenyl substituted flavone. The structural difference between 1 and 3 is the methyl cyclohexene in 1 and methylbenzene in 3. The methyl cyclohexene group of 1 was involved in specific alkyl interactions with Leu337, Ile335, and Met550 and this moiety is facing toward FAD at the hMAO-A active site (Figure 5). However, the methylbenzene of 3 was not involved in any interactions, which explains why 1 had better binding affinity and inhibition potency on hMAO-A than 3. Likewise, in hMAO-B inhibition, H-bond interaction of 1 with His115, Pro476, and Glu483 might explain for
better binding affinity and activity compared to 3. In addition, 1 and 2 bound in a similar pose and this might explain the same binding mode for both the ligands. The test ligand activity (Ki values) did not show a strong correlation with the docking score (binding energies). This variation might be attributed to the physicochemical properties of ligands, especially logP which was predicted high in the range of 6.7 to 7.3 from web-based software PreADMET (v2.0, YONSEI University, Seoul, Korea) (data not reported here). Overall, the results of the hMAO inhibition assay reveal that these Diels–Alder type adducts, especially 1, might have therapeutic value in managing PD. However, this treatment approach is just symptomatic, restoring dopaminergic function in the striatum [29]. Therefore, the discovery of new natural DA agonists is promising.

Depending on their stimulation or inhibition of adenyl cyclase and modulation of cAMP levels, DA receptors are categorized into two classes: D_{1}-like (D_{1}R and D_{3}R) and D_{2}-like (D_{2}R, D_{3}R, and D_{4}R). These DA receptors have specific anatomical distributions and specifically mediate DA action [30]. Several studies have pointed to DA receptor antagonists as a promising approach to managing heroin addiction. For instance, a D_{1}R antagonist (SCH 23390) and D_{2}R antagonists (haloperidol and raclopride) attenuate heroin-induced reinstatement [31,32], and a D_{3}R antagonist (SB-277011A) blocks the acquisition and expression of the conditioned place preference response to heroin [33]. Similarly, a natural alkaloid 1-tetrahydropalmatine is a D_{1}R/D_{2}R antagonist with an anti-addiction property [34], and govadine (D_{1}R/D_{2}R antagonist) demonstrated antipsychotic properties in conjunction with pro-cognitive effects in rats [35]. The extent of D_{2}R binding affinity and antagonizing ability represent the clinical efficacy of antipsychotic drugs [36]. Previously, a root ethanol extract of *M. alba* mediated skin wound healing by upregulating the mRNA levels of chemokine receptor 4, one of the GPCRs [37]. Other than that, no previous studies have reported on GPCR modulation by an *M. alba* root extract or its metabolites.

To evaluate the functional effects of adducts 1-3 on DA (D_{1}, D_{2}, D_{3}, and D_{4}) receptors, we conducted a cell-based GPCR functional assay. As shown in Figure 8c,d, 1–3 potently and concentration-dependently inhibited the agonist response of DA at D_{1}R and D_{2L}R. Even at 25 µM, 1–3 inhibited the DA response on D_{1}R/D_{2L}R by 92.32/97.16, 91.09/33.69, and 66.82/81.11%, respectively. Unlike sigmoidal dose–response curves of 1 and 2 at D_{1}R, compound 3 showed an unusual non-sigmoidal curve (Figure 8c). A higher standard deviation in response at 50 µM concentration led to unusual appearance. While self-association into colloidal particles at a higher concentration or multi-target actions [38–40] explains the possible reason for the observed non-sigmoidal dose–response curve of the compound 3. Likewise, in the D_{2L}R agonist assay, 2 showed nonspecific interference (NSI) in the assay system. This NSI might be attributed to aggregation/colloid formation or chemical reactivity because these are significant sources of nonspecific bioactivity particularly in high throughput screening (HTS) [41]. NSI by aggregates and colloids is detergent sensitive, so it will be confirmed in the coming report. We compared the binding affinity and interacting residues of test compounds 1–3 with those of a reference agonist (DA) and antagonist (SCH 23390) via a molecular docking simulation. As shown in Tables 5 and 6, 1–3 showed a high binding affinity (the binding energies of 1–3 were lower than those of the reference drugs at D_{1}R and D_{2}R, except for risperidone at D_{2}R). Test ligands 2 shared common H-bond interaction residues, Asp103 and Ser202 with the reference antagonist (SCH 23390). Furthermore, 1–3 displayed additional H-bond interactions with serine residues (Ser107, Ser188, and Ser198) and aspartic acid residues (Asp314 and Asp187). Similarly, at D_{2}R, 1–3 interacted with the key interacting residues Asp114, Trp100, and Phe389 [42]. All three ligands bound to D_{2L}R with high affinity and the binding energy was lower than that of the reference antagonist butaclamol. Interactions with Asp114, Cys118, Phe198, Phe389, Trp386, and Tyr416 were common among the test ligands and butaclamol (Table 6). Additional H-bond interactions between Ser197 and 1, Tyr408 and 2, and Trp100 and 3 were also observed. Residue Ser197 is a conserved-essential residue within the binding site for binding the D_{2}R antagonist risperidone [43], which was also observed for test ligand 2 binding. Tyr408 is located deep in the binding site, whereas Trp100 is at the periphery of the binding site of D_{2}R [44], and they were both involved in the binding of test ligands 2 and 3. According to Salmas et al. [45],
Phe389, Phe390, and Trp386 in TM6 are main residues for D2R-antagonists. Meanwhile, Phe189, Phe198, and Val190 are necessary as key residues for agonist ligands binding. Here, Phe389 and Val190 are interacting with ligand 2 whereas Val190 and Phe189 are bound to ligand 3 as hydrophobic bond. Using those findings, we characterized 1–3 as D1R/D2L antagonists. In a previous study, M. alba leaf extract possessed D2R-mediated anti-dopaminergic activity, suggesting a possible clinical application for M. alba leaves in psychiatric disorders [46]. Our findings suggest that 1–3 could have antipsychotic effects.

The test compounds showed an agonist effect on D1R and D4R. As shown in Figure 8a, b, 1–3 showed a potent agonist effect on D1R and D4R. D2R is prominently distributed within the limbic system and mediates the psychiatric manifestation of DA receptor stimulation. Therefore, DA receptor agonists with high affinity for D2R have an antidepressant effect [47]. Similarly, Levant et al. suggested that D3R-stimulation (rather than D2R-stimulation) might mediate the antiparkinsonian effects of DA receptor agonists with a high preference for D2R [48]. Rotigotine is an FDA-approved, full DA agonist (rank order: D3R > D2L > D1R = D3R > D4R) developed as a transdermal patch for the treatment of PD [1,49].

A previously conducted survey reported that more than 110 patent applications had been submitted concerning selective D1R ligands [50]. Unfortunately, none of them has yet received clinical approval due to failures of the pharmacokinetics or safety profiles [51]. Similarly, D1R agonism has been implicated in the management of cognitive deficits associated with schizophrenia [52] and attention-deficit/hyperactivity disorder [53] and also to reduce the adverse effects of opioids [54].

The results of the functional assays in this study show that the ligands 1–3 have concentration-dependent agonist effects on D1R and D4R (rank order: D3R > D4R). Even at 25 µM, 1–3 showed potent agonist responses on DA D3R/D4R of 71.92/63.00, 64.99/58.66, and 94.93/-%, respectively. The agonist effect of 3 on D4R was mild (% stimulation of agonist response of 44.85% at 100 µM). The antagonist effect on these receptor subtypes was negligible. We also used molecular docking simulations to compare the binding affinity and interacting residues between test compounds 1–3 and D3R (Table 7) with those of reference agonists DA and antagonists (eticlopride and (+)-butaclamol). Likewise, docking simulations of 1–3 and D4R was compared with those of reference agonists DA and nemonapride, and an antagonist clozapine (Table 8). As tabulated in Tables 7 and 8, the binding energies of 1–3 on D3R/D4R were comparable to the reference ligands. Interestingly, our prediction demonstrated that they had lower binding energy at D4R than at D3R. Interaction with Asp110 on D3R and Asp115 on D4R was in common with the agonist DA. It was reported earlier that a salt-bridge to the carboxylic acid group of the Asp110 on D3R and the Asp115 on D4R is critical to high-affinity ligand binding to dopaminergic receptors [55]. In this study, though ligands 1–3 did not form a salt-bridge with those receptors, they did form strong electrostatic interactions (Pi-Anion). In addition to their electrostatic interactions with Asp115 on D4R, 2 and 3 formed H-bond interactions with carboxylic acid group of Asp115.

At a molecular level, D1-like (D1 and D2) receptor signaling is mediated chiefly by the heterotrimeric G proteins Goαs/olf, which cause sequential activation of adenylate cyclase, cyclic AMP-dependent protein kinase, and the protein phosphatase-1 inhibitor DARPP-32 [56]. A recent study showed that hypersensitivity of D1R is responsible for 1-DOPA-induced activation of mTORC1 signaling, and D1R antagonist (SCH23390) blocked the 1-DOPA-induced phosphorylation of p70 S6 kinase (S6K), ribosomal protein S6, and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) in 6-OHDA-lesioned mice [57]. Moreover, DA through D1R induces ERK stimulation via a cAMP/protein kinase A (PKA)/Rap1/B-Raf/MAPK/ERK kinase (MEK) pathway and SCH 23,390 completely blocks the p-ERK1/2 levels induced by DA [58].

Likewise, D2-like (D2, D3, and D4) receptor signaling is mediated by the heterotrimeric G proteins Goαs/olf, which causes inhibition of adenylate cyclase thereby decreasing the phosphorylation of PKA substrates. Binding of DA to DA receptors regulate signaling via cAMP response element-binding protein (CREB), glutamate receptors, GABA receptors, and ion channels (e.g.,
calcium and potassium) [59]. Previous study reports that stimulation of D2-like receptors decreases PKA-stimulated phosphorylation of DARPP-32 at Thr34 and increases phosphorylation at Thr75 [60,61]. Even though DARPP-32 is an important modulator and/or effector of DA receptors signaling, it is not the only modulator of DA-mediated activities [62]. The test compounds of the present study showed a unique profile, i.e., moderate hMAO inhibition with good D₁R/D₂L antagonist and D₃R/D₄R agonist effect. So, what could be the underlying mechanism and in vivo effect is very interesting and need to be studied shortly.

4. Materials and Methods

4.1. Chemicals and Reagents

Mulberrofuran G (1), kuwanon G (2), and albanol B (3) were isolated and identified from the root bark of M. alba Linn following a method described previously [63]. The purity of these compounds was considered to be >98% as evidenced by spectral data. A MAO-GloTM assay kit was purchased from Promega (Promega Corporation, Madison, WI, USA). Transfected Chinese hamster ovary (CHO) cells were obtained from Eurofins Scientific (Le Bois l’Eveque, France). Hank’s balanced salt solution (HBSS), Dulbecco’s modified Eagle medium, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer were obtained from Invitrogen (Carlsbad, CA, USA). The hMAO isozymes and reference drugs selegiline, DA, serotonin, butaclamol, SCH 23390, clozapine, and (S)-WAY-100635 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2. In Vitro Human MAO Inhibition and Enzyme Kinetics

The potential of the test compounds for human MAO inhibition was evaluated via a chemiluminescence technique using the MAO-Glo kit (Promega, Madison, WI, USA). Detailed experimental conditions and procedures were reported previously [64,65]. The test compounds were evaluated at a concentration of 6, 30, and 120 µM. Selegiline was used as a positive control. The kinetic analysis of hMAO inhibition was analyzed at different concentrations of hMAO substrate depending on the isozyme (40, 80, and 160 µM for hMAO-A and 4, 8, and 16 µM for hMAO-B) following the same method of enzyme inhibition. The concentrations of the test compounds for the kinetic study are presented in Figures 2 and 3. Kinetic parameters were analyzed using SigmaPlot (v12.0, SPP Inc., Chicago, IL, USA).

4.3. Cell-Based Functional GPCR Assay

Cell-based functional GPCR assays were conducted in CHO cells transfected with a plasmid containing the GPCR gene of interest. The functional activity of the test compounds (agonist or antagonist) was evaluated by measuring their effects on cAMP modulation or Ca²⁺ ion mobilization, depending on the receptor type. All assays were performed at Eurofins Cerep (Le Bois l’Eveque, France) following their in-house protocol, as stated in our previous reports [66–68].

4.4. Measurement of cAMP Level

The functional activity of the test compounds on D₁R, D₃R, and D₄R was assessed by evaluating the effect on cAMP modulation. For this, stable transfectants (CHO-D₁R, CHO-D₃R, and CHO-D₄R) were suspended in HBSS (Invitrogen, Carlsbad, CA, USA) containing 20 mM HEPES buffer and 500 µM 3-isobutyl-1-methylxanthine, distributed into microplates (5 × 10³ cells/well), and incubated for 30 min at room temperature (RT) in the absence (control) or presence of the test compounds (6.25, 12.5, 25, 50, and 100 µM) or reference agonist (DA). In the D₃R and D₄R assays, the adenylyl cyclase activator NKH 477 was added at a final concentration of 1.5 and 0.7 µM and incubated for 30 and 10 min, respectively, at 37 °C. Then, the cells were lysed and a fluorescence acceptor (D2-labeled cAMP) and fluorescence donor (anti-cAMP antibody with europium cryptate) were added. The fluorescence transfer was measured at λex = 337 nm and λem = 620 and 665 nm using a microplate reader (Envision,
Perkin Elmer, Waltham, MA, USA) after 60 min of incubation at RT. Agonist effects are expressed as the % of the control response to 10 µM DA for \( D_1R \) and 300 nM DA for \( D_3R/D_4R \). Similarly, antagonist effects are expressed as the % inhibition of the control response to DA 300 nM for \( D_1R \), 10 nM for \( D_3R \), and 100 nM for \( D_4R \). The reference agonist DA and antagonists SCH 23390, (+)-butaclamol, and clozapine were used to validate the study.

4.5. Measurement of Intracellular \([Ca^{2+}]\) Levels

The functional activity of the test compounds on \( D_2R \) was tested by fluorimetrically evaluating their effect on cytosolic \( Ca^{2+} \) ion mobilization. In brief, CHO-\( D_2L \)R cells were separately suspended in HBSS (Invitrogen, Carlsbad, CA, USA) complemented with 20 mM HEPES buffer and distributed into microplates (1 × 10^5 cells/well). Then, a fluorescent probe (Fluo8, AAT Bioquest) mixed with probenecid in HBSS (Invitrogen, Carlsbad, CA, USA) supplemented with 20 M HEPES (Invitrogen) (pH 7.4) was added to each well, and the cells were allowed to equilibrate for 60 min at 37 °C. Thereafter, the plates were positioned in a microplate reader (FlipR Tetra, Molecular Device), and compounds 1–3 (6.25, 12.5, 25, 50, and 100 µM), reference agonist, or HBSS (basal control) were added. We then measured the fluorescent intensity, which varied in proportion to the free cytosolic \( Ca^{2+} \) ion concentration. Agonist effects are expressed as the % of the control response to 10 µM DA. Similarly, antagonist effects are expressed as the % inhibition of the control response to 700 nM DA. Reference agonist (DA) and antagonist (butaclamol) were used to validate the study.

4.6. Homology Modeling

The primary sequence of the human DA \( D_1 \) receptor was obtained from UniProt (ID: P21728, DRD1_HUMAN). The \( \beta_2R \) (\( \beta_2 \) adrenergic receptor) has a higher similarity to DA \( D_1R \) in the binding site region and sequence identity [28]. Hence, the model was built on the template of the \( \beta_2R \) crystal structure from the RCSB protein data bank (PDB) using ID 2RH1 with SWISS-MODEL. Refining the model was conducted using the ModRefiner sever [69].

4.7. In Silico Molecular Docking Simulation

Automated single docking simulations were carried out with AutoDock 4.2 [70]. X-ray crystallographic structures of \( hMAO-A \), \( hMAO-B \), \( hD_2L \), \( hD_3 \), and \( hD_4 \) were obtained from the PDB with IDs 2BXR, 2BYB, 6CM4, 3PBL, and 5WIV, respectively. The 3D chemical structures of the three test compounds were obtained from PubChem Compound (NCBI, CIDs 196583, 5281667, and 480,819 for compounds 1–3, respectively). The crystal structures of the reference compounds, selegiline, harmine, DA, SCH 23390, risperidone, butaclamol, eticlopride, nemonapride, and clozapine were also obtained from NCBI under CIDs 26758, 5280953, 681, 5018, 5073, 37461, 57267, 156333, and 135398737, respectively. Water and ligand molecules were removed using Discovery Studio (v17.2, Accelrys, San Diego, CA, USA). In the case of the \( hMAO \) isozymes, the cofactor flavin adenine dinucleotide (FAD) was retained. The Lamarckian genetic algorithm method in AutoDock 4.2 was applied. For the docking calculations, Gasteiger charges were added by default, and all the torsions were allowed to rotate. The grid maps were generated with the AutoGrid program. The docking protocol for rigid and flexible ligand docking consisted of 10 independent genetic algorithms, and other parameters were set using the defaults in the AutoDock Tools. The docking results were visualized using Discovery Studio.

5. Conclusions

This study is the first to report the therapeutic potential of natural Diels–Alder type adducts, mulberrofuran G (1), kuwanon G (2), and albanol B (3) from \( M. alba \) root bark in neurodegenerative diseases. Our investigations identified 1–3 as novel multi-target-directed ligands for the management of neurodegenerative diseases via \( hMAO \) inhibition and dopaminergic receptor modulation. Specifically,
cell-based GPCR functional assays in vector-transfected CHO cells expressing DA receptors characterized 1-3 as potent D₁R/D₂L antagonists and D₃R/D₄R agonists. The assay results were further supported by molecular docking studies, which predicted tight binding between the test ligands and the receptors. Overall, the results of this study provide evidence that ligands 1-3 from M. alba could be developed into neuronal drugs targeting DA receptors. Further in vivo studies are warranted to fully and precisely characterize the mechanism of action via a signal transduction pathway.

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