In Vitro and in Silico Human Monoamine Oxidase Inhibitory Potential of Anthraquinones, Naphthopyrones, and Naphthalenic Lactones from *Cassia obtusifolia* Linn Seeds

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**ABSTRACT:** In recent years, Cassia seed extract has been reported as a neuroprotective agent in various models of neurodegeneration, mainly via an antioxidant mechanism. However, no one has previously reported the effects of Cassia seed extract and its phytochemicals on human monoamine oxidase (hMAO) enzyme activity. The seed methanol extract, the solvent-soluble fractions, and almost all isolated compounds displayed selective inhibition of hMAO-A isozyme activity. Interestingly, compounds obtusin (3), alaternin (8), aloe-ermodin (9), questin (12), rubrofusarin (13), cassiaside (15), toralactone 9-O-β-gentiobioside (26), and (3S)-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-3,4-dihydro-1H-benzo[g]-isochromene-3-carboxylic acid 9-O-β-D-glucopyranoside (38) showed the most promising inhibition of the hMAO-A isozyme with IC$_{50}$ values of 0.17–11 μM. The kinetic study characterized their mode of inhibition and molecular docking simulation predicted interactions with Ile-335 and Tyr-326 in support of the substrate/inhibitor selectivity in respective isozymes. These results demonstrate that Cassia seed extract and its constituents inhibit hMAO-A enzyme activity with high selectivity and suggest that they could play a preventative role in neurodegenerative diseases, especially anxiety and depression.

**INTRODUCTION**

The typical structural and physiological properties of brain neurons decline with aging, accompanied by variable degrees of cognitive decline (Alzheimer’s disease; AD), movement disorder (Parkinson’s disease; PD), and excitotoxicity (Huntington disease; HD), which are often grouped together as neurodegenerative diseases (NDs). The etiopathology and affected brain regions differ among the NDs; however, they all share progressive dysfunction, collapse the neuronal network, neuronal cell death, neuroinflammation, and oxidative damage to the brain. Among the greatest health threats of the 21st century, cognitive frailty demands a great deal of attention. The incidence of NDs increases with age, and the population in developed countries is rapidly aging. The number of people with PD is expected to reach 8.7 million by 2040, so it will claim an increasing portion of world healthcare budgets, with an estimated current cost in excess of US$600 billion worldwide. PD is a chronic and progressive disorder of the central nervous system that affects the motor system. It is the second most frequent neurodegenerative disorder and is characterized by resting tremor, rigidity, and bradykinesia due to the loss of dopaminergic (DA) neurons of the midbrain. Though PD is a subject of great concern, its exact etiology remains unclear. Researchers have postulated that oxidative stress, mitochondrial dysfunction, neuroinflammation, and apoptosis lead to DA neuronal damage. In PD, progressive loss of DA neurons occurs in the substantia nigra pars compacta. Human monoamine oxidase (hMAO) is a flavoenzyme that catalyzes the oxidative deamination of various bioamines; its inhibitors were the first developed antidepressants. Because the major depressive disorder is estimated to be the second leading cause of disease worldwide, the need for hMAO inhibitors is urgent. hMAO has two isozymes (hMAO-A and -B) that differ in their sensitivity to inhibitors and substrate specificity.

MAO is an enzyme of crucial interest because it catalyzes the major inactivation pathway for the catecholamine neurotransmitters: adrenaline, noradrenaline, dopamine, and even 5-hydroxytryptamine. MAO is responsible for alterations in the level of neurotransmitters in the central nervous system, and imbalanced neurotransmitter levels are linked to the...
biochemical pathology of many neurogenic disorders, including depression, AD, and PD. Studies of the therapeutic effect of MAO enzyme inhibitors in depression have characterized two isomeric forms, hMAO-A and hMAO-B. The selectivity of a molecule to these isoforms determines its therapeutic activity. hMAO-A is responsible for the metabolism of norepinephrine, serotonin, and tyramine. Therefore, hMAO-A selective inhibitors have antidepressant activity. On the other hand, hMAO-B selectively metabolizes dopamine, so selective hMAO-B inhibitors have been used to treat PD.

Because MAO involves the oxidative deamination of primary, secondary, and tertiary amines to their corresponding aldehydes and free amines, which generates hydrogen peroxide (H2O2), oxidative stress is believed to be involved in a variety of NDs, including AD, PD, and amyotrophic lateral sclerosis, because neurons are sensitive to H2O2. Similarly, amyloid β protein toxicity in AD is caused by increased levels of H2O2 and the accumulation of lipid peroxides. Therefore, the development of new hMAO inhibitors from natural sources has attracted significant attention. Cassia obtusifolia Linn seed is well-known in traditional Chinese medicine (TCM) for its pronounced effects, including as a vision improver, aperient, diuretic, antiasthenic, cholesterol-lowering agent, and blood-pressure reducer. The seed extract has also been reported as a therapy for NDs. In a current report, Cassia obtusifolia seed extract protected the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced degeneration of DA neurons in the substantia nigra and striatum of PD mice. Furthermore, Cassia seed extract exhibited neuroprotective effects via its anti-inflammatory effects, upregulation of BDNF expression, and CREB phosphorylation. Cassia seed also has a long history of use against inflammation, type II diabetes, hepato toxicity,25 bacterial infections,27 oxidative stress,29 tumor progression,30 and mutagenesis.31 Recently, we have reported the possible role of rubrofusarin in comorbid diabetes and depression via PTP1B and hMAO inhibition. However, despite many reports about the different pharmacological actions of the seed extract, reports on the specific compounds that produce particular activities are insufficient. Therefore, to be more specific, we evaluated the hMAO inhibition potential of isolated compounds (Figure 1) and performed enzyme kinetics and molecular docking simulations.

**RESULTS**

**In Vitro Human MAO Inhibition by a MeOH Extract and Solvent-Soluble Fractions of *C. obtusifolia* Linn Seeds.** The methanol extract of Cassia obtusifolia Linn seeds and its different solvent-soluble fractions were evaluated for their potential to inhibit hMAO. As presented in Table 1, the methanol extract of Cassia seeds demonstrated good inhibition against both isozymes of the hMAO enzyme, hMAO-A (IC50: 86.89 ± 3.80 μg/mL), and hMAO-B (IC50: 196.00 ± 6.82 μg/mL). Among the solvent-soluble fractions, the EtOAc fraction showed the most potent inhibition of hMAO-A activity (IC50: 20.82 ± 5.04 μg/mL), followed by the CH2Cl2 fraction (IC50: 40.06 ± 3.58 μg/mL) and the n-BuOH fraction (IC50: 93.49 ± 0.27 μg/mL).

![Figure 1. Chemical structures of compounds isolated from *C. obtusifolia* Linn seeds.](image1)

### Table 1. Human MAO-A and MAO-B Inhibition by the Methanol Extract and Solvent-Soluble Fractions of *C. obtusifolia* Seeds

| samples                        | IC50 value for MAO isozymes (μg/mL ± SD) |
|-------------------------------|-----------------------------------------|
|                               | hMAO-A (n = 3)                          | hMAO-B (n = 3)                          | SI† |
| methanol extract              | 86.89 ± 3.80                            | 196.00 ± 6.82                           | 2.25 |
| CH2Cl2 fraction               | 40.06 ± 3.58                            | 98.51 ± 12.53                           | 2.46 |
| EtOAc fraction                | 20.82 ± 5.04                            | 96.28 ± 3.13                            | 2.70 |
| n-BuOH fraction               | 93.49 ± 0.27                            | 246.50 ± 10.31                          | 2.64 |
| deprenyl HCl                  | 9.19 ± 0.54                             | 0.12 ± 0.03                             | 0.013 |
| clorgyline                   | 0.00397 ± 0.75                         | 63.41 ± 1.20                           | 15 972.29 |

†The SI (selectivity index) is defined as the hMAO-B to hMAO-A ratio of IC50 values.

**Additional Notes:**

- The IC50 values for various compounds and fractions are provided in Table 1. The table includes the IC50 values for the methanol extract and different solvent-soluble fractions of *C. obtusifolia* seeds for hMAO-A and hMAO-B.
- The selectivity index (SI) is calculated by dividing the IC50 of hMAO-B by the IC50 of hMAO-A.
- The selectivity index for each fraction is also provided in the table.
The selective index (SI) was determined by the ratio of hMAO-B IC50/hMAO-A IC50.

Selective hMAO-B reference inhibitor, and values were extracted from the literature.54

The 50% inhibitory concentration (IC50) values (μM) were calculated from a log dose inhibition curve and expressed as the mean ± SD of triplicate experiments. bThe selective index (SI) was determined by the ratio of hMAO-B IC50/hMAO-A IC50. cSelective hMAO-A reference inhibitor, and values were extracted from the literature.44

In Vitro Human MAO Inhibition by Anthraquinones. A total of twenty-three anthraquinones were isolated in the form of either aglycones or glycosides. As tabulated in Table 2, all anthraquinones except for 1, 2, and 7 showed moderate to potent inhibition of hMAO-A isozyme activity. Potency varied depending on the nature, number, and position of the functional groups in the anthracene-9,10-dione (anthraquinone) skeleton. The most promising inhibitor of hMAO-A was 12, which had an IC50 value of 0.17 ± 0.01 μM, followed by 9 (IC50: 2.47 ± 0.14 μM), 8 (IC50: 5.35 ± 0.09 μM), and 4 (IC50: 11.12 ± 0.60 μM). In addition to their hMAO-A

Table 2. Human MAO-A and MAO-B Inhibition by Compounds from Cassia obtusifolia Seeds

| compounds | hMAO-A (n = 3) | hMAO-B (n = 3) | SI | IC50 (μM)a |
|-----------|----------------|----------------|----|------------|
| naphthopyrones |  |  |  |  |
| rubrofusarin (13) |  |  |  |  |
| cassioside (15) |  |  |  |  |
| isorubrofusarin 10-O-β-D-glucopyranoside (20) |  |  |  |  |
| rubrofusarin 6-O-β-D-glucopyranoside (22) |  |  |  |  |
| rubrofusarin 6-O-β-D-gentiobioside (28) |  |  |  |  |
| isorubrofusarin gentiobioside (29) |  |  |  |  |
| rubrofusarin 8-O-β-D-glucopyranoside (32) |  |  |  |  |
| rubrofusarin triglucoside (33) |  |  |  |  |
| rubrofusarin tetroglucoside (cassioside B2) (35) |  |  |  |  |
| naphthalenes and naphthalenic lactones |  |  |  |  |
| cassitrose (16) |  |  |  |  |
| 1-hydroxy-2-acetyl-3,8-dimethoxy-naphthalene 6-O-β-D-glucopyranosyl (1 → 2)-β-D-glucopyranoside (19) |  |  |  |  |
| toralactone 9-O-β-D-gentiobioside (26) |  |  |  |  |
| cassialactone 9-O-β-D-gentiobioside (27) |  |  |  |  |
| (3R)-cassialactone 9-O-β-D-glucopyranoside (37) |  |  |  |  |
| (3S)-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-3,4-dihydro-1H-benzo[g]isochromene-3-carboxylic acid 9-O-β-D-glucopyranoside (38) |  |  |  |  |
| deprenyl c |  |  |  |  |
| clorgline d |  |  |  |  |

The 50% inhibitory concentration (IC50) values (μM) were calculated from a log dose inhibition curve and expressed as the mean ± SD of triplicate experiments. The selective index (SI) was determined by the ratio of hMAO-B IC50/hMAO-A IC50. Selective hMAO-A reference inhibitor, and values were extracted from the literature.44

4.06 ± 3.58 μg/mL) and n-BuOH fraction (IC50: 93.49 ± 0.27 μg/mL). Similarly, the EtOAc fraction showed the most potent hMAO-B inhibition (IC50: 56.28 ± 3.13 μg/mL), followed by the CH2Cl2 fraction (IC50: 98.51 ± 12.53 μg/mL) and n-BuOH fraction (IC50: 246.50 ± 10.31 μg/mL). All of the solvent-soluble fractions inhibited hMAO-A better than hMAO-B, with a selectivity index (SI) of approximately 2.5. From each fraction, we isolated compounds with different skeletons (anthraquinone, naphthopyrone, naphthalene, and naphthalenic lactone) and evaluated their ability to inhibit hMAO. The inhibition result is presented in Table 2.
Figure 2. Michaelis–Menten plots, Lineweaver–Burk plots, and secondary plots of compound 3 (A, B, C, and D), 8 (E, F, G, and H), and 9 (I, J, K, and L) for hMAO-A inhibition.

Figure 3. Michaelis–Menten plots, Lineweaver–Burk plots, and secondary plots of compound 12 (A, B, C, and D), 15 (E, F, G, and H), 26 (I, J, K, and L), and 38 (M, N, O, and P) for hMAO-A inhibition.

Isozyme inhibition, 8 and 12 exhibited the best inhibition of hMAO-B among these compounds. Only a few compounds were active against hMAO-B, indicating that the anthraquinones have preferential/selective inhibition of hMAO-A. Glycosylation of the active aglycones retarded activity; the pattern of activity was aglycones > glucopyranoside >
In Vitro Human MAO Inhibition by Naphthalenes and Naphthopyrones. Six naphthalenes and naphthopyrones were evaluated for their hMAO inhibitory potential. Unlike the activity pattern for the anthraquinone glycosides, inhibition of hMAO-B activity was moderate only for gentiobioside > triglucoside > tetraglucoside. Interestingly, for inactive aglycones 1 and 2, glycosylation induced inhibition of isozyme-A but not isozyme-B.

In Vitro Human MAO Inhibition by Naphthalenes and Naphthopyrones. The list of isolated naphthalenes (Table 2), 13, 15, and 20 were the most active against the hMAO-A isozyme, with IC$_{50}$ values of 5.90, 11.26, and 16.32 μM, respectively. Inhibition of hMAO-B activity was moderate only for 20 and was mild for the other glycosides. As presented in Table 2, glycosylation of 13 retarded its inhibition of the MAO enzyme.

In Vitro Human MAO Inhibition by Naphthalenes and Naphthopyrones. Six naphthalenes and naphthopyrones were evaluated for their hMAO inhibitory potential (Table 2). These compounds were obtained as glycosides. Compounds 26 and 38 had IC$_{50}$ values < 10 μM for hMAO-A. Interestingly, 26 did not show any observable inhibitory effect on hMAO-B up to 400 μM, whereas 38 moderately inhibited isozyme-B, with an IC$_{50}$ value of 96.15 ± 3.35 μM. Compounds 16, 19, 27, and 37 moderately inhibited isozyme-A, but their activity toward isozyme-B was negligible.

Overall, the enzyme inhibition assay showed that compounds 3, 8, 9, 12, 13, 15, 26, and 38 were the most potent (IC$_{50}$: 0.17 to 11.26 μM) and highly selective for hMAO-A inhibition.

Enzyme Kinetics of hMAO Inhibition. Because compounds 3, 8, 9, 12, 15, 26, and 38 most potently inhibited the hMAO isozymes, we selected them for the enzyme kinetics study. Enzyme kinetics of 13 has been reported in our recent report. The results of the kinetic study are presented using Michaelis–Menten plots, Lineweaver–Burk plots, and secondary plots (Figures 2–4) and summarized in Table 3. As tabulated in the table, 26 and 38 showed selective and noncompetitive inhibition of hMAO-A, with $K_i$ values of 4.30 and 9.57 μM, respectively, and 9 showed a competitive mode of hMAO-A inhibition, with a $K_i$ value of 0.5 μM. Similarly, the mode of selective hMAO-A isozyme inhibition by 15 was mixed-type, with a $K_i$ value of 6.26 μM and a $K_{su}$ value of 12.32 μM. The mode of inhibition of the equipotent compound 8 over both isozymes was also a mixed-type. For hMAO-A inhibition, it showed a $K_i$ value of 3.97 μM and a $K_{su}$ value of 9.33 μM, and for hMAO-B inhibition, it had a $K_i$ value of 2.41 μM and a $K_{su}$ value of 4.46 μM. The mode of hMAO-A inhibition by 3 and 12 was competitive. The anthraquinone 12 exhibited the most potent inhibition of hMAO-A, with a $K_i$ value of 0.13 μM. In addition, it showed competitive inhibition of hMAO-B, with a $K_i$ value of 4.14 μM.

In Silico Molecular Docking Simulation of hMAO Inhibition. Small changes in a parent structure affected the...
potency and selectivity of hMAO isozyme inhibition in the in vitro assay. Therefore, to explain the variations in potency and selectivity over the two isozymes, we used AutoDock 4.2 to study the docking of the active compounds in the site cavities of hMAO-A (2z5z) and hMAO-B (2v5z) (Figure 5). The most active compounds (3, 8, 9, 12, 13, 15, 26, and 38) were selected to predict their binding energies and the residues that interact with the enzyme. The reference inhibitors harmine and safanimide were used to validate the docking results for the A- and B-isozymes, respectively. The molecular docking simulation results are tabulated in Tables 4 and 5 and depicted in Figures 5–8.

Molecular Docking Simulation of hMAO Inhibition by Anthraquinones. As tabulated in Table 4, ligands 3, 8, 9, and 12 bound competitively to the catalytic site with the best pose, as indicated by their low binding energies. The lowest binding energy was predicted for the most potent compound 12 (−9.37 kcal/mol), followed by 8 and 9 (approx. −8.80 kcal/mol) and 3 (−8.51 kcal/mol). In addition to the catalytic site, 8 bound to the allosteric site with a binding energy of −8.30 kcal/mol, demonstrating that it is a mixed-type inhibitor.

Ligands 3, 8, 9, and 12 have a 9,10-dioxoantracene scaffold in common, with different substituents in the peripheral aromatic rings that played a key role in the ligand–enzyme interaction, thereby affecting the potency and selectivity of their hMAO inhibition. The 2-OH group of 3 was involved in the formation of H-bonds that interacted with Cys323 and Thr336 at the active catalytic site of hMAO-A (Figure 6A,B). Similarly, other predicted interacting residues were Ile-335, Tyr-407, Phe208, and Leu180, and Leu337, which possibly stabilized the enzyme–ligand complex. As depicted in the enzyme kinetics, 8 bound to both the catalytic (−8.82 kcal/mol) and allosteric (−8.30 kcal/mol) sites of the hMAO-A enzyme. At the catalytic site, OH groups at the 1-, 3-, and 8-positions formed H-bonds with Phe208, Thr336, and Asn181, respectively (represented by the green dotted lines in Figure 6D).

Similarly, the 6-methyl group was involved in the interaction with FAD, Tyr-407, and Tyr-444. In contrast, at the allosteric site, the 7-OH group interacted with Ala111 and Phe112 via H-bonding (Figure 7B). In addition, OH groups at the 1- and 3-positions displayed H-bond interactions with His488 and Asn125. However, no interaction was observed with the 8-OH group at the allosteric site. Compound 9 bound to the catalytic site of the hMAO-A enzyme with −8.80 kcal/mol binding energy by forming two H-bond interactions. Specifically, the 1-OH group and the 3-hydroxymethyl group showed H-bond interactions with FAD and Tyr197, respectively (Figure 6E,F). In addition, three aromatic rings interacted with Tyr-407 and Ile-335, and the 8-OH group interacted with Ile180. The most active compound (12, which had a submicromolar IC<sub>50</sub> value) displayed the lowest binding energy (−9.37 kcal/mol) among the test and reference compounds, forming H-bond interactions with Tyr-444, FAD, and Phe208. In addition, Tyr-444 and FAD were involved in π–σ and π–alkyl interactions (Figure 6G,H).

Similarly, two compounds (8 and 12) exhibited potent inhibition of hMAO-B enzyme activity. Therefore, these two compounds were docked in the crystal structure of the hMAO-B enzyme (2v5z) (Figure 8), and the binding energies and predicted interactions with amino acid residues are presented in Table 5. Safanimide, a coligand of 2v5z, was used as a reference ligand to validate the docking results. As shown in Table 5, compound 8 bound to the catalytic site with the minimum binding energy (−8.85 kcal/mol), and its key interactions involved H-bonds (Tyr-435, Cys172, Tyr-326), π–σ (Leu171, Tyr-398, FAD), π–π stacked (Tyr-398, Tyr-435, Tyr-326), and π–alkyl (Leu171, Cys172, and Ile199) interactions (Figure 7A,D). Similarly, at the allosteric site, 8 formed five H-bonds (Gln206, Pro102 × 2, Ile199, and Leu171) and two π–σ (Leu171 and Ile199), alkyl (Pro104 and Leu164), and π–alkyl (Trp199 and Ile199) interactions with a binding energy of −8.42 kcal/mol (Figure 7B,E). Compound 12, on the other hand, bound to the catalytic site by forming major H-bond interactions with FAD, Cys172, and Tyr-435 (Figure 7C,F). Other interacting residues that possibly stabilized the hMAO-B–12 complex were Tyr-435, FAD, Tyr-398, Tyr-326, Leu171, Ile199, Leu171, Cys172, and Ile198. Compounds 8 and 12 shared common interacting...
Table 4. Molecular Interaction of the hMAO-A (2z5x) Active Site with Active Compounds and the Reported Inhibitor Harmine

| compounds | energy (kcal/mol) | interaction residues |
|-----------|------------------|----------------------|
| 3         | −8.51            | H-bond: Cys323, Thr336 |
|           |                  | pi-sigma: Ile335, Tyr444, FAD, pi-sulfur: Cys323, pi-pi stacked: Tyr407, pi-pi T-shaped: Phe208, alkyl: Cys323, Ile325, pi-alkyl: phe208, Ile180, Ile335, Leu337 |
| 8         | −8.82            | H-bond: Asn181, Phe208, Thr336 |
|           |                  | pi-sigma: Ile335, Tyr407, pi-sulfur: Cys323, pi-pi stacked: Tyr407, pi-alkyl: Tyr444, FAD, Ile335, Leu337 |
| 9         | −8.30            | H-bond: Ala111, Tyr121, His488, Phe112, Asn125 |
|           |                  | pi-anion: Gln492, pi-sigma: Phe112, pi-pi T-shaped: Trp128 |
| 12        | −8.80            | H-bond: Tyr197, FAD |
|           |                  | pi-pi stacked: Tyr407, pi-alkyl: Ile180, Ile335 |
| 15        | −8.42            | H-bond: Glu215, Cys323, Ile180, Asn181 |
|           |                  | pi-sigma: Tyr444, pi-pi stacked: Tyr407, pi-alkyl: Tyr407, FAD, Ile180, Ile335 |
|           | −7.59            | H-bond: Arg172, Asp328, Gla327, Gla329 |
|           |                  | pi-pi T-shaped: Tyr173, alkyl: Pro186, pi-alkyl: His187, Pro186, Arg172, Leu176 |
| 26        | −6.06            | H-bond: Thr276, Met300, Tyr410, Gln188 |
|           |                  | pi-alkyl: Ala302, Cys398 |
| 38        | −7.83            | H-bond: Asp328, Glu329, Lys357, Gln327 |
|           |                  | pi-anion: Gln329, alkyl: Pro186, pi-alkyl: His187 |
| harmine   | −8.5             | H-bond: Asn181 |
|           |                  | pi-sigma: Tyr444, pi-pi stacked: Tyr407, alkyl: Ile180, pi-alkyl: Phe208, Ile180, van der Waals: FAD |

Binding energy, which indicates binding affinity and capacity for the active site of hMAO-A. All amino acid residues from the enzyme–inhibitor complexes were determined using AutoDock 4.2 and Discovery studio. Catalytic inhibition mode. Allosteric inhibition mode. Reported hMAO-A inhibitor (coligand of 2z5x).
residues (Tyr-435, FAD, Ile199, Tyr-398, Cys172, and Leu171) with the reference ligand (safinamide).

Molecular Docking Simulation of hMAO Inhibition by Naphthopyrones. The active glycoside 15 bound to the allosteric cavity of hMAO-A with a binding energy of −7.59 kcal/mol by forming H-bond interactions with Asp328, Arg172, Glu327, and Glu329 and having other interactions with His187, Leu176, Pro186, and Tyr175. Interestingly, multiple H-bond interactions were observed between Glu327, Glu329, and the −OH groups of the sugar moiety of this glycoside. Furthermore, 15 bound to the catalytic site with higher affinity by forming four H-bond interactions (Gln206 and Cys323 with the glycosyl −OH group and Ile180 and Asn181 with the 10-OH group), as shown in Figure 6IJ.

Molecular Docking Simulation of hMAO Inhibition by Naphthalenes and Naphthalenic Lactones. Compounds 26 and 38, two active glycosides, bound to the allosteric cavity of hMAO-A with binding energies of −6.06 and −7.83 kcal/mol, respectively. With higher binding energy, compound 38 bound to the allosteric site by forming H-bond interactions with Asp328, Glu327, Glu329, and Lys357 and other interactions with His187 and Pro186. Interestingly, multiple H-bond interactions were observed between Glu327, Glu329, and the −OH groups from the sugar moieties at the C-9 position, the −OH group at the C-10 position, and the ketone group at the C-1 position displayed multiple H-bond interactions with four residues (Thr276, Met300, Tyr410, and Glu188) with a binding energy of −6.06 kcal/mol. In addition, an aromatic ring was involved in a π–alkyl interaction with Ala302 and Cys398. Though both of these inhibitors have a common parent structure and bound to the allosteric pocket, their interacting residues were

| compounds | energy (kcal/mol) | interaction residues |
|-----------|------------------|---------------------|
| 8         | −8.85            | H-bond: Tyr435, Cys172, Tyr326 |
|           | −8.42            | pi-sigma: Leu171, Tyr398, FAD, pi-sulfur: Cys172, pi-π stacked: Tyr398, Tyr435, Tyr326, pi-alkyl: Leu171, Cys172, Ile199 |
| 12        | −9.46            | H-bond: FAD, Cys172, Tyr435 |
| safinamide| −9.9             | H-bond: Tyr435, FAD, Glu206 |

aBinding energy, which indicates binding affinity and capacity for the active site of hMAO-B. All amino acid residues from the enzyme–inhibitor complexes were determined using AutoDock 4.2 and Discovery studio. Catalytic inhibition mode. Allosteric inhibition mode. Reported hMAO-B inhibitor (coligand of 2v5z).

Figure 6. Close-up of the compounds 3 (A and B), 8 (C and D), 9 (E and F), 12 (G and H), and 15 (I and J) binding sites showing the interaction between the inhibitors and catalytic site residues of hMAO-A. The chemical structures of 3, 8, 9, 12, 15, harmine, and FAD are shown in pink, green, orange, yellow, purple, blue, and black, respectively.
completely different. Both inhibitors showed an equal number of H-bond interactions.

**DISCUSSION**

With aging, the incidence of NDs increases, making the neurodegenerative disease a common diagnosis in the elderly. Therefore, developing disease- or gene-modifying drugs to counteract the progression of NDs is a hot research topic and one of the biggest challenges of modern pharmacology. Despite much primary research on the causes and pathogenic features of NDs, progress toward effective treatments has been frustratingly slow. The most probable barriers hindering the development of neuronal drugs are (a) timely relapse of symptomatic relief from treatments using the enzyme inhibition approach already approved by the FDA, (b) potential side effects and food and drug interactions, (c) high R&D expenditures, and (d) a small success rate in clinical trials (Phase II and Phase III). Therefore, research on TCM is trending in the hope of discovering novel, safe, and better-tolerated therapeutics that take advantage of different treatment strategies. In this study, we have evaluated the role of *C. obtusifolia* Linn seeds, a well-known treatment in TCM, in...
neurodegenerative diseases by screening the seed extract against hMAO activity following bioassay-guided isolation. *C. obtusifolia* seeds contain mainly anthraquinones, naphthopyrones, and their glycosides, along with other fatty acids and amino acids. The MeOH-seed extract and three solvent-soluble fractions exhibited good inhibition of the hMAO enzyme, and the selectivity of inhibition was toward the hMAO-A isozyme. Upon bioassay-guided isolation, eleven anthraquinone aglycones (1−6, and 8−12), twelve anthraquinone glycosides (7, 14, 17, 18, 21, 23−25, 30, 31, 34, and 36), one naphthopyrone aglycone (13) and its glycosides (22, 28, 32, 34, and 36), and other glycosides (15, 16, 19, 20, 26, 27, 29, 37, and 38) were isolated in pure form and evaluated for their inhibitory potential against hMAO enzyme activity. Most of the isolated compounds displayed prominent inhibition of the hMAO-A isozyme.

After the bioassay-guided isolation, we sought to draw some insights about the structure−activity relationship of the isolated compounds. Numerous 9,10-anthraquinone, naphthopyrone, and naphthalene analogues (aglycones and their glycosides) were evaluated for their inhibitory effect on hMAO enzymes, and the structure−activity relationship was investigated. From the list of anthraquinone analogues available (Table 2), it was suggestive that simple dihydroxyanthraquinone (1 and 2) did not show anti-hMAO activity. However, O-methylation of the hydroxyl groups favorably influenced inhibitory activity (1 and 2 vs 3−6). Interestingly, trihydroxyanthraquinone (5 and 10) and tetrahydroxyanthraquinone (8) exhibited potent activity. Compound 10 is a 1,6,8-trihydroxy-3-methylanthracene-9,10-dione that is present in relatively high quantity in Cassia seeds. In the enzyme inhibition assay, 10 displayed moderate inhibition of hMAO-A (IC_{50}: 23.27 ± 1.16 μM) and mild inhibition of hMAO-B (IC_{50}: 5.67 ± 0.74 μM). An additional 7-hydroxy group in 10 enhanced the potency of hMAO-A inhibition by four times and the potency of hMAO-B inhibition by 14 times (as depicted by the activity of 8). Similarly, the substitution of an 8-OH group in 10 by an −OCH3, as in 12, further elevated the potency toward both isozymes. In particular, 8-OCH3 greatly enhanced hMAO-A inhibition (10 vs 12). Interestingly, masking the 6-OH group of 10 and replacing the 3-methyl group with a hydroxymethyl group produced compound 9, which was 10-fold more potent in hMAO-A inhibition. However, these changes completely masked the hMAO-B inhibition potential. Furthermore, replacing the −OH groups at the 1-, 6-, and 7-positions of 8 with an −OCH3 group and adding an −OH group at the 2-position (as in 3), reduced the hMAO-A inhibition activity by half and completely abolished the hMAO-B inhibition effect (8 vs 3). Compound 17, the fully O-methylated analogue of 14, had different positions for the O-glucoside and had approximately five times more inhibitory activity against hMAO-A than 14.

To further understand the stereochrometry between the active anthraquinones and the hMAO enzyme, we performed structure-based molecular modeling. The free binding energy of 8 and 9 at the catalytic site of the hMAO-A enzyme was similar (−8.8 kcal/mol); however, the potency of 9 was twice that of 8. A greater number of interactions was predicted for 8 when these two compounds were docked at the active site cavity of hMAO-A. A greater number of interactions was predicted for 8 when these two compounds were docked at the active site cavity of hMAO-A. On the contrary, 9 had two unique H-bond interactions with FAD and Tyr197 that were not observed in the binding of 8. Because MAO is a flavin-containing enzyme, FAD is vital for enzyme activity, and tyrosine residues are critical for FAD binding, enzyme folding, and enzyme activity. Therefore, the H-bond interaction of 9 with FAD and Tyr197 concomitantly explains its higher potency. Similarly, 12 was the most potent hMAO-A inhibitor tested here. Compared with the reference inhibitor and other test inhibitors, it had the lowest binding energy (−9.37 kcal/mol). All of the inhibitors interacted with at least one of the tyrosine residues. However, 12 had H-bond and π–σ interactions with the most functional tyrosine residue (Tyr-444) and two additional H-bond interactions with FAD and Phe208. Because the hydroxyl groups of Tyr-444 and Tyr-435 are more important for substrate binding than those of Tyr-407 and Tyr-398, 12 displayed potent inhibition by preventing the substrate from binding to the active site cavity of the enzyme. In hMAO-B inhibition, 8 and 12 exhibited potent inhibition, and the potency of 8 was twice that of 12. Compound 8 bound to catalytic and noncatalytic sites of hMAO-B. Though the predicted binding energy for 12 was lower than that of 8, multiple bond interactions with Tyr-435, cys172, and Leu171 might have enhanced the stability of the compound 8−enzyme complex. Most of the hMAO inhibitors interacted with Ile-335 at the binding site, indicating why the Cassia compounds selectively inhibit the hMAO-A enzyme: Ile-335 in hMAO-A and Tyr-326 in hMAO-B play a crucial role in substrate/inhibitor selectivity. Other than the dual inhibitors (8 and 12), other drugs in this study selectively inhibited the hMAO-A isozyme. Overall, the structural insights and molecular docking prediction concluded that (a) the most potent inhibition of hMAO-A (by 9 and 12) was because they had the most favorable interactions (H-bond interactions with functional tyrosine residues and cofactor FAD) in the reactive site of the enzyme, (b) compounds with low activity and binding affinity lacked a H-bond interaction with FAD, and (c) the bulky group sugar in the glycosides hampered their interaction with the enzyme, producing weak inhibition.

In a previous report, Fujiwara and colleagues evaluated four anthraquinones (emodin, chrysophanol, questin, and physcion) from the fungi *Anixiella micropertusa* against the mouse liver MAO enzyme and found that emodin was the only moderate inhibitor (IC_{50}: 37 μM). However, in another study, six anthraquinones (emodin, rhein, chrysophanol, aloesin, emodin, physcion, and 1,8-dihydroxyanthraquinone) were inactive against rat MAO enzyme. These two studies demonstrated the biased effect of emodin on mouse MAO inhibition. Interestingly, here, we found potent inhibition of hMAO-A activity by 9 and 12 and moderate inhibition by 10. The type of MAO enzyme, i.e., human vs mouse, must be the reason for these discrepant findings. Though hMAO-A and mouse MAO-A have 92% sequence identity, differential sensitivity to phentermine inhibition suggests that structural and functional differences exist between them.

Oxidative deamination by hMAO liberates H2O2, a powerful oxidizer, and induces oxidative stress, a root cause of several NDs. As a neuroprotective agent, Cassia seed extract protects neuronal cells from scopolamine- or bilateral common carotid artery occlusion-induced cell damage via anti-inflammatory and antioxidant responses (by attenuating iNOS and COX-2 levels and increasing the expression of pCREB and BDNF) and acetylcholinesterase inhibition. Similarly, an ethanol extract protected hippocampal cells against mitochondrial toxin (3-nitropropionic acid; 3-NP) and reduced N-methyl-D-aspartate-induced cell death by attenuating dysregulated Ca2+. Excitotoxicity and oxidative stress are two pathways
that lead to neurodegeneration due to glutamic acid, and hMAO-A isozyme inhibitors (but not hMAO-B inhibitors) prevent this glutamate toxicity.44 Our results in this study reveal selective hMAO-A inhibition by Cassia seed constituents. Whether these inhibitors protect neuronal cells from toxic 3-NP and glutamic acid or are responsible for the previously reported neuroprotective activity of the seed extract remains to be explored. In addition, how these inhibitors act on G-protein coupled receptors for the management of NDs requires urgent study.

In conclusion, C. obtusifolia Linn seed is a well-known TCM treatment whose neuroprotective activity has recently been described. In this study, we have explored the role of Cassia seed and its metabolites against neurodegenerative diseases, particularly focusing on the hMAO enzyme. The seed extract and its metabolites selectively inhibited the hMAO-A isozyme. In particular, aglycons 3, 8, 9, 12, and 13 and glycosides 15, 26, and 38 showed promising inhibition of hMAO-A. Compounds 8 and 12 were active against hMAO-B, too. The enzyme kinetic study revealed the mode of enzyme inhibition, and an in silico molecular docking simulation predicted Ile-335 as the determinant interacting residue for hMAO-A selectivity, and Tyr-407, Phe208, and Ile180 predicted Ile-335 as the determinant interacting residue for hMAO-A selectivity.

MATERIALS AND METHODS

Chemicals and Reagents. We purchased hMAO isozymes, deprenyl HCl, and dimethyl sulfoxide from Sigma-Aldrich Co. (St. Louis, MO). A MAO-Glo assay kit was purchased from Promega (Promega Corporation, Madison, WI). All other chemicals and solvents used were purchased from Merck and Fluka, unless otherwise stated.

Plant Material. Raw C. obtusifolia Linn seeds were purchased from Omni Herb Co. (Daegu, Korea) and authenticated by Prof. J.-H. Lee (Dongguk University, Gyeongju, Korea). A voucher specimen (no. 20130302) has been deposited in the laboratory of Prof. J. S. Choi.

Extraction and Fractionation. The dried C. obtusifolia Linn seeds (3.0 kg) were refluxed in methanol (MeOH) for 3 h (6 L × 3 times). After filtration, the total filtrate was concentrated to dryness in vacuo at 40 °C to acquire the MeOH extract (430 g). Then, the MeOH extract was suspended in distilled H2O: MeOH (9:1) and successively partitioned with dichloromethane (CH2Cl2), ethyl acetate (EtOAc), and n-butanol (n-BuOH) to yield the CH2Cl2 (107 g), EtOAc (147 g), and n-BuOH (76.8 g) fractions, respectively, as well as the H2O residue (89 g).

Isolation of Compounds from the CH2Cl2 Fraction. The CH2Cl2 fraction (107 g) was chromatographed over a silica gel column (15 × 100 cm, 63–200 μm particle size, Merck) eluted with CH2Cl2–MeOH (100:0 → 1:1, gradient), which yielded 10 subfractions (CF1–CF10). Fraction CF1 (5.4 g) was chromatographed on a silica gel column (5 × 80 cm) and eluted with n-hexane-acetone (500:1, gradient system) to yield chrysophanol (1, 168 g) and physcion (2, 250 g). Fraction CF2 (8.75 g) was chromatographed over a silica gel column (3 × 60 cm) and eluted with n-hexane–EtOAc (50:1) to afford obtusin (3, 230 mg) and obtusifolin (4, 190 mg). Fraction CF8 (9.5 g) was chromatographed over a silica gel column (3 × 60 cm) and eluted with n-hexane–EtOAc (10:1) to obtain three subfractions (CF8.1–CF8.3). Fraction CF8.1 (1.08 g) was chromatographed over a silica gel column (2 × 80 cm) using n-hexane–EtOAc (10:1), which produced aurantiobutin (5, 410 mg). Fraction CF8.3 (800 mg) was chromatographed over a silica gel column (1 × 60 cm) and eluted with CH3Cl2–MeOH (100:1) to afford chryso-obtusin (6, 250 mg). Fraction CF10 (12 g) was chromatographed over a silica gel column (5 × 80 cm) using CH3Cl2–MeOH (10:1) to yield gluco-obtusifolin (7, 50 mg).

Isolation of Compounds from the EtOAc Fraction. The EtOAc soluble fraction (147 g) was subjected to a silica gel column (15 × 100 cm) and eluted with CH3Cl2–MeOH (30:1 → 1:1, gradient), which produced 20 subfractions (EFr1–EFr.20). Fraction EFr2 (6.4 g) was subjected to a silica gel column (5 × 80 cm) using a gradient solvent system of n-hexane–EtOAc (10:1) to yield alaternin (8, 50 mg), aloesin (9, 60 mg), and emodin (10, 170 mg). Similarly, EFr4 (2.2 g) was chromatographed on a silica gel column (5 × 80 cm) and eluted with n-hexane–EtOAc (5:1) to yield 2-hydroxyemodin 1-methyl ether (11, 68 mg), questin (12, 40 mg), and rubrofusarin (13, 54 mg). Subfraction EFr7 (2.6 g) was chromatographed on a silica gel column (3 × 80 cm) and eluted with CH3Cl2–MeOH–H2O (15:1:0.1) to yield chrysophanol-2-O-glucoside (14, 50 mg), cassiaside (15, 275 mg), and cassiaside (16, 30 mg). Subfraction EFr16 (200 mg) was chromatographed on a silica gel column (2 × 80 cm) and eluted with EtOAc–MeOH–H2O (24:3:2) to yield glucourantiobutin (17, 65 mg).

Isolation of Compounds from the n-BuOH Fraction. The n-BuOH soluble fraction (76.8 g) was chromatographed on a Diaion HP-20 using an H2O-MeOH gradient solvent system to give H2O (44.6 g), 40% MeOH (3.5 g), 60% MeOH (25.7 g), and 100% MeOH (2.5 g) fractions.

The 60% MeOH fraction (25.7 g) was chromatographed on a silica gel column and eluted with CH3Cl2–MeOH–H2O = 10:1:0.1 to yield 11 subfractions (B60M1–B60M11). Fraction B60M3 (570 mg) was chromatographed over a silica gel column (3 × 80 cm) and eluted with EtOAc–MeOH (20:1) to yield emodin 1-O-β-D-glucopyranoside (18, 18 mg). Fraction B60M3 (920 mg) was subjected to a silica gel column (3 × 80 cm) with EtOAc–MeOH–H2O (24:3:2) and yielded 1-hydroxyl-2-acetyl-3,8-dimethoxy-naphthalene 6-O-β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside (19, 16 mg), isorubrofusarin 10-O-β-D-glucopyranoside (20, 95 mg), and physcion 8-O-β-D-glucopyranoside (21, 24 mg).

Similarly, fraction B60M6 (1.06 g) was chromatographed over a silica gel column (4 × 80 cm) with EtOAc–MeOH–H2O (24:3:2) and yielded rubrofusarin 6-O-β-D-glucopyranoside (22, 11 mg).

Fraction B60M3 (9.0 g) was chromatographed on a silica gel column with EtOAc–MeOH–H2O (24:3:2) as the eluent to yield alaternin 1-O-β-D-glucopyranoside (23, 7.5 mg) and 1-desmethylaurantiobutin 2-O-β-D-glucopyranoside (24, 23 mg) along with a large amount of precipitate and mother liquor in some subfractions. The subfractions with similar thin layer chromatography patterns were combined, and the precipitate and mother liquor were separated by filtration. The precipitate was dissolved in MeOH–H2O (2:1) and chromatographed on a silica gel column (4 × 80 cm) eluted with EtOAc–MeOH–H2O (24:3:2) to obtain physcion 8-O-β-gentiobioside (25, 13 mg), toralactone 9-O-β-gentiobioside (26, 85 mg), cassialactone 9-O-β-gentiobioside (27, 23 mg), and rubrofusarin gentiobioside (28, 76 mg). Similarly, repeated
chromatography of the mother liquor in the silica gel column with EtOAc–MeOH–H₂O (24:3:2) yielded isorubrofusarin gentiobioside (29, 15 mg), chrysophanol 1-β-gentiobioside (30, 85 mg), emodin-1-β-gentiobioside (31, 15 mg), and rubrofusarin 6-0-β-D-apiofuranosyl (1 → 6)-O-β-D-glucopyranoside (32, 65 mg). The last subfraction B60M11 (2.7 g) was chromatographed on a Si gel column eluted with EtOAc–MeOH–H₂O (21:5:3); repeated chromatographic steps yielded rubrofusarin triglucoside (33, 65 mg), chrysophanol triglucoside (34, 65 mg), cassiaside B2 (35, 14 mg), and chrysophanol tetraglucoside (36, 17 mg).

Similarly, the 40% MeOH fraction (3.5 g) was chromatographed over a Si gel column with EtOAc–MeOH–H₂O, 600:99:81, and yielded 20 subfractions. Subfraction 7 (174 mg) was subjected to a Si gel column with EtOAc–MeOH–H₂O (30:2:1) and yielded 12 subfractions (M7-1–M7-12). M7-8 (78 mg) was further subjected to a reversed phase (RP)-column using 40% EtOAc−MeOH and yielded (R)-3,4-dihydro-10-hydroxy-3-hydroxy-7-methoxy-3-methyl-1H-naphtho[2,3-c]pyran-1-one 9-O-β-D-glycopyranoside or (3R)-cassialactone 9-O-β-D-glucopyranoside (37, 15 mg). Similarly, subfraction 16 (200 mg) was subjected to repeated Si gel chromatography eluted with EtOAc–MeOH–H₂O, 21:4:3, and purified by an RP-18 (40% MeOH), which produced (35S)-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-3,4-dihydro-1H-benzol[g]isochromene-3-carboxylic acid 9-O-β-D-glucopyranoside (38, 20 mg).

All of the compounds isolated from the different solvent-soluble fractions (the CH₂Cl₂, EtOAc, and n-BuOH fractions of the MeOH extract of C. obtusifolia seeds) were identified by comparing their spectral data with the published spectral structures of all isolated compounds are shown in Figure 1.

In Vitro Human MAO Inhibitory Assay. The hMAO inhibitory potential of the compounds was evaluated via a chemiluminescent assay in a white, opaque, 96-well plate using the MAO-Glo kit (Promega, Madison, WI). All of the experimental conditions and procedures we used in this study were similar to those reported in our previous paper.50 The percent of inhibition (%) was obtained using the following equation: % inhibition = (A₀ − Aₙ)/A₀ × 100, where A₀ is the absorbance of the control, and Aₙ is the absorbance of the sample.

Kinetic Parameters in hMAO-A Inhibition: Michaels–Menten Plots, Lineweaver–Berk Plots, and Secondary Plots. Michaels–Menten plots, Lineweaver–Berk plots, and secondary plots were used to determine the kinetic mechanisms.51,52 The reaction mixtures contained three different concentrations of the MAO substrate (320, 160, and 80 μM) for hMAO-A and 32, 16, and 8 μM for hMAO-B) in the presence or absence of the test compounds. The Michaelis–Menten constant (Kₘ) and maximum velocity (Vₘₐₓ) for each enzyme inhibition were measured by Lineweaver–Berk plots, and the inhibition constant (Kᵢ) was calculated from secondary plots using SigmaPlot 12.0 software (SPCC, Inc., Chicago, IL).

Molecular Docking. The docking of the target enzyme and active compounds was successfully simulated using the AutoDock 4.2 program.38 X-ray crystallography of the hMAO A-harmin complex (PDB ID:2ZSX) and hMAO-B-safinamide (PDB ID:2VSZ) was obtained from the RCSB Protein Data Bank (PDB) website (http://www.rcsb.org/), with respective resolutions of 2.2 and 1.6 Å.53,54 The three-dimensional (3D) structures of 3, 8, 9, 12, 15, and 26 were obtained from the PubChem Compound database (NCBI), with compound IDs of 155380, 12548, 10207, 160717, 164146, and 14189968, respectively. Similarly, the 3D structure of 38 was constructed using Chem3D Pro v12.0 and adjusted to pH 7.0 using MarvinSketch (ChemAxon, Budapest, Hungary). To assess the appropriate binding orientations and conformations of the ligand molecules with different protein inhibitors, an automated docking simulation was performed using AutoDockTools (ADT). For the docking calculations, Gasteiger charges were added by default, the rotatable bonds were set by the ADT, and all torsions were allowed to rotate. The grid maps were generated using AutoGrid. The docking protocol for rigid and flexible ligand docking consisted of 10 independent genetic algorithms; the other parameters used were the ADT defaults. The results were visualized and analyzed using Discovery Studio (v17.2, Accelrys, San Diego, CA).

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Notes

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AD, Alzheimer’s disease; ADT, AutoDockTools; FAD, flavin adenine dinucleotide; HD, Huntington disease; hMAO, human monoamine oxidase; NDs, neurodegenerative diseases; PD, Parkinson’s disease; TCM, traditional Chinese medicine

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