SUPPLEMENTRY MATERIAL

A new arsenal of polyphenols to make Parkinson's disease extinct: HPLC-MS/MS profiling, very interesting MAO-B inhibitory activity and antioxidant activity of *Otostegia fruticosa*

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A new arsenal of polyphenols to make Parkinson's disease extinct: HPLC-MS/MS profiling, very interesting MAO-B inhibitory activity and antioxidant activity of *Otostegia fruticosa*

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ABSTRACT

Fifteen compounds belong to phenolic acids, derivatives of phenolic acids, iridoids, xanthones and flavonoids were characterized in the methanolic extract of *Otostegia fruticosa* leaves using HPLC-MS/MS. Extract has been also investigated for its MAO-B inhibitory activity, antioxidant activity, total phenolic and total flavonoid content. The extract exhibited interesting MAO-B inhibitory activity (IC50; 2.24 ± 0.08) compared to the reference compound selegiline (0.55 ± 0.02 µg/mL). It also showed a potent antioxidant activity proven in both DPPH and ORAC assay methods. The extract showed an IC50 of 3.64 ± 1.22 µg/mL in the DPPH test which was significantly lower than that of the standard ascorbic acid which attained an IC50 of 18.3 ± 1.41 µg/mL. Moreover, in the oxygen radical absorbance capacity assay (ORAC) the extract showed a decline in the IC50 to 3.48 ± 1.16 µg/mL as compared to the standard Trolox which exhibited an IC50 of 27.0 ± 13.41.

Keywords: *Otostegia fruticosa*, MAO-B, anti-oxidant, HPLC-MS/MS, Parkinson's disease

Experimental Section

Plants materials

Leaves of *O. fruticosa* were collected from Bani Matar village, Sana'a Governate, Yemen, in July 2020. Plant materials were kindly authenticated by Dr. Abdul Wali Ahmed Al-Khulaidi, associate professor of flora and vegetation at the Agricultural Research and Extension Authority "Southern Upland Station" Taiz/Ibb, Yemen). Voucher specimen of the authenticated plant was deposited at Pharmacognosy Department, Faculty of Pharmacy, Cairo University with voucher number; 13-10-2021I. The collected samples were air dried in shed and stored in tightly closed containers till use.

Extraction

Ten grams of *O. fruticosa* leaves were extracted by 90% HPLC grade methanol using ultrasonic-assisted extraction method till exhaustion. The extracts were filtered and solvent was totally evaporated using rotary evaporator at 40ºC. The dried extracts were weighted and the required weight of samples were sent for investigation.

Chemicals and Reagents

For HPLC-ESI-MS/MS experiment, 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 bioactive compounds were supplied by Sigma -Aldrich (Milan, Italy). 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 filter, Phenomenex (Castel Maggiore, Italy).

For total phenolics and flavonoids content determination, Folin-Ciocalteu reagent, methanol, gallic acid, and (+)-catechin were purchased from Sigma Aldrich (Milan, Italy). Sodium carbonate, sodium nitrite, sodium hydroxide, and aluminium chloride were purchased from Merck (Darmstadt, Germany).

For MAO-B Inhibition Screening, Fluorometric monoamine oxidase B inhibitor screening kit was purchased from Biovision® (San Francisco, CA, USA). These kits include ready-to-use solutions, such as enzyme assay buffer solutions, OxiRed™ Probe solutions in DMSO, MAO-B enzymes (lyophilized), MAO-B substrates (lyophilized), developers (lyophilized), inhibitor control, selegiline, (lyophilized) solutions.

For antioxidant activity evaluation, 2,2-Diphenyl-l-picrylhydrazyl (DPPH), fluorescein, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), and Trolox were from Sigma Aldrich (Milan, Italy). All other chemicals used were of analytical grade.

Methods

HPLC-ESI-MS/MS method

The quantification of 30 bioactive compounds in different extracts has been carried out by following a previous analytical procedure (Nzekoue et al. 2020). Individual stock solutions of each compound, at a concentration of 1000 mg L^{-1}, were prepared by dissolving pure standard compounds in LC-MS grade methanol and storing them in glass-stoppered bottles at 4°C. Following that, standard working solutions at varied concentrations were generated on a regular basis by dilution of the stock solution with methanol. Dried extracts have been dissolved (10 mg/mL) in methanol. Sonication was employed to aid extract dissolution, and the samples were filtered using a 0.2 μm filter prior to HPLC-MS/MS analysis. In brief, HPLC-MS/MS experiments were carried out using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionisation (ESI) source that operated in both negative and positive ionisation modes. 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 **Table S1**. The most abundant product ion was used for quantitation, and the other for qualification.

HPLC-MS/MS analytical method validation

The analytical method for the quantification of 30 bioactive compounds has been validated by testing some parameters such as linearity, sensitivity and repeatability taking the cue from a previous paper (Nzekoue et al. 2020). The linearity has been measured by analysing seven different concentrations

(0.001−5 µg/mL) of the standard mixture and plotting the resulting calibration curves with their determination coefficient (R^2). All the analytes showed a good linearity since the R^2 ranged from 0.9935 to 0.9998. The sensitivity has been measured by testing the limit of detection (LOD) and the limit of quantification (LOQ) for each monitored compound. The signal-to-noise (SNR) ratio was calculated by injecting different concentrations of the standard mixture and the LOD and LOQ were estimated as the concentrations of the analytes giving the SNR of 3:1 and 10:1, respectively. For all the analytes the LOD were from 0.3 to 50 µg/L and LOQ ranged from 1 to 200 µg/L. Intraday repeatability (intraday precision) and interday repeatability (interday precision) were calculated by evaluating the relative standard deviation (% RSD) of peak areas by injecting three different concentrations of the analytes three times in the same day for three consecutive days. The intraday precision ranged from 1.2 to 6.2% while interday precision were from 4.4 to 10.3%.

Total phenolics and flavonoids determination

The total phenolic content was calculated as gallic acid equivalents (GAE) per g of sample using the Folin-Ciocalteu reagent and a gallic acid calibration curve. In brief, 100 μL of tested extracts were transferred into test tubes, and their volumes were increased to 500 μL with distilled water before being oxidised with the addition of 250 μL of Folin-Ciocalteau reagent. After 5 minutes of reaction, the mixture was neutralised with 1.25 μL of 20% aqueous Na_2CO_3 solution. The mixtures were allowed to stand at room temperature for 40 minutes until the characteristic blue colour developed before centrifugation at 4000g for 5 minutes. At 725 nm, the absorbance of the clear supernatants was measured in comparison to a blank containing an extraction solvent instead of sample. Total phenolic content of each sample was determined by means of a calibration curve prepared with gallic acid and expressed as mg of gallic acid equivalents (GAE) per kg of dry matter (Žilić et al. 2012). Moreover, the total flavonoid content was determined as catechin equivalents (CE) per g of sample using an aluminium chloride ($AlCl_3$) colorimetric assay (Žilić et al. 2012). Briefly, 50 μL of 5% $NaNO_2$ was mixed with 100 μL of the appropriate extracts. After 6 min, 500 μL of a 10% $AlCl_3$ solution was added to form a flavanoid−aluminum complex. After 7 min, 250 μL of 1 M NaOH was added, and the mixture was centrifuged at 5000g for 10 min. The absorbance of the supernatant was measured at 510 nm against the blank containing the extraction solvent instead of a sample. The total flavonoid content was expressed as mg of catechin equivalents (CE) per kg of dry matter.

Antioxidant activity evaluation

DPPH assay

The estimation was done according to the method of Brand-Williams and Cuvelier (Brand-Williams et al. 1995). All experiments were carried out in triplicate. Ascorbic acid was used as positive control. Antioxidant activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (IC_{50}).

Oxygen radical absorbance capacity (ORAC assay)

Tested samples were dissolved in phosphate buffered saline (10 mM, pH 7.4) and investigated for their antioxidant capacity. The antioxidant capacity was measured by determining the time course of the fluorescence decay of fluorescein (Sigma), induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) compared with Trolox, (6-hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid), used as a positive control (Brand-Williams et al. 1995; Lucas-Abellán et al. 2008).

Monoamine Oxidase-B Inhibitor Screening method

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 oftested 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.

Statistical Analysis

The data are expressed as means ± standard deviations (S.D.). Unpaired Student's t-test was used to examine the statistical significance. The statistical analyses and graphical presentations were created using the GraphPad Prism software (version 9; GraphPad Software, Inc., San Diego, CA, USA). For all statistical tests, the $p < 0.05$ level was used as the statistical significance criterion.

References

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Table S1. HPLC-MS/MS acquisition parameters, working as "dynamic-Multiple Reaction Monitoring" mode, including retention time (Rt) and delta retention time (Δ Rt) for each transition.

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

19	3,5-Dicaffeoylquinic acid	515	353[a] 191	Negative	9.82	3
20	Quinine	325	79[a] 81	Positive	10.1	5
21	Naringin	579	271[a] 151	Negative	10.17	3
22	Rutin	609	300[a] 271	Negative	10.34	3
23	Hyperoside	463	300[a] 271	Negative	10.43	3
24	*Trans*-cinnamic acid	149	131[a] 77	Positive	10.79	3
25	Resveratrol	227	185[a] 143	Negative	10.92	3
26	Amarogentin	585	227[a] 245	Negative	11.05	3
27	Kaempferol-3-glucoside	447	284[a] 227	Negative	11.24	3
28	Quercitrin	447	300[a] 301	Negative	11.24	3
29	Quercetin	301	151[a] 179	Negative	13.03	3
30	Isogentisin	257	242[a] 214	Negative	16.31	3

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

Table S2. Content (μg/g of dried extract) of different bioactive compounds found in the *O. fruticosa* leaves extract.

No.	Compound	*O. fruticosa* Leaves
1	Shikimic acid	0.14
2	Gallic acid	1.50
3	Loganic acid	35.75
4	3-Caffeoylquinic acid	381.05
5	Swertiamarin	n.d.
6	Gentiopicroside	n.d.
7	(+)-Catechin	n.d.
8	Delphinidin-3,5-diglucoside	n.d.
9	Sweroside	n.d.
10	5-Caffeoylquinic acid	231.57
11	Caffeine	n.d.
12	Cyanidin-3-glucoside	n.d.
13	Vanillic acid	0.90
14	Caffeic acid	17.09
15	(-)-Epicatechin	n.d.
16	Syringic acid	1.84
17	*p*-Coumaric acid	3.92
18	Ferulic acid	2.70
19	3,5-Dicaffeoylquinic acid	3.87
20	Quinine	n.d.
21	Naringin	n.d.
22	Rutin	0.30
23	Hyperoside	0.48
24	*Trans*-cinnamic acid	0.38
25	Resveratrol	n.d.
26	Amarogentin	n.d.
27	Kaempferol-3-glucoside	n.d.
28	Quercitrin	n.d.
29	Quercetin	0.08

30	Isogentisin	n.d.
Total identified compounds		**681.56**

* 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. Total Phenolic and flavonoids content in studied samples determined by the Folin-Ciocalteu assay (calculated as mg of gallic acid/ g of sample and mg of catechin/ g of sample, respectively).

Plant material	**Total phenolics (mg/g)**	**Total flavonoids (mg/g)**
O. fruticosa leaves extract	413 ± 2.01	168 ± 3.01

Values above are the means of triplicate experiments ± SD

Table S4. Anti-oxidant activity of *O. fruticosa* leaves extract tested by DPPH assay and expressed as IC_{50} μg/mL ± SD.

Parameter / **Treatment**	**DPPH IC_{50} [µg/ml]**
	Mean ± SD
O. fruticosa leaves extract	3.64 ± 1.22
Ascorbic acid	18.3[a] ± 1.41

Each cell reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-test, with the criterion for statistical significance set at the $P < 0.05$ level as follows: [a] versus *O. fruticosa* leaves extract.

Table S5. Anti-oxidant activity of *O. fruticosa* leaves extract testd by ORAC assay and expressed as IC_{50} μg/mL ± SD.

Parameter / **Treatment**	**ORAC IC_{50} [μg/ml]**
	Mean ± SD
O. fruticosa leaves extract	3.48 ± 1.16
Trolox	27.0[a] ±13.41

Each cell reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-test, with the criterion for statistical significance set at the $P < 0.05$ level as follows: [a] versus *O. fruticosa* leaves extract.

Table S6. Percentage of MAO-B inhibition of *O. fruticosa* leaves extract and standard inhibitor expressed as IC_{50} ± SD

Parameter / **Treatment**	**ORAC IC_{50} [μg/ml]**
	Mean ± SD
O. fruticosa leaves extract	2.24 ± 0.08
Selegiline (standard inhibitor)	0.55[a] ± 0.02

Each cell reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-test, with the criterion for statistical significance set at the $P < 0.05$ level as follows: [a] versus *O. fruticosa* leaves extract.

Figure S1.

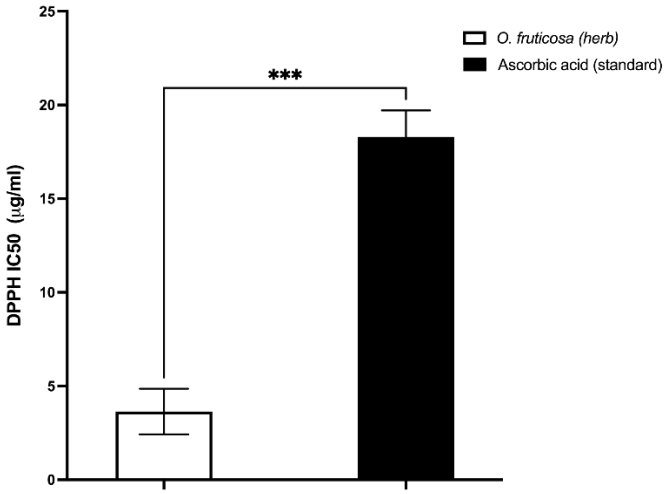
25
20
15
10
5
0
DPPH IC50 (µg/ml)
***
O. fruticosa (herb)
Ascorbic acid (standard)

Figure S2

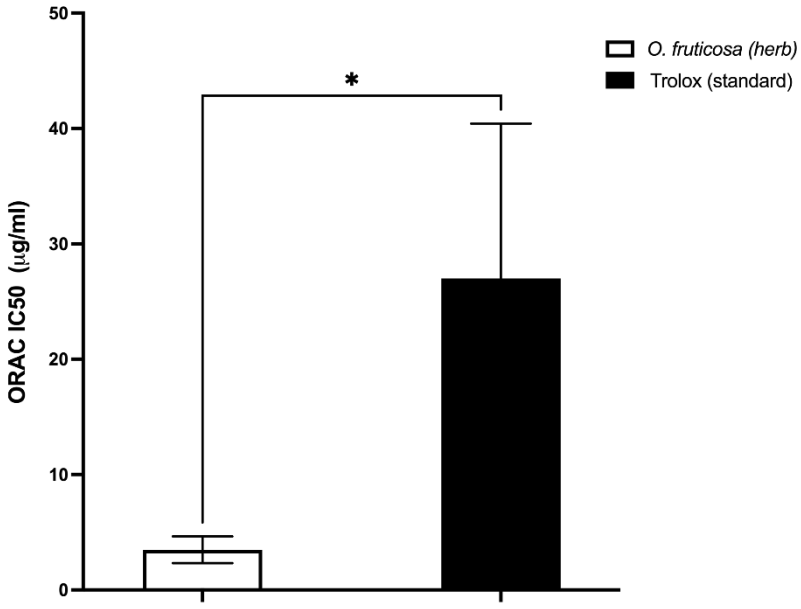
50
40
30
20
10
0
ORAC IC50 (µg/ml)
*
O. fruticosa (herb)
Trolox (standard)

Figure S3

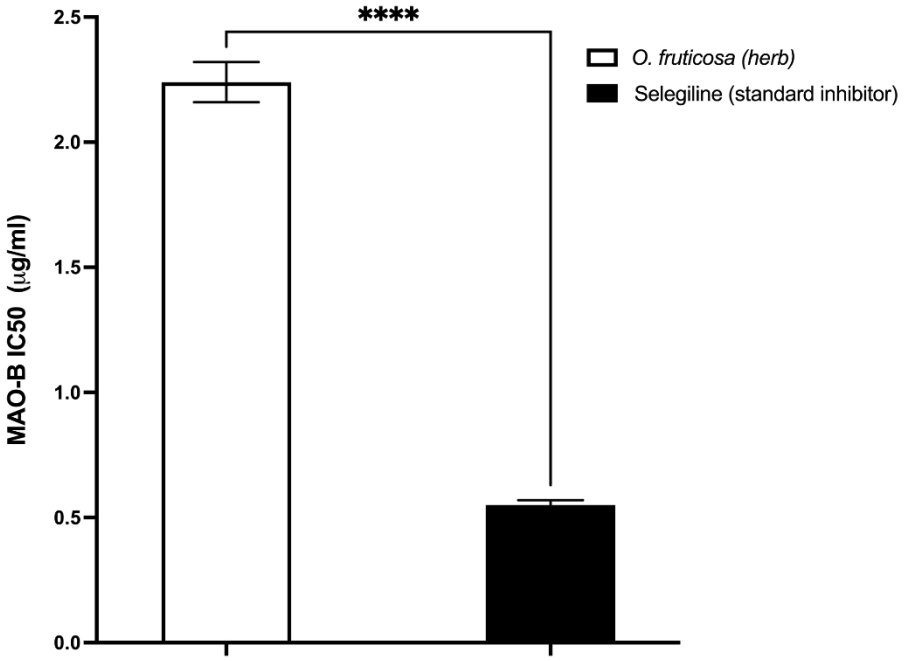
****
O. fruticosa (herb)
Selegiline (standard inhibitor)
MAO-B IC50 (μg/ml)
2.5
2.0
1.5
1.0
0.5
0.0

Figure legends

Figure S1. DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) assay

Figure S1: Determination of the antioxidant capacity of O. fruticosa leaves extract using DPPH assay as compared to the standard Ascorbic acid]t (4) = 13.62, p = 0.0002[. Each vertical-lined bar reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-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 S2. Oxygen radical absorbance capacity (ORAC assay)

Determination of the antioxidant capacity of *O. fruticosa* leaves extract using ORAC assay as compared to the standard Trolox [t (4) = 3.027, p = 0.0389]. Each vertical-lined bar reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-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. In-Vitro MAO-B Inhibitor Activity

Determination of the MAO-B inhibitor capacity of *O. fruticosa* leaves extract using an *in-vitro* fluorometric assay as compared to the standard Selegiline [t (4) = 35.5, p <0.0001]. Each vertical-lined bar reflects the mean of the experiments ± S.D. (n = 3). Statistical analysis was performed by Unpaired Student's t-test, with the criterion for statistical significance as follows: *p <0.05, **p <0.01, ***p <0.005, ****p <0.001, ns no significance.