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Original Research Article

Natural Products Screening for the Identification of Selective Monoamine Oxidase-B Inhibitors

ABSTRACT

Aims: Monoamine oxidase-B inhibitors(MAO-BI) are used for the initial therapy of Parkinson disease. Also, MAO-BI showed effective neuroprotective abilities in several neurodegenerative diseases. However, some concerns existed about their long-term use. Nevertheless, natural compounds showed potential MAO-B selective inhibitions. To date, few selective and natural MAO-B inhibitors have been identified. Therefore, the current study is designed to identify plants with potent and specific MOA-B inhibition.

Study Design: In this work, we utilized high throughput screening to evaluate the different plants ethanolic extract for their effectiveness to inhibit *recombinant human* (*h*)MAO-A and *h*MAO-B and to determine the relative selectivity of the top MAO-BI.

Methodology: Recombinant human isozymes were verified by Western blotting, and the 155 plants were screened. A continuous fluorometric screening assay was performed followed by two separate *h*MAO-A and *h*MAO-B microtiter screenings and IC_{50} determinations for the top extracts.

Results: From the screened plants, 9% of the extracts showed > 1.5-fold relative inhibition of *h*MAO-B (RI_B) and another 9% with > 1.5-fold relative inhibition of *h*MAO-A. The top extracts with the most potent RI_Bs were *Psoralea corylifolia* seeds, *Phellodendron amurense* bark, *Glycyrrhiza uralensis* roots, and *Ferula assafoetida* roots, with the highest RI_B of 5.9-fold. Furthermore, extensive maceration of the promising extracts led to an increased potency with a preserved RI_B as confirmed with luminescence assay. The four extracts *h*MAO-B inhibitions were equally potent (IC_{50}= 1.3 to 3.8 µg/ml) with highly significant relative selectivities to inhibit *h*MAO-B (4.1- to 13.4-fold).

Conclusion: The obtained results indicate that Psoralea *corylifolia* seeds, *Ferula assafoetida,* and *Phellodendron amurense* ethanolic extracts have potent and selective inhibition for MAO-BIs. Highlighting these extracts as natural sources for novel MAO-BIs to be investigated for their therapeutic use in neurodegenerative diseases including Parkinson's disease.

Keywords: Parkinson disease, selective monoamine oxidase-B inhibitors, *Psoralea corylifolia* seeds, *Phellodendron amurense*, *Glycyrrhiza uralensis, Ferula assafoetida*.

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ABBREVIATIONS

EEs	ethanolic extracts
RS_B	relative selectivity for *h*MAO-B inhibition
tyr. HCl	p-tyramine HCl
benz. HCl	benzylamine HCl
SNpc	substantia nigra pars compacta
SN	substantia nigra
RI	relative inhibition
RI_B	relative *h*MAO-B inhibitor
AD	Alzheimer's disease
DEP	selegiline (Deprenyl®)
H_2O_2	hydrogen peroxide
EOH	ethanol
PCS	*Psoralea corylifolia* seeds
PAB	*Phellodendron amurense* barks
FAR	*Ferula assafoetida* resins
GUR	*Glycyrrhiza uralensis* roots
PCSEE	*Psoralea corylifolia* seeds ethanolic extract
PABEE	*Phellodendron amurense* barks ethanolic extract
FAREE	*Ferula assafoetida* resins ethanolic extract
GUREE	*Glycyrrhiza uralensis* roots ethanolic extract
MAO	monoamine oxidase
*h*MAO-A	*recombinant human* monoamine oxidase-A
*h*MAO-B	*recombinant human* monoamine oxidase-B
HTS	high throughput screening
NE	norepinephrine

1. INTRODUCTION

In Parkinson's disease (PD) and depression, monoamine oxidase-A and B inhibitors (MAO-AIs and MAO-BIs) are currently used as effective drugs. MAO-A and MAO-B are two isozymes belong to the Flavin-containing amine oxidases that can be found in astrocytes and the substantia nigra pars compacta (SNpc) neurons to metabolize monoamine neurotransmitters. In PD, MAO-BIs are used to increase neurotransmitter dopamine (DA), reduce oxidative stress level and relieve the psychomotor

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68 disease symptoms [1]. Even though DA is well metabolized by both isozymes [2], MAO-B is more
69 specific in metabolizing the already depleted DA in the SNpc of the PD patients [3]. Additionally, the
70 activity of MAO-B is elevated up to three-fold in PD and Alzheimer's disease (AD) as compared to
71 normal levels [4]. That MAO-B elevation [1, 5] with the co-localized of active MAO-A isozyme can
72 potentially aggravate oxidative stress in aging patients. Both MAOs activities produce abnormally high
73 amounts of hydrogen peroxide (H_2O_2) and aldehydes that are neurotoxic. Those byproducts
74 potentially damage proteins, nucleic acids, lipids and activate apoptotic pathways [6]. Unfortunately,
75 the aldehyde dehydrogenase enzyme that metabolizes the neurotoxic aldehydes produced by the
76 active MAOs was found to be genetically deficient in PD patients' SNpc [7, 8]. Other oxidative stress
77 defense enzymes may also become limited with the overwhelming reactive species produced.
78 Consequently, these toxic byproducts, particularly of active MAO-B, can potentially accumulate in
79 neurons and astrocytes leading to cell death and aggravating neurodegeneration.

80 While MAO-AIs are usually associated with concerns about food and drug interactions that lead to
81 rare but serious side effects (the cheese effect and serotonin syndrome) [9, 10], MAO-BIs were found
82 ideal for the management of PD as in the case of selegiline (Deprenyl®) (DEP). These inhibitors were
83 proven to be clinically efficient for decades as they delayed the need for L-dopa in PD management.
84 Selective MAO-BIs may also inhibit the conversion of nontoxic xenobiotic substrates to neurotoxins in
85 the brain, such as the MPTP conversion to its neurotoxic product MPP+. MAO-BI also exerted anti-
86 apoptotic and other multifunctional neuroprotective activities [11] which led to extended PD patients'
87 life expectancy [8]. Moreover, MAO-BIs such as DEP were reported beneficial in other neurological
88 disorders such as cerebrovascular ischemia, Tourette syndrome, narcolepsy, and AD [12].

89 Although this may sound ideal for MAO-BI DEP in neurological diseases, some concerns exist with
90 their long-term use. Recent evidence of neurotoxic metabolite of DEP, L-methamphetamine, showed
91 contradictions in their antiparkinsonian action *in vitro* [13], and some attributed rare cases of tolerance
92 or dependence development on some MAOIs to their amphetamine-like metabolites structures [14].
93 Meanwhile, the currently available MAO-BIs are synthetic compounds that share common structures
94 such as DEP and rasagiline, clorgyline that contains N-propargyl, the responsible group for MAO
95 inhibition and neuroprotection [2]. On the other hand, it was reported that potent and selective MAO-
96 BIs in nature are commonly found to include flavonoids, β-carbolines, xanthines, and alkaloids [15].

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Therefore, new natural structures may promote the discovery of new lead compounds with unique properties as in the classical MAO-BIs. To recognize if the total phytochemical constituents of plant extracts also have the ability to selectively inhibit human MAO-B, high-throughput screening (HTS) was conducted on both isozymes. Pointing out the plants with the most selective MAO-B inhibitory properties may further reveal unique phytochemical structure properties with multifunctional neuroprotective and neurorescue properties beneficial to neurodegenerative such as PD.

2. METHODOLOGY

2.1. Materials

The *h*MAO-A and *h*MAO-B isozymes, produced in BTI-TN-5B1-4 insect cells containing human cDNA, and their analyzed active units (U), were purchased and identified by Sigma-Aldrich (St. Louis, MO, USA). Isozymes stocks were diluted with 1% of 1 M HEPES in Hank's Balanced Salt Solution (HBSS) (pH 7.4) and aliquots stored at -80℃ for single use. Standards of pirlindole, a reversible inhibitor for MAO-A (RIMA), deprenyl (DEP), an irreversible MAO-BI, and cell culture media and supplements were also purchased from Sigma-Aldrich. Different plant parts were purchased from and identified by their trades companies including, East Earth Trade Winds (Redding, CA, USA), Mountain Rose, Herbs (Eugene, OR, USA), Mayway Corp. (Oakland, CA, USA), Monterey Bay Spice Comp. (Watsonville, CA, USA). Western blotting equipment and reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and BCA Protein Assay Kit from Peirce (Rockford, IL, USA). Amplex™ Red MAO Assay Kit was purchased from Molecular Probes by Life technologies™ (Eugene, OR, USA), and tyramine HCl from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). MAO-Glo™ Kit was purchased from Promega Inc. (Madison, WI, USA).

2.2. Ethanolic Extraction

Plants natural products were extracted for screening for their *h*MAO-A and *h*MAO-B inhibiting potentials, and the top active extracts (potent and selective at 1mg/ml) were further extensively extracted. Briefly, 155 different plant dry parts were used (leaf, stem, root, petal, bark, resin, herb ..., etc.). Each defined amount of 250 mg was grounded to fine powders, homogenized in 99.95% ethanol and macerated once for 50 mg/ml extracts. The top four active plants, of 8 g each, were subject to repeated maceration with mild agitation as the used ethanol solvent was exchanged every

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125 24 h and evaporated in a fume hood for ten days to get the crude extract. Only *Ferula assafoetida*
126 resin (FAR) was subject to 80 ℃ evaporation for a short time using a rotary evaporator to speed
127 drying. All labeled ethanolic extracts (EEs) were stored in air tight glass containers at -20℃ in the
128 dark until use.

129 **2.3. Proteins Verification and Method Validation**

130 **2.3.1. Western Blotting**

131 Western blotting was used to verify MAO isozymes. Human dopaminergic neuroblastoma cell line of
132 SH-SY5Y was used as a positive control containing both isozymes, MAO-A [16], and MAO-B, as in
133 the anti-MAO-B datasheet. The cells were obtained from American Type Culture Collection (CRL-
134 2266) (Manassas, VA, USA) and were cultured in DMEM with 10% fetal bovine serum, 100 IU/mL
135 penicillin/streptomycin. To lysate the cells, we used RIPA buffer/protease inhibitor (4℃) with freezing
136 and thawing cycles.
137 To assure equally loaded amounts in micrograms, we performed the BCA protein assay, and the Bio-
138 Tek Synergy HTX Multi-Reader set to 562 nm for analysis. All samples were prepared with 2 x
139 Laemmli sample buffer-2.5% mercaptoethanol loading buffer for 12 µg per lane. Proteins were
140 denatured using heating block for 3-5 min at 100 ℃ before loading and separated using 1D SDS-
141 PAGE gel electrophoresis of 10%Tris-HCl gradient at 200 V for 55 min. Gels were wetly transferred to
142 nitrocellulose membranes at 100 V for 75 min. Primary antibodies used were rabbit monoclonal anti-
143 MAO-B antibody [EPR7103] (Abcam; ab125010), rabbit monoclonal anti-MAO-A antibody [EPR7101]
144 (Abcam; ab126751) with 1-2:1000 ratio each in cold skim milk. Rabbit anti-β-actin antibody (Abcam;
145 8227) was used for control. Secondary antibodies were goat anti-rabbit IgG H&L HRP-conjugated
146 probes (Abcam; ab6721). The signal was detected using Supersignal® West Pico Chemiluminescent
147 Substrate from Thermo Scientific, Peirce Biotechnology (Rockford, IL, USA) and VersaDoc imaging
148 system using CCD camera (Bio-Rad; Hercules, CA, USA)

149 **2.3.2. Substrate Metabolism with Time**

150 In this experiment, substrate concentrations and time required for maximum detectable *h*MAO-A and
151 MAO-B activities were validated; optimal parameters were determined using the continuous Amplex
152 Red fluorometric assay. In brief, *h*MAO-A and B (0.7 U/ml; 0.07 U per reaction) activities were

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153 assayed using p-tyramine HCl (tyr.HCl) and benzylamine HCl (benz. HCl) as substrates, respectively.
154 Different substrate volumes of 25 µL of 4 x the final concentrations were added into black opaque 96-
155 well microplates for their related isozyme assay. Added substrate final concentrations ranged from 0
156 to 0.8 mM with *h*MAO-A, and from 0 to 3 mM with *h*MAO-B, as buffers substituted substrates in
157 control wells. In the dark, the fluorometric reagent was prepared as 4 x the final concentration of 200
158 µM Amplex Red 1 U/ml and horseradish peroxidase (HRP type-II) in PBS (pH 7.4). Freshly prepared
159 reagent of 25 µL was added to each well and the reaction was initiated by adding 50 µL of 2 x
160 isozyme final concentration to the different related substrate concentrations and controls in the wells.
161 Immediately, the fluorescent signal (AFU) of the reactions kinetics with time was read at various time
162 intervals (minutes then hours) at RT. Pre-plate for time zero and post-plate readings for different time
163 intervals were obtained by subtracting the time zero pre-plate reading to monitor the increase as an
164 indicator for the product resorufin continuous accumulation. The AFU excitation resorufin was at 530
165 nm, and its read fluorescence detection was at 590 nm using Synergy HTX Multi-Reader (Bio-Tek).

166 **2.3.3. H_2O_2 Scavenging Activity, Autoxidation, and Resorufin Quenching**

167 Determining maximum H_2O_2 produced within 1 h of incubation at RT was accomplished by
168 interpolating maximum AFU from the H_2O_2 linear standard curve of ranged 0-5 µM (R^2 of 99.3%)
169 using GraphPad Prism software. Values of the blank wells without H_2O_2 or enzymes were subtracted
170 from all their corresponding test values. MAO total H_2O_2 production was at a maximum of 0.9 ± 0.01
171 nmol (4.5 ± 0.07 µM). Thus, the scavenging activities were tested for a maximum of 5 µM at RT.
172 Freshly prepared H_2O_2 was added as 4 x the final concentration to 2 x the final extract concentrations
173 equivalent to MAOs assays. The quenching ability of the Amplex Red product resorufin by the
174 extracts was tested. Based on preliminary studies, resorufin was added as 4 x the final concentration
175 of 20 µM to 1.3 x the final extract concentrations equivalent to MAOs assays. In autoxidation, the
176 reactions were measured with the same method as scavenging activities except substituting H_2O_2
177 with used reaction buffer and calculated separately as folds of signal increase. Extract tests with
178 high differences from controls are to be eliminated from the MAOs screen assay.

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181 **2.4. *h*MAO-A and *h*MAO-B Fluorometric Microtiter Screening**

182 MAOs activities were assayed using an extremely sensitive continuous fluorometric assay containing
183 Amplex Red (10-acetyl-3, 7-dihydroxyphenoxazine) reagent. The enzymatic H_2O_2 was measured with
184 and without extracts or standards. In addition to random plant selection, some plants were chosen
185 based on our previous work on *h*MAO-B natural inhibitors [17]. Briefly, each of the 155 EEs was
186 diluted in PBS (pH 7.4) in black 96-well microplates to equally make 4 x the final concentration of
187 1mg/ml (n= 2). *h*MAO isozymes on ice with 4 x the final concentration, 0.7 U/ml each, were used. The
188 *h*MAO-A and *h*MAO-B (25 µL) were separately added to 25 µL EEs or buffer for control and incubated
189 30-40 min at RT. For the top four extracts IC_{50}s determination, 8 x working solutions in PBS (pH 7.4)
190 were serially diluted for at least ten points before adding the enzymes as mentioned earlier. Control
191 groups were tested with and without maximum ethanol of 1.25%. Buffer solution substituted the
192 enzymes in the correspondent blank wells.
193 The 4 x working solution of Amplex Red reagent was freshly prepared as earlier mentioned in the
194 substrate metabolism optimization method. The previously optimized 4 x the final concentration of 0.5
195 mM tyr. HCl (for *h*MAO-A) and 3 mM benz. HCl (for *h*MAO-B) were prepared. Each substrate was
196 mixed with Amplex Red reagent at 1:1 ratio. A 50 µL of each mixed solution was added to its
197 corresponding enzyme/extract wells to make the required final extract concentrations. Fluorescent
198 resorufin product was quantified at different time intervals as plates were read at an
199 excitation/emission of 530/590 nm using Synergy HTX Multi-Reader (Bio-Tek, USA). Time zero pre-
200 plate and post-plate readings, at times of 60 min each, were obtained. Percent enzyme inhibition and
201 Relative inhibition (RI_B) were determined for all extracts. In comparison to the related control, any
202 extract that inhibited *h*MAO-B to less than 85% or showed > 1.5-fold ratio RI_B were pointed out. The
203 same was done to the top relative inhibitors against *h*MAO-A (RI_A). Only extracts that ranked the most
204 potent against *h*MAO-B were further evaluated for IC_{50}s as with DEP and pirlindole standard controls.

205 **2.5. Confirmation by a luminescence assay**

206 A luminescence assay, using the MAO-Glo™ Kit, was used with DEP standard to ensure preserved
207 RI_B. Briefly, 12.5 µL of 4 x the final concentrations of 20 µg/ml of each extensively extracted plant
208 (PCSEE, PABEE, FAREE, and GUREE) or 5 µg/ml DEP were added to white opaque 96-well
209 microplates. Fresh 25µL of 2 x the final concentration of 0.9 U/ml *h*MAO-A and *h*MAO-B isozymes in

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reaction buffer (pH 7.4) were incubated with the extracts for 30 min at RT. Controls used were with and without ethanol (0.1%). Reaction buffer substituted each corresponding isozyme to make the blank wells. Based on Valley's method [18] and our preliminary optimizations, 12.5 µL of 4 x the final concentration of 40 and 4 µM of luciferin derivative substrate for *h*MAO-A and *h*MAO-B reactions, were added respectively. The reaction was incubated for 60 min at RT. Reporter luciferase detects reagent of 50 µL per well was added. After 30 min of incubation, produced arbitrary light units (ALU) were detected using Synergy HTX Multi-Reader (Bio-Tek).

2.6. Statistical Analysis

Analysis performed by GraphPad Prism Software v6.02 (San Diego, CA, USA). Data points were presented as the mean ± SEM. IC_{50}s values were interpolated from normalized data by the asymmetric sigmoidal curve and averaged from at least two experiments. One-way and two-way ANOVA were performed followed by multiple comparisons tests to determine the significance of the difference between each two or more groups. In this investigation, relative inhibition (RI_B) which is the ratio of % *h*MAO-A activity/%*h*MAO-B activity at a particular concentration and relative selectivity (RS_B) which is the ratio of *h*MAO-A IC_{50}/*h*MAO-B IC_{50} were measured.

3. RESULTS

3.1. *h*MAO-A and *h*MAO-B Verification

Both *h*MAO-A and *h*MAO-B identities were verified using Western blotting. The human MAOs antibodies and β-actin Western blotting successfully identified both *h*MAO-A and *h*MAO-B sample proteins at about ~60, ~59, and ~42 KDa, respectively (**Fig. 1**). High intensity detected bands for *h*MAO-A and hMAO-B matched the human neuroblastoma SH-SY5Y cells positive controls at their molecular weights.

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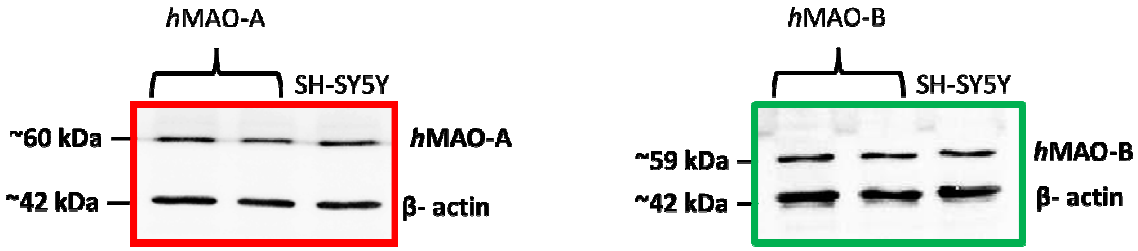

Fig. 1. Verification of the used enzymes *h*MAO-A and *h*MAO-B identities by Western blotting using equally loaded proteins, and rabbit monoclonal anti-MAO-A, anti-MAO-B and anti-β-actin antibodies; SH-SY5Y cells (12 µg) were used as positive control for both isozymes. Bands were detected by HRP-conjugated anti-rabbit secondary antibody.

3.2. *h*MAO-A and *h*MAO-B Assay Method Validation

To optimize the required time of incubation and substrates concentrations for the used isozymes amounts, an enzyme-progression curve with different substrate concentrations was performed before the screening. A proportional increase of AFUs was detected by the used 0.07 U isozyme (0.7 U/ml) at its initial linear rate of reaction (**Fig. 2**) at RT**.** AFU, as an H_2O_2 indicator, increased linearly (R^2= 99.33%) with a maximum of 6304 ± 25 AFU with time and substrates concentrations within 2 h by *h*MAO-A (**Fig. 2 A**), and 1 h by *h*MAO-B (**Fig. 2 B**). For optimum isozymes activities, tyr. HCl concentrations of 0.5 to 0.8 mM (**Fig. 2 A**), and benz. HCl up to 3 mM were required (**Fig. 2 B**). Using the optimized conditions with standard selective inhibitors of MAO-AI pirlindole and MAO-BI DEP (**Fig. 2 C** and **D**); DEP and pirlindole selectively and dose-dependently inhibited their isozymes (DEP *h*MAO-A IC_{50}= 1.2 ± 0.5 µM and *h*MAO-B IC_{50}= 10 ± 10 nM, and pirlindole *h*MAO-A IC_{50}= 0.24 ± 0.05 µM and *h*MAO-B IC_{50}= 262.2 ± 5.8 µM). To exclude other possible interactions that may interfere with the *h*MAOs assays, H_2O_2 scavenging, autoxidation, and quenching activities were pre-tested. Any extract with ≥ 50% scavenging or ≥ 30% quenching activities were excluded from the *h*MAO-A and *h*MAO-B inhibition extract screenings. Thus, 30 extracts exclusion from the screen.

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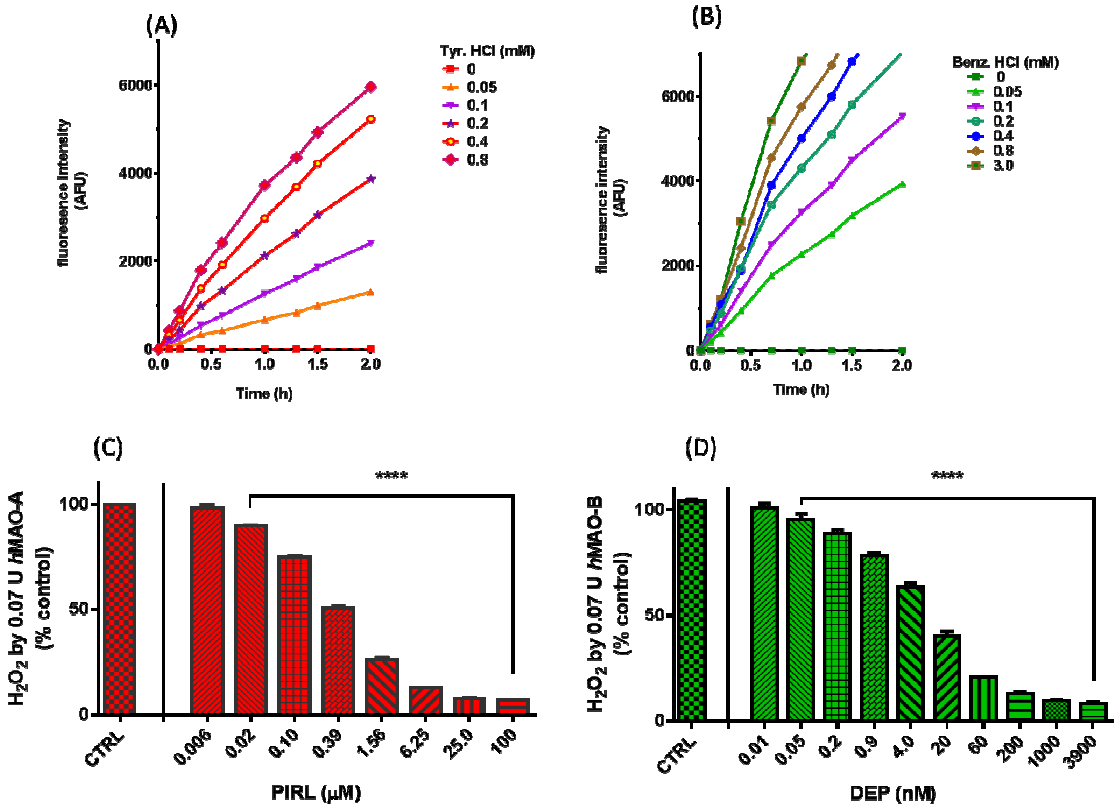

Fig. 2. Fluorometric assay validation for screening; time and substrate concentration optimization with **(A)** *h*MAO-A and **(B)** *h*MAO-B isozymes at RT. AFU: Arbitrary Fluorescence Units. Progression curves with best maximum linearity in the presence of different substrate concentrations. Optimized conditions of isozymes were inhibited dose-dependently by selective standard inhibitors: **(C)** MAO-AI pirlindole (PIRL) and **(D)** MAO-BI deprenyl (DEP). Statistical analysis was presented as the mean ± SEM, n= 3. The significance of difference between a standard and its control reaction was determined using one-way ANOVA followed by Dunnett's multiple comparisons test. **** $p < 0.0001$.

3.3. Microtiter screening for *h*MAO-B Relative Inhibition (RI)

To determine the potential of the different plant extracts to exhibit potent RI_B, two separate *h*MAO-A, and *h*MAO-B inhibition microtiter screenings were conducted using the continuous fluorometric assay (**Fig. 3**) as previously recommended for HTS [19]. After excluding extracts with the H_2O_2 scavenging or quenching activities, 132 out of 155 EEs total were tested for both isozymes and ranked as *h*MAO-BIs from low to high (**Fig. 3 A**). The figure shows the different inhibition efficacies and relative inhibitions; extracts were effective against *h*MAO-B (green dots curved down), *h*MAO-A (red dots scattered away lower than the green curve), both, or no inhibitions. Interestingly, the screening elucidated 9% of the 132 plants extract with >1.5-fold *h*MAO-B relative inhibition (RI_B) (**Table 1**), and

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271 other 9% of the extracts exerted >1.5-fold *h*MAO-A relative inhibition (RI_A) (**Table 2**). The screen
272 results indicated plants potentials to have significantly collective selective *h*MAO-A and *h*MAO-B
273 inhibiting activities (at least p = 0.05). These particular plants may contain more selective *h*MAO-B or
274 *h*MAO-A inhibitors than the ones without different significant inhibitions.
275 The first step in our *h*MAO-B inhibition selectivity screen was to determine potency against the *h*MAO-
276 B activity. The most potent inhibitors of >85% *h*MAO-B activity in **Figure 3 B** ranks were
277 *Phellodendron amurense* barks (PAB) > *Psoralea corylifolia* seeds (PCS) > *Baptisia tinctoria* roots
278 (BTR) > *Glycyrrhiza uralensis* roots (GUR) > *Paeonia suffructicose* roots and barks (PSB) > *Ferula*
279 *assafoetida* resins (FAR). Further in the determination of RI_B, the ranked top RI_B six extracts were
280 partially different (**Fig 3 C**). Although PAB showed the most potent *h*MAO-B inhibition, the extract with
281 the highest RI_B was GUR (5.9-fold). That was followed by PAB, *Camellia sinensis* leaves (CSL), FAR,
282 *Piper nigrum* fruits (PNF), and PCS. From **Figure 3 A** & **B**, the screened extracts with shared
283 characters of potency against *h*MAO-B and RI_B (PAB, PCS, GUR, and FAR) were selected for further
284 selectivity determination. That method of selection based on the top six ranked screen plants potency
285 and RI_B is to include selective *h*MAO-BIs properties that are hidden by extract potency.

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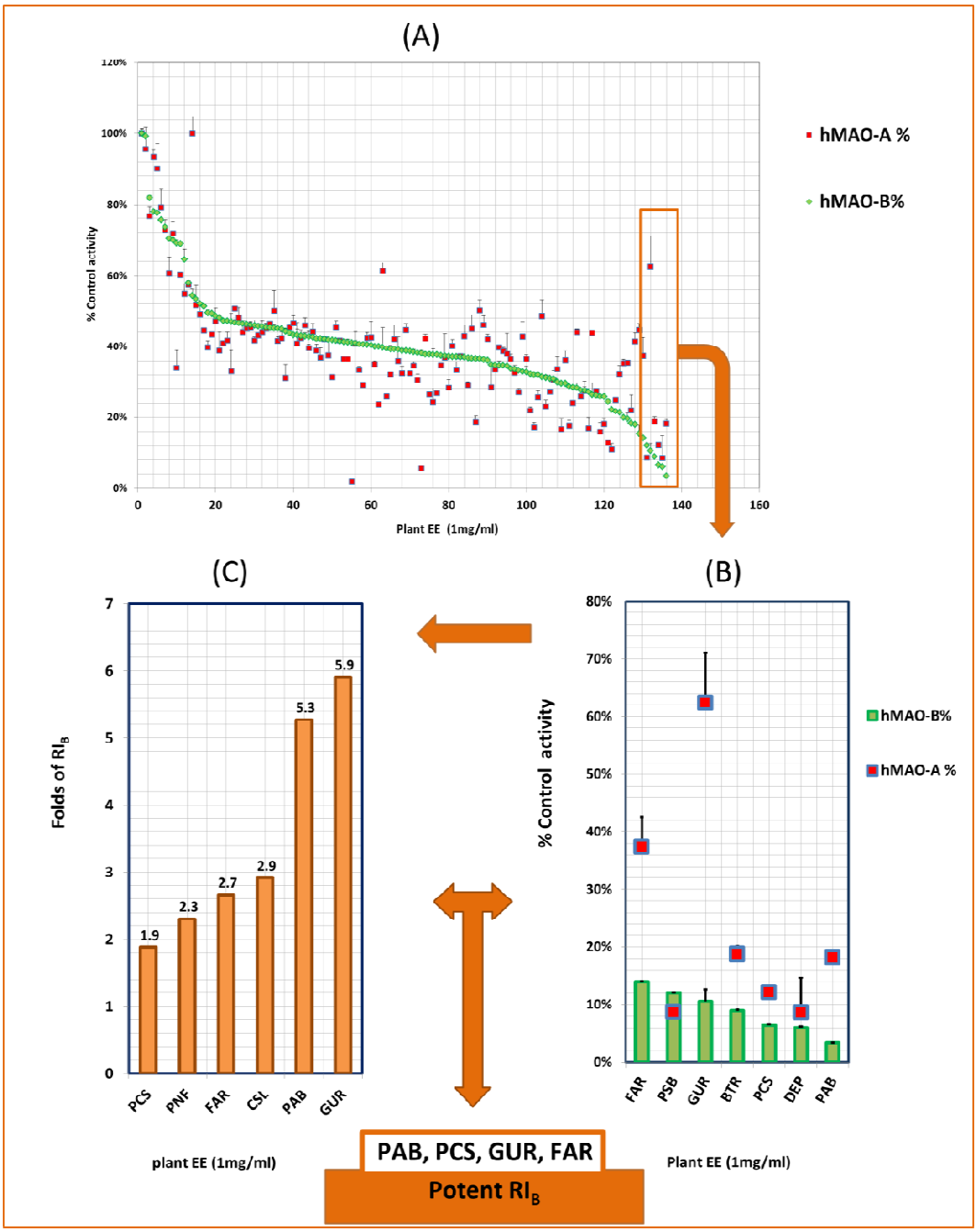

Fig. 3. High Throughput Screening of plants for potent relative inhibitors of recombinant human monoamine oxidase-B (*h*MAO-B) (RI_B): **(A)** 132 out of 155 extracted plants with ethanol (1 mg/ml) after hydrogen peroxide scavengers and quenchers excluded. **(B)** The top potent six extracts inhibited > 85% of *h*MAO-B activity. **(C)** The top six extracts with RI_B (>1.8-fold). The most potent RI_B extracts in this screen were. *Glycyrrhiza uralensis* (GUR), *Psoralea corylifolia* seeds (PCS) *Phellodendron amurense* barks (PAB), and *Ferula assafoetida* resin (FAR). Data points compared to standard deprenyl (DEP) are expressed as mean ± SEM, with n= 2. RI_B= *h*MAO-A/*h*MAO-B.

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296 **Table 1.** The top relative inhibitors against *h*MAO-B (RI_B) with > 1.5-fold at 1mg/ml plant
297 ethanolic extract are 12 out of 132 extracted plants. RI_B= %*h*MAO-A/%*h*MAO-B. Significance
298 of difference between *h*MAO-A and *h*MAO-B% was determined using two-way ANOVA
299 followed by Sidak's multiple comparisons test. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p <$
300 0.0001.

***Botanical name* - part used**	***h*MAO-A ± SEM (%)**	***h*MAO-B ± SEM (%)**	***Rank*ed RI_B (fold)**	**P Level**
***Glycyrrhiza uralensis* - root**	62.4 ± 8.6	10.6 ± 2.0	**5.9**	****
***Phellodendron amurense* - bark**	18.2 ± 1.2	3.5 ± 0.01	**5.3**	**
***Camellia sinensis*- leaf**	44.6 ± 1.8	15.3 ± 2.5	**2.9**	****
***Ferula assafoetida* - resin**	37.3 ± 5.2	14.0 ± 0.1	**2.7**	****
***Piper nigrum* - fruit**	41.4 ± 2.3	18.0 ± 0.3	**2.3**	****
***Baptisia tinctoria* - root**	18.7 ± 1.3	8.9 ± 0.2	**2.1**	*
***Psoralea corylifolia* - seed**	12.1 ± 0.7	6.5 ± 0.2	**1.9**	*
Phoenix dactyliferav- fruit	100.0 ± 4.9	54.3 ± 2.0	**1.8**	****
***Origanum majorana* - herb**	35.4 ± 0.2	19.6 ± 1.7	**1.8**	**
***Magnolia denudate* - flower**	35.3 ± 1.1	19.9 ± 0.1	**1.8**	**
***Lycopus lucidus* - rhizome**	43.8 ± 0.7	26.3 ± 3.3	**1.7**	***
***Curcuma longa* - rhizome**	44.0 ± 0.8	28.3 ± 1.4	**1.6**	**

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Table 2. The top relative inhibitors against *h*MAO-A (RI_A) with > 1.5-fold at 1mg/ml plant ethanolic extract are 11 out of 132 extracted plants. RI_A= %*h*MAO-B/%*h*MAO-A. Significance of difference between *h*MAO-A and *h*MAO-B% was determined using two-way ANOVA followed by Sidak's multiple comparisons test. ** $p < 0.01$,*** $p < 0.001$, **** $p < 0.0001$.

***Botanical name* - part used**	***h*MAO-A ± SEM (%)**	***h*MAO-B ± SEM (%)**	***Rank*ed RI_A (fold)**	**P Level**
***Clematis trifoliate* - fruits**	1.8 ± 0.1	41.1 ± 0.3	**23.0**	****
***Dryopteris crassirhizoma* - rhizome**	5.5 ± 0.1	38.1 ± 0.8	**6.9**	****
***Tilia europaea* - leaf**	18.5 ± 1.8	36.6 ± 0.5	**2.0**	****
***Zanthoxylum bungeanum* - seed**	12.8 ± 0.5	24.5 ± 0.2	**1.9**	***
***Lindera aggregata* - root**	17.1 ± 1.5	32.0 ± 0.2	**1.9**	****
***Laurus nobilis* - leaf**	16.7 ± 3.0	29.6 ± 0.4	**1.8**	****
***Agrimonia pilosa* - herb**	23.7 ± 0.2	39.9 ± 0.4	**1.7**	****
***Helichrysum foetidum* - flower**	17.7 ± 1.4	28.7 ± 0.01	**1.6**	***
***Sargentodoxa cuneate* - stem**	15.9 ± 2.7	25.8 ± 0.1	**1.6**	**
***Caesalpinia sappan* - bark**	16.8 ± 3.0	27.2 ± 0.5	**1.6**	**
***Salvia apiana* - leaf**	24.2 ± 4.0	37.7 ± 1.6	**1.6**	****

3.4. Confirmation of Relative *h*MAO-B Inhibition (RI)

To confirm the screening results and the preservation of RI_B of the selected top four extracts (GUR, PAB, PCS, and FAR) after extensive maceration, we used a non-H_2O_2-dependent luminescence assay (**Fig. 4**). Used ethanol had no the assay. All tested extensively extracted EEs of only 20 µg/ml exerted an equally potent *h*MAO-B inhibition ($p > 0.05$) by > 70% of the 0.4 U isozymes activities. Moreover, the extracts showed very significant high RI_B activities (8.5-, 5.6-, 3.3-, 2.8-fold for PCS, PAB, FAR, and GUR, respectively ($p \leq 0.05$ and 0.0001). The results indicate that the screen was successful in finding potent RI_Bs. Also, extracts potencies of inhibition are relatively high and their selectivities to inhibit *h*MAO-B had not been altered nor masked by the extensive extraction. Notably, extensive extraction showed an alteration in rankings of extracts with PCS higher potency against *h*MAO-B ($p < 0.01$) than GUR.

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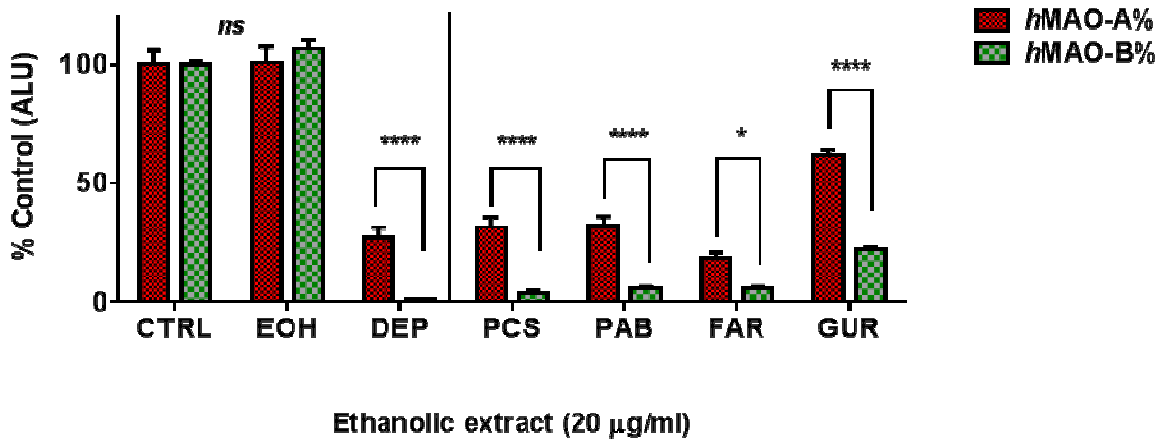

Fig. 4. Luminescence assay confirmation of relative inhibition of *h*MAO-B (RI_B) by extensively extracted plants of *Psoralea corylifolia* seeds (PCS), *Phellodendron amurense* (PAB), *Ferula assafoetida* resin (FAR) and *Glycyrrhiza uralensis* (GUR). ALU: arbitrary light units. Controls activities were compared with ethanol (EOH) and standard MAO-B inhibitor selegiline (DEP) at 5 µg/ml. All four extracts potently inhibited *hMAO*-B more than *h*MAO-A. Data points were presented as the mean ± SEM, with at least n= 3. The significance of difference between the two isozymes was determined using two-way ANOVA followed by Sidak's multiple comparisons test. * $p \leq 0.05$, **** $p < 0.0001$.

3.5. *h*MAO-B Relative Selectivity (RS_B)

To determine the most selective extract among the four extensively macerated plants with ethanol, the Relative selectivity (RS_B) of each of GUREE, PCSEE, PABEE, and FAREE was investigated using Amplex Red assay of both isozymes (**Fig. 5**). No significant difference was observed between controls with and without the used ethanol concentrations. With a similar X-axes scale, all tested EEs showed a concentration-dependent *h*MAO-A and *h*MAO-B inhibitory potencies with clear RS_Bs. The extracts showed no significant different *h*MAO-B inhibitory potencies from each other ($P > 0.05$). Nonetheless, the RS_B of each of the four extracts was highly significantly different ($p < 0.01$ and 0.001). Specifically, the most selective *h*MAO-B inhibitors among the four tested EE were PCSEE and FAREE, with more significant difference RS_Bs ($p < 0.001$) than GUREE and PABEE ($p < 0.01$). The results obtained also indicate preserved RS_Bs with increased potencies against *h*MAO-B with the extensive maceration.

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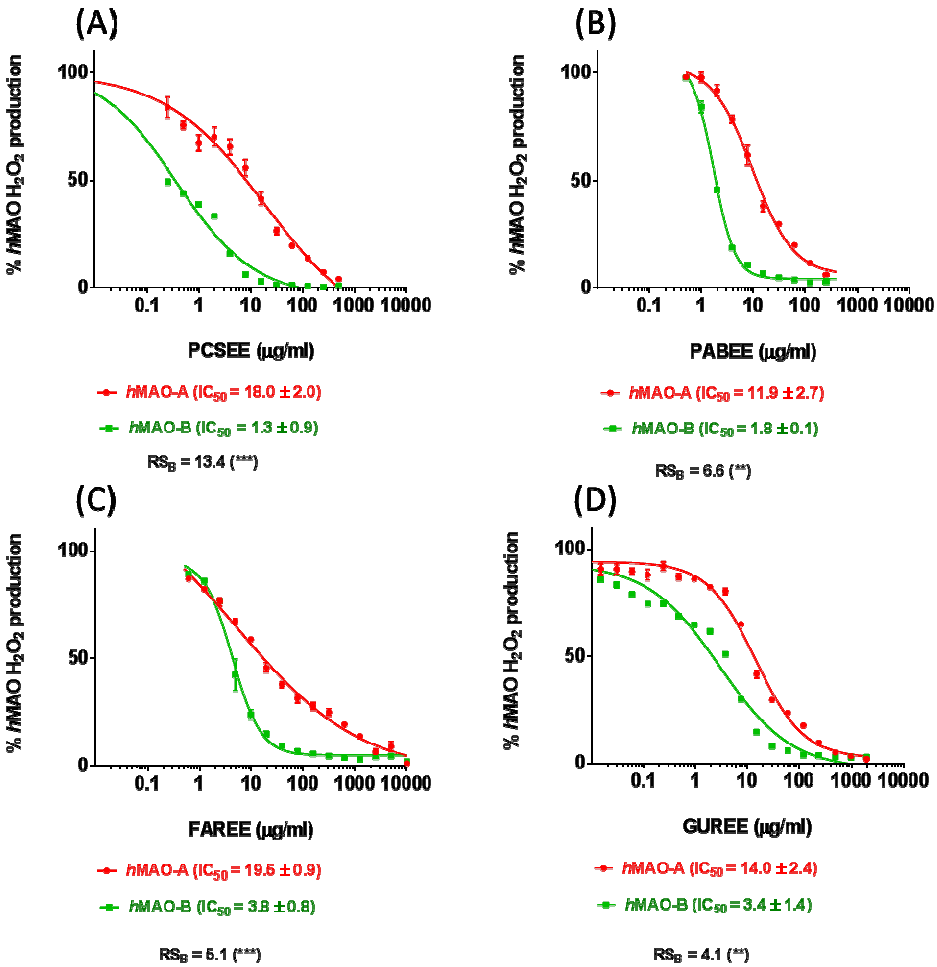

Fig. 5. *h*MAO-A and *h*MAO-B inhibitory potencies and *h*MAO-B relative selectivities (RS_B) of the extensively macerated ethanolic extracts of **(A)** *Psoralea corylifolia* (PCSEE), **(B)** *Phellodendron amurense* barks (PABEE), **(C)** *Ferula assafoetida* (FAREE), and **(D)** *Glycyrrhiza uralensis* (GUREE). All extracts were likewise potent MAOs inhibitors with a significantly high RS_B. The percent points were presented as the mean ± SEM, n= 4. IC_{50} ± SEM values were averaged from two experiments. Significance of difference between the two isozymes IC_{50}s for each extract was determined using two-way ANOVA followed by Sidak's multiple comparisons test. ** $p < 0.01$,*** $p < 0.001$.

4. DISCUSSION

Plant extracts ability to inhibit human MAO-B selectively was investigated by microtiter screening of 132 ethanolic plant extracts out of the 155 extracts. The initial screen indicates the high potential of plant extracts that contain varieties of selective *h*MAO-BIs, *h*MAO-AIs, and non-selective *h*MAOIs. The screen designated the abundance of common selective MAO-A and MAO-B inhibitors in nature. While it is less relevant for PD and thus beyond the scope of this work investigate *h*MAO-A inhibitors, our focus was on the plants that specifically inhibit *h*MAO-B. *Psoralea corylifolia* seeds, *Phellodendron*

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366 *amurense* barks, *Glycyrrhiza uralensis* roots*, and Ferula assafoetida* resin ethanolic extracts stood out
367 as potent and selective *h*MAO-B inhibitors. Regardless of their extensive extraction and the used
368 assay, the four extracts consistently showed higher relative *h*MAO-B inhibitions more than *h*MAO-A,
369 which indicates an intrinsic selectivity to inhibit *h*MAO-B. On the other hand, the further extensive
370 extraction dramatically enhanced the potency, particularly at high concentrations. Similar to the used
371 standards of DEP and pirlindole, the high extracts potencies concealed, but did not alter, their
372 preserved *h*MAO-B relative inhibition.
373 The obtained four plant ethanolic extracts preliminary RI_Bs and conclusive RS_Bs were not due to their
374 effects on H_2O_2 as confirmed with the luminescence assay. H_2O_2 scavenging activities or redox
375 properties would equivalently reduce the total H_2O_2 in both assayed isozymes at the same extract
376 concentration. Also, the H_2O_2 scavenging activity can alter the inhibition selectivity from *h*MAO-B to
377 *h*MAO-A which produces less H_2O_2 at 1 h reaction. The Amplex Red assay used for this screen is a
378 highly sensitive one-step reaction method with a stable detection reagent product. It was previously
379 evaluated for HTS and proposed over other conventional HPLC method for its convenience and
380 continuity [19]. The use of the endogenous substrate tyramine and measuring the cytotoxic enzymes
381 product H_2O_2 is advantageous as it mimics the biological reactions within the body. In contrast with
382 the luminescence assay, *h*MAO-B very high luciferin derivative substrate affinity (4 μM) may not
383 represent natural neurotransmitters affinities as benz. HCl does in the fluorometric assay. However,
384 our used fluorescence assay led to eliminating many extracts from the screening as it was not suitable
385 to detect MAOIs in extracts with H_2O_2 scavenging activities because of the extract direct interferences
386 with the enzymatic H_2O_2.
387 In this work, the investigated *Ferula assafoetida* resin (*aka* stinking assa; family Apiaceae) showed
388 high potency and selective inhibition of *h*MAO-B. This resin is used as a spice and a phytomedicine
389 around the globe for centuries. In the folklore medicine, it is mostly used in asthma, gastrointestinal
390 disorders, and neuronal disorders [20]. In recent reports, the resin improved memory and learning in
391 rats [21], and exhibited neuroprotection and nerve stimulation in mice peripheral neuropathy [22], and
392 anticonvulsant properties [23]. FAR contains bioactive phytochemicals such as polysulfides,
393 sesquiterpenes, sesquiterpene-coumarins, diterpenes, phenolics, and flavonoids [20, 24]. Its
394 coumarin umbelliprenin showed anti-inflammatory properties [20], while ferulic acid showed anti-

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395 atherosclerotic, antioxidant, and neuroprotective properties [25] and became a candidate for AD [26].
396 Therefore, investigations on the resin concerning PD need to be considered.
397 In addition, the seeds of *Psoralea corylifolia* (*aka*, Bu Gu Zhi or Babchi; family Leguminosae) are
398 important in traditional Chinese and Ayurvedic medicines [27]. PCSEE was one of the most potent
399 and selective *h*MAO-BI using our fluorometric screening assay. Our PCS findings are supported by
400 our previous investigations on its *h*MAO-B inhibitory potency tested spectrophotometrically [17], and
401 its selectivity for *h*MAO-B using a luminescence assay [28]. Previous PCS screened extracts for
402 active constituents revealed that the ethanolic extract composes more medically active compounds
403 than some other PCS extracts, which makes it a better candidate for novel phytomedicines [29].
404 PSCEE is rich in benzopyrone structure constituents including coumarins and flavonoids. PCS
405 furocoumarins psoralen and isopsoralen showed *rat* MAOs activities inhibitions [30], which was
406 supported by total furocoumarins potent antidepressant effects on mice [31]. PCS also contains
407 isoflavones which have been used as dietary supplements in various diseases, including
408 osteoporosis, cognitive dysfunction, cardiovascular disease, and inflammation [32], which are close to
409 PCS multifaceted properties [33-35]. We previously investigated bavachinin and genistein flavonoids
410 constituents of PCS. Bavachinin exhibited a selective *h*MAO-B inhibition [28] while isoflavone
411 genistein was similarly potent but less selective against *h*MAO-B [36]. Moreover, PCSEE contains
412 monoterpenes that protected against the MAO-B substrate 1-methyl-4-phenyl-1,2,3,6-
413 tetrahydropyridine (MPTP) SN cell damage and MPTP-induced motor deficits in PD model [37],
414 inhibited DA and norepinephrine (NE) transporters [38], and showed antidepressant effects with
415 catecholamine neurotransmitters regulation [39, 40]. The PCS extracts were also neuroprotective
416 against the MPTP precursor MPP+ [38] and the nitropropionic acid (3-NP) induced cytotoxicity and
417 mitochondrial dysfunction [41]. Although the seeds are used in dermatological disorders health
418 supplements [33] and increasingly investigated on *in vitro* and animal models, the extract and its
419 phytochemicals clinical effects on degenerative diseases are yet to be clinically considered. From our
420 results, the observed association between PCS constituents MAO-B inhibitions and the extracts
421 neuroprotection in the previous reports suggests more investigations for potential beneficial PCS
422 phytochemicals for PD.

423 Also, *Phellodendron amurense* (*aka* Amur cork tree; family Rutaceae) is a meagerly investigated
424 Chinese medicinal plant. In our study, its bark ethanolic extract clearly was a selective *h*MAO-BI as its

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potent inhibition was previously spectrophotometrically confirmed [17]. The plant constituted alkaloids such as phellodendrine, palmatine, jatrorrhizine, and berberine [42, 43] where the later displayed safe antidepressant-like activities in mice by the possible mechanism MAO-A inhibition and increasing DA, NE and serotonin brain levels [44, 45]. PAB is high in the flavone tetramethyl-o-scutellarin, and the triterpenoids limonoids [42]. Limonoid obacunone was found neuroprotective in glutamate-induced neurotoxicity in vitro [46]. In clinical studies, PAB extract supplement safely reduced cortisol [47], and relieved mild anxiety in women [48]. Also, PAB inhibited pro-inflammatory cytokines [49, 50] and protected from prostate tumors progression [51], property found in some MAO-AIs [52]. Based on our results and literature, there is a lack of knowledge on MAO-B inhibition and selectivity benefits of PAB extracts and phytochemicals. Further studies on PABEE as MAO-BI source for PD are highly recommended.

The roots of *Glycyrrhiza uralensis* (aka Chinese licorice; family Leguminosae) is another commonly used medicinal plant in traditional Chinese and natural medicine. Our new finding that GUREE inhibits hMAO-B selectively is supported by our previous finding for its *h*MAO-B inhibition [17]. Interestingly, *GUR* was more selective than Glycyrrhiza glabra in our screen. Reported *Glycyrrhiza uralensis* different active constituents from other *Glycyrrhiza* genuses may influence its MAO-B selective inhibition [53]. GUR contains unique phytochemicals including isoprenylated phenolics [54] flavonoids, chalcones, and triterpene saponins [55]. Chalcone isoliquiritigenin, is an inhibitor for MAO-B [56] with multifunctional anti-inflammatory, antioxidant, cytoprotective [57] cellular detoxification system activator [58] and anti-apoptotic [59] anti-amyloid-β toxicity [60] neuroprotective properties. GUR total flavonoids extracts showed neurogenesis protective effect in depressed rats model [61]. The flavonoid liquiritin showed antioxidant and antiapoptotic neuroprotective effects in mice [62] and ameliorated depression in rat model [63]. Its benzopyran dehydroglyasperin-C also showed neuroprotection [64]. Xiaoyaosan, a traditional herb combination containing GUR for chronic depression, was effective in both animal models and clinical trials [65, 66]. Other multifunctional properties of GUR constituents included reducing pro-inflammatory cytokines, nitric oxide, reactive oxygen species, lipid peroxidation [67], and mitochondrial impairment [68]. Interestingly, GUREE reports covered its chemopreventive [69] and anti-diabetic properties [70]. Specifically investigating GUREE as a selective MAO-BI could be beneficial.

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5. CONCLUSION

Natural products are abundant of MAOIs with MAO-B selectivity and PCSEE, PABEE, GUREE, and FAREE are sources of yet to define MAO-B specific natural inhibitors. These plants contain high varieties of pharmacologically unique active phytochemicals such as coumarins, terpenes, flavonoids, and alkaloids. Therefore, the current findings may lead to the discovery of novel selective MAO-B inhibitors to benefit PD patients and beyond. Future research is required to elucidate and understand the pharmacological actions of these extracts and their phytochemicals which are responsible for the selectivity of *h*MAO-B inhibition and, consequently, finding safe therapeutic compounds for neurodegenerative diseases such as PD.

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