SUPPLEMENTRY MATERIAL

Dismantling Parkinson's disease with herbs: MAO-B inhibitory activity and quantification of chemical constituents using HPLC-MS/MS of Egyptian local market plants

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ABSTRACT

Withania somnifera, *Angelica sinensis*, *Glycyrrhiza glabra*, and *Simmondsia chinensis* were acquired from the Egyptian market, profiled for their chemical constituents, screened for the *in-vitro* MAO-B inhibitory activity and evaluated for the total phenolic content. Thirty compounds were characterized in the selected herbs using HPLC-MS/MS. *In-vitro* MAO-B inhibitory activity and total phenolic content of the acquired herbs were compared with those of a prepared herbal formula consisting of a mixture of equal amounts of the four mentioned herbs. The most potent MAO-B inhibitory activity was exerted by the methanol extract of the prepared formula (IC_{50} of 712.19 ± 13.90 ng/ml) compared to selegiline (IC_{50} of 581.69 ± 11.35 ng/ml). The highest value of the total phenolic content was shown by *Angelica sinensis* methanolic extract (76.15 ± 0.1 mg/g) followed by *Glycyrrhiza glabra* methanolic extract (65.74 ± 0.1 mg/g), then the mixture's methanolic extract of the four herbs (37.04 ± 0.1 mg/g).

Keywords: HPLC-MS/MS, Ashwagandha, Angelica, Licorice, Jojoba, MAO-B, Parkinson's disease

Experimental Section

Plant materials

The plant materials *Withania somnifera* roots, *Angelica sinensis* roots, *Glycyrrhiza glabra* roots and *Simmondsia chinensis* fruits were purchased in dry form in October 2020 from Egyptian market, kindly identified and authenticated by Prof. Dr. Abdel-Halim Mohammed; Professor of Agriculture, Flora department, Agricultural museum, Dokki, Giza, 12611 Egypt, and kept in the Museum of Pharmacognosy Department, Faculty of Pharmacy, Cairo University, voucher specimen numbers are 11-06-2021I for *Glycyrrhiza glabra,* 12-06-2021I for *Angelica sinensis,* 13-06-2021I for *Withania somnifera* and 13-06-2021II for *Simmondsia chinensis*.

Extraction

20 g of each plant powder (*Withania somnifera* root, *Angelica sinensis* root, *Glycyrrhiza glabra* root and *Simmondsia chinensis* fruits) were weighed and sonicated three times each with 200 ml of methanol for 30 minutes at room temperature (25 ◦C) using ultrasonic water bath (53 KHz). Extracts were filtered through Whatman filter paper, then evaporated to dryness under reduced pressure using rotatory evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at 40 ˚C and used for HPLC-MS/MS analysis and for biological screening.

Five g of each plant powder were weighed. Mixed powders were sonicated three times each with 200 ml of methanol for 30 minutes at room temperature (25 ◦C) using ultrasonic water bath (53 KHz), dried by using rotavapor at 40 0C and kept for HPLC-MS/MS analysis and biological screening. Extracts were filtered through Whatman filter paper, then evaporated to dryness under reduced pressure using rotatory evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at 40 ◦C and used for HPLC-MS/MS analysis and for biological screening.

Chemicals and Reagents

Cyanidin-3-glucoside chloride, delphinidin-3,5-diglucoside chloride, kaempferol-3-glucoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The other 27 analytical standards of the 30 authentic compounds were supplied by Sigma-Aldrich (Milan, Italy). Individual stock solutions of each analyte, at a concentration of 1000 mg L^{-1}, were prepared by dissolving pure standard compounds in LC-MS grade methanol and storing in glass-stoppered bottles at 4 °C. Afterwards, standard working solutions at various concentrations were prepared daily by appropriate dilution of the stock solution with methanol. HPLC-grade formic acid 99–100% was purchased from Merck (Darmstadt, Germany) and LC-MS grade methanol were supplied by Sigma-Aldrich (Milano, Italy). Ultra-pure water was obtained from a Milli-Q Reagent Water System (Bedford, MA, USA). All other solvents and chemicals were analytical grade. Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 µm syringeless filters, Phenomenex (Castel Maggiore, Italy).

HPLC-ESI-MS/MS

Quantification of the 30 bioactive compounds in different extracts carried out by following a previous analytical procedure (Nzekoue et al. 2020). Dried extracts have been dissolved (10 mg/mL) in methanol. Ultra-sonication used to facilitate the extract dissolution before HPLC-MS/MS analysis, the samples were filtered with 0.2 µm filter. Briefly, HPLC-MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in negative and positive ionization mode. In fact, the instrument allowed to perform a one run with polarity switching without any problems. The separation of target compounds was achieved on a Kinetex PFP analytical column

(100 mm x 2.1 mm i.d., particle size 2.6 µm) from Phenomenex (Torrance, CA, USA) at a flow rate of 0.2 mL min^{-1} in gradient elution mode. The mobile phase was a mixture of water (A) and methanol (B) both with formic acid 0.1% and its composition varied as follows: 0–2 min, isocratic condition, 20% B; 2–15 min, 80% B; 15–18 min, isocratic condition, 80% B; 18–23 min, 100% B; 23–35 min, 20 % B. The injection volume was 2 µL. The temperature of the column was 30°C and the temperature of the drying gas in the ionization source was 350°C. The gas flow was 10 L/min, the nebulizer pressure was 25 psi and the capillary voltage was 4000 V. Detection was performed in the "dynamic-Multiple Reaction Monitoring" (dynamic-MRM) mode and the dynamic-MRM peak areas were integrated for quantification. The most abundant product ion was used for quantitation, and the other for qualification. For all analytes eight different concentrations, at the following range 0.001−5 µg mL−1, have been used for plotting the calibration curves. The peaks of dynamic-MRM product ions of each analyte have been integrated using MassHunter Software (Agilent Technologies) and data were processed using Microsoft Excel (Microsoft Office 2019). The selected ion transitions and the mass spectrometer parameters are reported in Table S1. For quantification all samples were injected in triplicate (n=3) and standard deviations have been added in table S2.

In vitro MAO-B inhibitor activity

Inhibitory potency was estimated using commercial screening kits (Biovision, USA) following the user's manual guidelines. The assay is based on the fluorometric screening of hydrogen peroxide, produced during the oxidative deamination of MAO-B substrate (Tyramine). The assay was carried in a 96-well black opaque microplate with flat bottom in duplicate. For each well, freshly prepared 50 µl of MAO-B enzyme solution: (49 µl of MAO-B assay buffer mixed with 1 µl of diluted MAO-B enzyme) were added into wells containing 10 µL of tested extracts, inhibitor control and enzyme control. Then 40 µl of MAO-B substrate solution, prepared by mixing 37 µl of MAO-B Assay Buffer, 1 µl of tyramine, 1 µl of developer and 1 µl OxiRedTM probe, was added to each well to start the enzymatic reaction. The reaction mixtures were incubated for 10 min at 37°C.The positive control was the reaction of MAO-B enzyme with the substrate (in absence of inhibitors), while the negative control was the reaction of MAO-B enzyme and the inhibitors selegiline (in the absence of substrate).The obtained fluorescence was measured (Excitation/Emission = 535/587 nm) kinetically at 37°C for 10-40 min. Concentration response curves showing the fluorescence values against time were plotted from which IC_{50} values (the half maximal inhibitory concentration) were calculated and results are expressed as mean ± SD. The percentage Relative Inhibition was calculated as follows:

$$\% \text{ Relative Inhibition} = \frac{(\text{Slope of EC} - \text{Slope of S}) \text{ X } 100}{\text{Slope of EC}}$$

Where; EC, the enzyme control and S, substrate EC. performed from concentration–response curves, plotted by the GraphPad Prism software.

Determination of total phenolic content (TPC)

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method (Saboo et al. 2010). Briefly, 200 µL of extracts (10 mg/mL) and 2.0 mL of solution A (mix 10 mL of 2% Na2CO3 with 0.1 mL of $CuSO_4$ and 0.1 mL of sodium and potassium tartrate) were mixed and after 4 min, 0.4 mL of 0.5 M sodium hydroxide was added. After 10 min 0.2 mL of Folin-Ciocalteu reagent (1:1 v/v with water) was added. The solution was left for 30 min and its absorbance was measured with a UV–Vis. spectrophotometer at 765 nm. The total phenolic content was calculated from the standard calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Statistical Analysis

Results were expressed as a mean of three experiments ± standard error of the mean. All results are presented as mean values of three replicates. Standard debiations (SD) and one-way ANOVA with Tukey LSD post hoc test was used to estimate the overall significance. P values below 0.05 were considered statistically significant.

References

Nzekoue, F.K., Angeloni, S., Navarini, L., Angeloni, C., Freschi, M., Hrelia, S., Vitali, L.A., Sagratini, G., Vittori, S. and Caprioli, G., 2020. Coffee silverskin extracts: Quantification of 30 bioactive compounds by a new HPLC-MS/MS method and evaluation of their antioxidant and antibacterial activities. *Food Research International*, *133*, p.109-128.

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Table S1. HPLC-MS/MS acquisition parameters, including retention time (Rt) for each transition.

No.	Compounds	Precursor ion (*m/z*)	Product ion (*m/z*)	Polarity	Retention time (Rt) (min)
1	Shikimic acid	173	173	Negative	1.40
			-	-	
2	Gallic acid	169	125[a]	Negative	2.37
			51		
3	Loganic acid	375	213[a]	Negative	3.13
			113		
4	3-Caffeoylquinic acid	353	191[a]	Negative	3.58
			179		
5	Swertiamarin	419	179[a]	Negative	4.89
			89		
6	Gentiopicroside	357	177[a]	Positive	5.33
			73		
7	(+)-Catechin	289	245[a]	Negative	5.48
			109		
8	Delphinidin-3,5-diglucoside	463	300[a]	Negative	5.64
			271		
9	Sweroside	403	125[a]	Negative	5.95
			179		
10	5-Caffeoylquinic acid	353	191[a]	Negative	6.22
			85		
11	Caffeine	195	138[a]	Positive	6.50
			110		
12	Cyanidin-3-glucoside	449	287[a]	Positive	6.50
			403		
13	Vanillic acid	167	108[a]	Negative	6.70
			152		
14	Caffeic acid	179	135[a]	Negative	6.87
			134		
15	(-)-Epicatechin	289	245[a]	Negative	7.03
			109		
16	Syringic acid	197	182[a]	Negative	7.48
			123		
17	*p*-Coumaric acid	163	119[a]	Negative	8.47
			93		
18	Ferulic acid	193	134[a]	Negative	9.16
			178		
19	3,5-Dicaffeoylquinic acid	515	353[a]	Negative	9.82
			191		
20	Quinine	325	79[a]	Positive	10.1
			81		
21	Naringin	579	271[a]	Negative	10.17
			151		
22	Rutin	609	300[a]	Negative	10.34
			271		

23	Hyperoside	463	300[a] 271	Negative	10.43
24	*Trans*-cinnamic acid	149	131[a] 77	Positive	10.79
25	Resveratrol	227	185[a] 143	Negative	10.92
26	Amarogentin	585	227[a] 245	Negative	11.05
27	Kaempferol-3-glucoside	447	284[a] 227	Negative	11.24
28	Quercitrin	447	300[a] 301	Negative	11.24
29	Quercetin	301	151[a] 179	Negative	13.03
30	Isogentisin	257	242[a] 214	Negative	16.31

[a]These product ions were used for quantification, the others to confirm the analytes

Table 2. Total identified phenolic acids/flavonoids (μg/g of dried extract) found in the various extracts.

No.	Compound	Mixture of 4 herbs	*S. chinensis*	*G. glabra*	*W. somnifera*	*A. sinensis*
1	Shikimic acid	0.16 ± 0.005	0.04 ± 0.002	n.d.*	n.d.	0.31 ± 0.015
2	Gallic acid	0.08 ± 0.003	1.06 ± 0.074	0.69 ± 0.034	n.d.	0.13 ± 0.003
3	Loganic acid	n.d.	n.d.	n.d.	n.d.	n.d.
4	3-Caffeoylquinic acid	12.22 ± 0.733	n.d.	0.48 ± 0.029	4.94 ± 0.247	80.71 ± 3.228
5	Swertiamarin	0.04 ± 0.003	n.d.	n.d.	n.d.	0.47 ± 0.009
6	Gentiopicroside	29.40 ± 2.352	77.92 ± 7.013	n.d.	n.d.	n.d.
7	(+)-Catechin	n.d.	n.d.	1.41 ± 0.085	n.d.	n.d.
8	Delphinidin-3,5-diglucoside	n.d.	n.d.	n.d.	n.d.	n.d.
9	Sweroside	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.007
10	5-Caffeoylquinic acid	189.70 ± 9.485	0.22 ± 0.004	8.44 ± 0.169	40.67 ± 1.627	578.05 ± 23.122
11	Caffeine	n.d.	n.d.	n.d.	n.d.	n.d.
12	Cyanidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.
13	Vanillic acid	10.76 ± 0.323	0.38 ± 0.011	28.78 ± 2.302	2.31 ± 0.069	3.61 ± 0.217
14	Caffeic acid	0.73 ± 0.029	n.d.	1.28 ± 0.064	0.25 ± 0.005	2.49 ± 0.199
15	(-)-Epicatechin	n.d.	n.d.	n.d.	0.23 ± 0.012	n.d.
16	Syringic acid	1.56 ± 0.094	n.d.	4.32 ± 0.259	0.37 ± 0.022	1.30 ± 0.065
17	*p*-Coumaric acid	0.88 ± 0.061	0.79 ± 0.056	1.53 ± 0.077	0.14 ± 0.006	n.d.
18	Ferulic acid	3.51 ± 0.316	7.19 ± 0.575	0.64 ± 0.026	1.63 ± 0.081	2.60 ± 0.026
19	3,5-Dicaffeoylquinic acid	80.82 ± 4.041	0.35 ± 0.017	0.38 ± 0.015	0.15 ± 0.009	251.10 ± 5.022
20	Quinine	n.d.	n.d.	n.d.	n.d.	n.d.
21	Naringin	0.20 ± 0.014	n.d.	0.85 ± 0.009	n.d.	0.37 ± 0.033
22	Rutin	0.05 ± 0.002	1.74 ± 0.052	0.06 ± 0.006	0.02 ± 0.001	0.83 ± 0.050
23	Hyperoside	0.36 ± 0.021	6.69 ± 0.134	0.53 ± 0.016	n.d.	8.22 ± 0.740
24	*Trans*-cinnamic acid	0.09 ± 0.001	n.d.	0.12 ± 0.002	0.20 ± 0.016	0.31 ± 0.025
25	Resveratrol	n.d.	n.d.	0.08 ± 0.004	n.d.	n.d.
26	Amarogentin	n.d.	n.d.	n.d.	n.d.	n.d.
27	Kaempferol-3-glucoside	0.05 ± 0.004	n.d.	0.20 ± 0.006	n.d.	0.11 ± 0.008
28	Quercitrin	0.02 ± 0.002	2.21 ± 0.088	0.32 ± 0.006	n.d.	0.59 ± 0.023
29	Quercetin	n.d.	7.82 ± 0.391	n.d.	n.d.	n.d.
30	Isogentisin	n.d.	n.d.	n.d.	n.d.	n.d.
	Total phenolic acids	300.35 ± 15.017	9.99 ± 0.599	46.66 ± 3.733	50.66 ± 2.533	920.29 ± 46.015
	Total identified flavonoids	0.67 ± 0.027	18.46 ± 0.554	3.37 ± 0.101	0.25 ± 0.008	10.12 ± 0.405
	Total compounds	**330.61** ± 19.837	**106.42** ± 4.257	**50.11** ± 2.506	**50.91** ± 2.036	**931.42** ± 46.571

* n.d., not detectable; Relative Standard Deviation (RSD %) were 3.2−9.9% for all compounds; Total phenolic acids are referred to the total concentration of chlorogenic acids and phenolic acids.

Table S3. Effect of the different extracts versus the mixture and selegiline on MAO-B enzyme were evaluated using an *in-vitro* fluorometric assay

Parameter / Treatment	IC_{50} [ng/ml]
	Mean ± SD
Withania somnifera	1543.45±30.13
Glycyrrhiza glabra	810.43^{a}±15.82
Simmondsia chinensis	7229.60^{ab}±141.17
Angelica sinensis	2720.39^{abc}±53.12
Mixture	712.19^{acd}±13.90
Selegiline	581.69^{abcd}±11.35

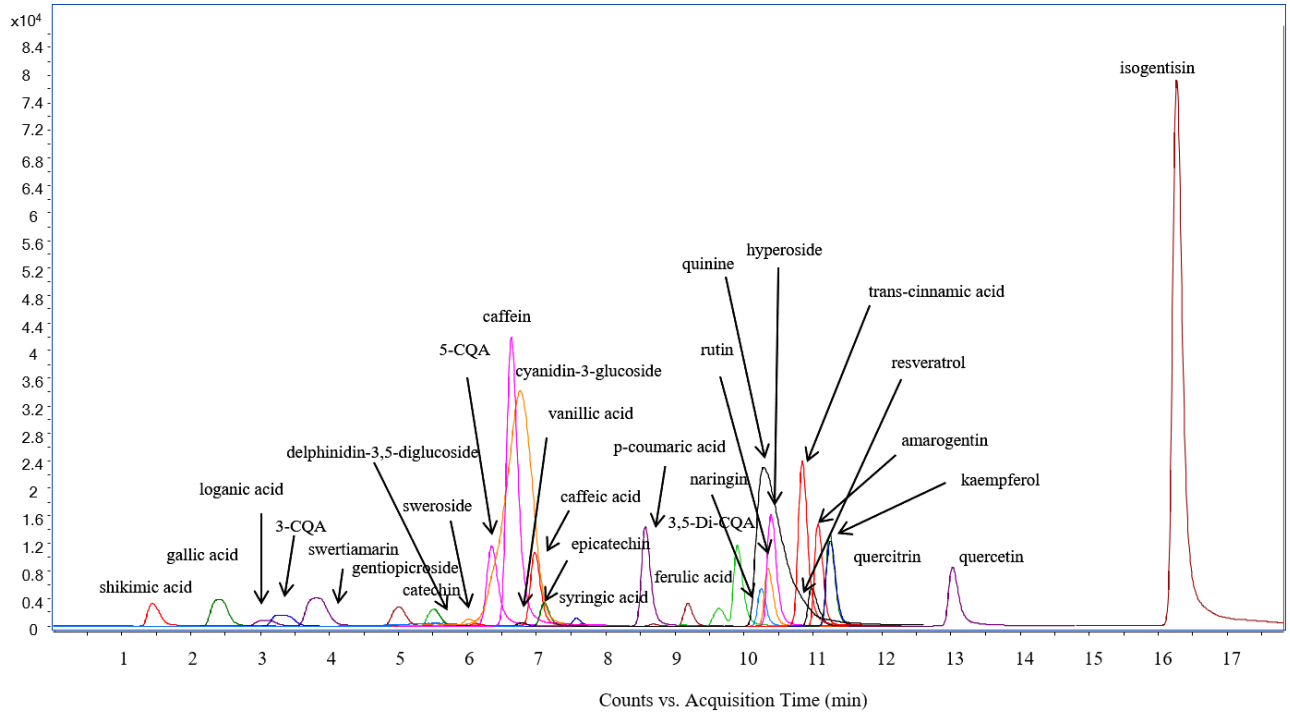
Each cell reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance set at the $P < 0.05$ level as follows: [a] versus *Withania somnifera*, [b] versus *Glycyrrhiza glabra*, [c] versus *Simmondsia chinensis* and [d] versus *Angelica sinensis*.

Table S4. Determination of the total phenolic content of the different extracts as well as the prepared mixture

Parameter / Treatment	Total Phenolic content [mg of gallic acid /g of sample]
	Mean ± SD
Withania somnifera	**24.11±0.1**
Glycyrrhiza glabra	**65.74[a]±0.1**
Simmondsia chinensis	**16.15[ab]±0.1**
Angelica sinensis	**76.15[abc]±0.1**
Mixture	**37.04[abcd]±0.1**

Each cell reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance set at the $P < 0.05$ level as follows: [a] versus *Withania somnifera*, [b] versus *Glycyrrhiza glabra*, [c] versus *Simmondsia chinensis* and [d] versus *Angelica sinensis*.

Figure S1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2
1.6
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0
isogentisin
quinine
hyperoside
trans-cinnamic acid
caffein
5-CQA
rutin
resveratrol
cyanidin-3-glucoside
vanillic acid
amarogentin
delphinidin-3,5-diglucoside
p-coumaric acid
kaempferol
loganic acid
naringin
sweroside
caffeic acid
3,5-Di-CQA
3-CQA
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quercetin
gallic acid
swertiamarin
gentiopicroside
ferulic acid
shikimic acid
catechin
syringic acid
1
2
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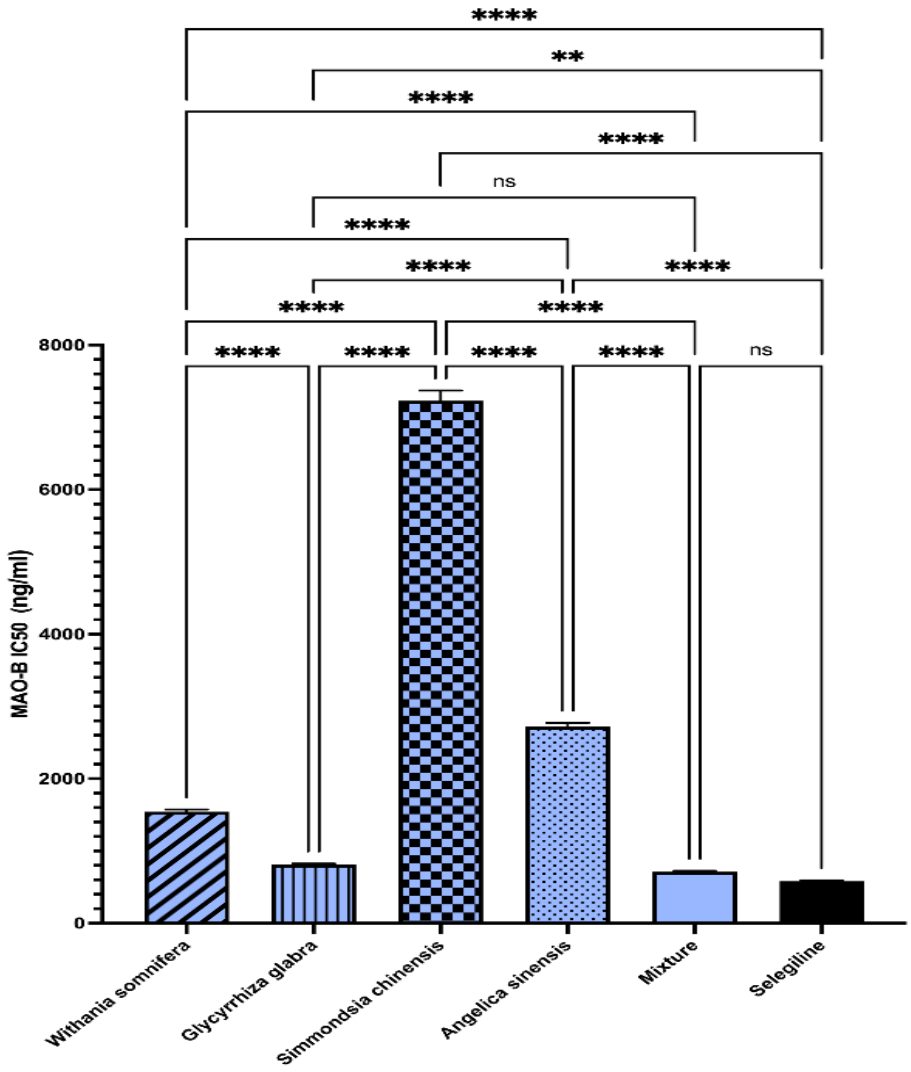
16
17
Counts vs. Acquisition Time (min)

Figure S2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4000
2000
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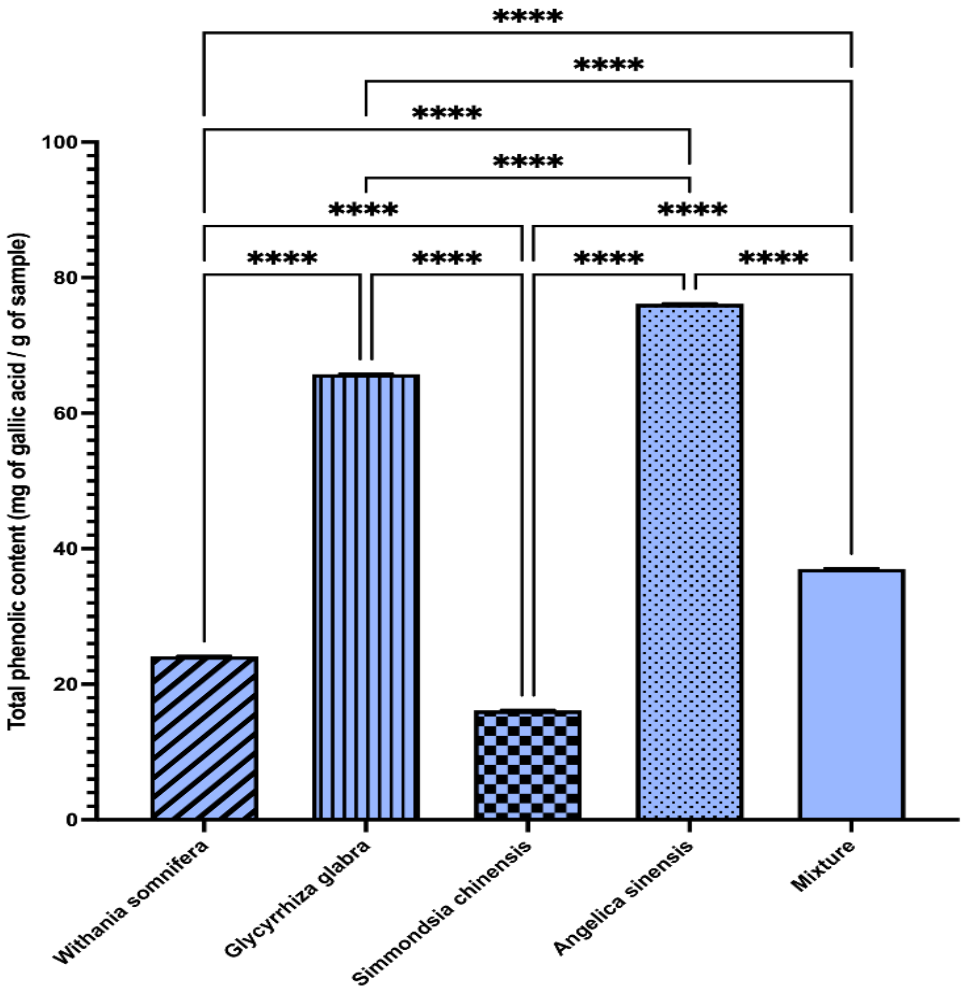
MAO-B IC50 (ng/ml)
Withania somnifera
Glycyrrhiza glabra
Simmondsia chinensis
Angelica sinensis
Mixture
Selegiline

Figure S3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Total phenolic content (mg of gallic acid / g of sample)
100
80
60
40
20
0
Withania somnifera
Glycyrrhiza glabra
Simmondsia chinensis
Angelica sinensis
Mixture

Figure legends

Figure S1. HPLC-MS/MS chromatogram of a standard mixture of 30 bioactive compounds plotted as overlapped MRM transition of each analyte

Figure S2. Effect of the different extracts versus the mixture and selegiline on MAO-B enzyme were evaluated using an in-vitro fluorometric assay]$F(5, 12) = 4864$, $p < 0.0001$[. Each vertical-lined bar reflects the mean of the experiments ± S.D. ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: *p <0.05, **p <0.01, ***p <0.005, ****p <0.001, ns no significance.

Figure S3. Determination of the total phenolic content of the different extracts as well as the prepared mixture [$F(4, 10) = 204435$, $p < 0.0001$]. Each vertical-lined bar reflects the mean of the experiments ± S.D. ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: *p <0.05, **p <0.01, ***p <0.005, ****p <0.001, ns no significance.