SUPPLEMENTARY MATERIAL

Antidepressant Potential of *Mesembryanthemum cordifolium* Roots assisted by Metabolomic Analysis and Virtual Screening

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Abstract

Depression is a common mental disturbance that can be categorized as mild, moderate or severe. Mesemberine alkaloids, the main recognized phytoconstituents of some plants belonging to family Mesembryanthemaceae, are well-known as serotonin reuptake inhibitors. Therefore, the objective of this study is to evaluate the antidepressant activity of the alkaloidal fraction of *Mesembryanthemum cordifolium* L.f. (*Aptenia cordifolia*) roots, family Mesembryanthemaceae using forced swimming test, assisted by metabolomic analysis and *in silico* ligand-based and structure-based screening. Results showed that the alkaloidal fraction displayed an antidepressant activity superior to imipramine hydrochloride, a standard antidepressant agent. Nine alkaloids were annotated from the metabolomic analysis. Interestingly, among the dereplicated constituents, mesembrane (**5**) displayed strong binding affinity to SERT protein, which is slightly higher than the antidepressant drug venlafaxine. In conclusion, the alkaloidal fraction of the *M. cordifolium* (*A. cordifolia*) root exhibits an antidepressant activity which can be attributed in part to mesembrane (**5**).

Keywords: *Mesembryanthemum cordifolium*; *Aptenia cordifolia*; Mesembryanthemaceae; Metabolomics; Antidepressant; *in silico* screening

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Experimental

Plant material

Mesembryanthemum cordifolium L. f. (syn: *Aptenia cordifolia* L. f.) was identified by Prof. Naser Barakat, Department of Botany and Microbiology, Faculty of Science, Minia University. A voucher specimen (Mn-Ph-Cog-032) was stored in Pharmacognosy Department, Faculty of Pharmacy, Minia University, Minia, Egypt. The roots of *M. cordifolium* (*A. cordifolia*) (250 g) were extracted using 95% ethyl alcohol, then the dried ethanol extract (21.5 g) was further suspended in the least amount of distilled water, transferred to separating funnels, then defatted with light petroleum ether. The remaining aqueous solution was then acidified and extracted with ethyl acetate to give acidic ethyl acetate fraction (2.8 g) (non-alkaloidal fraction). The mother liquor was then completely basified and extracted again with ethyl acetate to give basic ethyl acetate fraction (1.8 g)(alkaloidal fraction)(Gaffney 2008).

Metabolomic analysis

The metabolomic analysis is a reliable and selective analytical method required to identify secondary metabolites in complex mixtures (Raheem et al. 2019). Secondary metabolites of the alkaloidal fraction were tentatively identified with the aid of existing high resolution mass and from the online and in-house databases. Molecular formula prediction was done utilizing MZmine's algorithm, which employs a combination of empirical techniques that included isotope pattern matching using the positive and negative mode electrospray ionization spectral data at a MW tolerance within 10 ppm. Known compounds were tentatively identified with the help of METLIN and DNP databases for plants natural products. The total ion chromatograms of positive and negative modes are shown in Supplementary Fig. S1.

The mass ion peak at *m/z* 165.116 $[M+H]^+$ (RT, 1.39 min.) for predicted molecular formula (MF) $C_{10}H_{15}NO$ was dereplicated as hordenine (**1**), that was previously isolated from *A. cordifolia* and, *A. lancifolia* (Gaffney 2008). Likewise, *m/z* 259.142 $[M+H]^+$ (RT, 2.80 min.) for suggested MF $C_{16}H_{21}NO_2$ was dereplicated as 4,5-dihydro-4'-O-methylsceletenone (**2**) which was previously obtained from *A. cordifolia* and *A. lancifolia* (Gaffney 2008). Also, *m/z* 257.158 $[M+H]^+$ (RT, 2.81 min.), corresponding to predicted MF $C_{16}H_{19}NO_2$, was dereplicated as 4'-O-methylsceletenone (**3**) that was isolated before

from *A. cordifolia* and *A. lancifolia* (Gaffney 2008). Another alkaloid with MF $C_{15}H_{17}NO_2$, in agreement with *m/z* 243.126 $[M+H]^+$ (RT, 2.91 min.) was dereplicated as sceletenone (**4**), formerly reported from *Sceletum strictum* (Gaffney 2008). Likewise, another alkaloid with MF $C_{17}H_{25}NO_2$ and *m/z* 275.189 $[M+H]^+$(RT, 3.08 min.) was characterized as mesembrane (**5**) and/or O-methyldihydrojoubertiamine (**8**), which were formerly isolated from *Sceletium* species (Gericke & Viljoen 2008). Moreover, *m/z* 261.173 $[M+H]^+$ (RT, 3.27 min.), corresponding to predicted MF $C_{16}H_{23}NO_2$, was dereplicated as dihydro-joubertiamine (**6**), an alkaloid previously isolated from *S. tortuosum* (Gaffney 2008), Whereas, *m/z* 273.173 $[M+H]^+$ (RT, 3.30 min.) was dereplicated as O-methyl-joubertiamine (**7**), with MF $C_{17}H_{23}NO_2$, which was reported from *Sceletium* species (Gericke & Viljoen 2008). Finally, *m/z* 326.190 $[M-H]^-$ (RT, 9.77 min.) for suggested MF $C_{20}H_{26}N_2O_2$ was dereplicated as touruosamine (**9**) which was isolated from *S. tortuosum* and *S. subvelutinum* (Gericke & Viljoen 2008).

Chemicals

All chemicals and solvents were of analytical grades and were purchased from Merck (Germany) and Sigma Chemical Co. (USA), respectively. Imipramine hydrochloride® (Richmond Pharmaceuticals Inc) was used as a standard antidepressant drug.

Animals

Adult male Wistar rats, weighing 180 ± 20 g, were obtained from El Nahda University (NUB), Faculty of Pharmacy, BeniSuef, Egypt. Rats were maintained on standardized laboratory conditions (temperature 23 ± 2 °C, 55% ± 5% humidity on a 12-h light/dark cycle) and were provided with free access to standard rat chow and water. The experimental method of the current study was approved by the Animal Care and Use Committee of Department of Pharmacology, Faculty of Pharmacy, Minia University. All animal experiments adhere to the National institutes of Health guidelines for the care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals 2011).

Forced swimming test (FST)

After arrival to the local animal house facility, all animals were allowed to acclimatize for at least one week. The FST was performed according to a previously described method by Yankelevitch-Yahav (Yankelevitch-Yahav et al. 2015). This test is used as a standard

test for screening of antidepressant activity. Each rat was placed in an open cylindrical container (diameter 15 cm, height 35 cm), containing 20 cm of water (depth) at 25 ± 2 °C. Each rat was forced to swim for 6 min, and only when it stopped struggling and floated on the water, the rat was considered immobile. The total duration of the test was video recorded and the duration of immobility and struggling was measured for each rat.

In order to determine the best time for testing the antidepressant activity, rats were divided into three groups consisting of control (vehicle treated rats) and rats treated with the alkaloidal fraction (100 mg/kg dissolved in a vehicle consisting of dimethyl sulfoxide (DMSO) : Tween: distilled water (1:1:8), via intraperitoneal; i.p. route). The dose was selected based on a previous study (Loria et al. 2014). The volume injected was 100 μL/100 g rat weight. After 30 min of dose administration, the FST was applied to group 1 but group 2 was allowed to wait for 1 hour after dose administration. Finally, the FST was applied on group 3 after 90 min. The struggling and immobility times were calculated for the three groups to determine the best time interval between the injection time and performing the test, which was deduced from the experiment as 1 hour where the alkaloidal fraction showed a longer struggling time and a shorter immobilization time as shown in supplementary Fig. S2 A and B. For ease of comparison, data from the control group was combined together as there were no significant difference between 30, 60 and 90 min groups.

Accordingly, all the FST tests were performed at a one-hour interval between the injection time and the FST. Rats were divided into three groups. Group 1 acted as control and received only the vehicle (DMSO: Tween: distilled water (1:1:8)) and group 2 (positive control) received a single-dose of imipramine hydrochloride® (15 mg/kg) dissolved in the vehicle (Silva et al. 2016). Group 3 received the alkaloidal fraction (100 mg/kg dissolved in the vehicle). After the FST, the brain tissues were carefully dissected for LC/ MS analysis to determine the ability of the identified alkaloids to cross the blood brain barrier.

Exploratory activity in the open field test in rats:

Exploratory activity was assessed in the elevated open field test as previously prescribed (Shehata et al. 2020). Briefly, control or treated rats were injected by vehicle or the alkaloidal extract, respectively. One hour later, each rat was individually placed in the corner of a wooden box (60x60x30 cm) and the performance of animal was evaluated by

counting the number of crossed square (ambulation), the number of times the animal cleans itself (grooming) and the number of times the animal stands on his hind limb (rearing) using a video camera for 5 min. The open field box was cleaned after each animal to remove any olfactory cues.

Determination of the ability of alkaloids to cross the blood brain barrier

Brain tissues were dissected immediately after the experiment and homogenized with the ethyl acetate and centrifuged, then the recovered ethyl acetate extracts of the rats' brains obtained from the previously described groups in addition to another brain of a non-treated rat, on which 4 mg of alkaloidal fraction was added after the dissection. They were allowed to dry in open air. The sample (100 µg/mL) solution was prepared then subjected to LC-ESI-MS analysis. Samples injection volumes (10 µL) were injected into the UPLC instrument equipped with reverse phase C-18 column (ACQUITY UPLC - BEH C18 1.7 µm particle size - 2.1 × 50 mm Column). Mobile phase elution was made with the flow rate of 2 mL/min using gradient mobile phase comprising two eluents; eluent A is H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid. The parameters for analysis were carried out using positive ion mode as follows: (source temperature 150 °C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h). Mass spectra were detected in the ESI between *m/z* 100–1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its mass spectrum with reported data. Total ion chromatogram of the samples in supplementary Figure S3.

Statistical analysis

Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was applied and Tukey-cramer was used as a post hoc test. Graph Pad Prism 7 was used for statistical calculations (Graph Pad Software, San Diego, California, USA). Results were considered significant at *p* values less than 0.05.

Ligand-based virtual screening and Molecular docking simulation

The ligand-based virtual screening for possible molecular targets to the obtained compounds, identified from the metabolomic analysis, are based on 2D/3D ligand similarity search using the "SwissTargetPrediction" method (http://www.swisstargetprediction.ch)(Gfeller et al. 2013). The "SwissSimilarity" online

tool (http://www.swisssimilarity.ch) was used to investigate the structural similarity of the identified compounds to the FDA approved Drug database and the bioactive ligands databases (Zoete et al. 2016). The X-ray structure of SERT (using the PDB entry 5I6X co-crystallized with paroxetine) was retrieved from protein data bank and used in the present docking study (Coleman et al. 2016). The structure preparation module in MOE 2019.01 (Chemical Computing Group, Montreal, QC, Canada) was applied in the preparation of the protein structure. All ligands (except paroxetine and sodium) and water were removed from the structure before docking. The co-crystallized ligand was defined as the center of the binding site (Radwan et al. 2019). Compounds **2** (4,5 dihydro-4'-O-methylsceletenone), **4f**, **5** (mesembrane) and **8** (O-methyl dihydrojoubertiamine) as well as venlafaxine were docked using the rigid-receptor method, with thirty docking poses were calculated for each compound. All other options were left at their default standards. The co-crystallized ligand was also docked with other compounds for validation of the docking method. The resulting docking poses were visualized using MOE 2019.01. The binding free energy (ΔG) in kcal/mol of all docked compounds was calculated using the top-scored docking poses (Abdelkader et al. 2018).

In silico Drug-likeness, ADME profiling, and toxicity risk assessment

The drug-likeness and pharmacokinetic properties of all identified compounds were predicted using Swiss ADME database (Daina et al. 2017). The ability of the compounds to passively diffuse through human gastrointestinal tract and to infiltrate the blood-brain barrier as well as to be a P-glycoprotein substrate were predicted via Boiled-Egg method (Daina & Zoete 2016).

Supplementary Tables

Table S1. IC_{50} to SERT in nM, binding free energy (ΔG) in kcal/mol, amino acid residues which form interactions and the type, distance and energy of these interactions for compounds 2, 4f, 5 and 8 compared to the antidepressant drug venlafaxine and the co-crystallized ligand paroxetine.

Ligand	*IC_{50} (nM) SERT*	*ΔG (kcal/mol) SERT*	*Amino acid residues*	*Interaction*	*Interaction distance (A)*	*Energy (kcal/mol)*
Paroxetine	2[a]	-8.6	Tyr95	H-donor	2.79	-12.0
			Asp98	H-donor	2.82	-10.0
			Asp98	Ionic	2.82	-5.8
			Tyr176	H-π	3.75	-0.6
Venlafaxine	27[b]	-7.4	Tyr95	H-donor	2.97	-0.9
			Tyr95	Cation-π	3.83	-0.7
			Asp98	Ionic	3.80	-1.0
			Tyr176	H-π	3.49	-0.5
2	ND[c]	-6.5	Phe335	H-donor	3.01	-8.6
4f	35[d]	-7.5	Asp98	H-donor	3.15	-1.1
			Asp98	Ionic	3.20	-3.3
5	ND[c]	-7.5	Tyr95	H-donor	3.31	-0.9
8	ND[c]	-7.1	Asp98	H-donor	2.95	-1.0
			Tyr176	H-π	3.76	-0.8

[a] Paroxetine IC_{50} value shown as reported (Davis et al. 2016)

[b] Venlafaxine IC_{50} value shown as reported (Sabatucci et al. 2010) [c] ND, not determined

[d] Compound 4f IC_{50} value shown as reported (Shao et al. 2011)

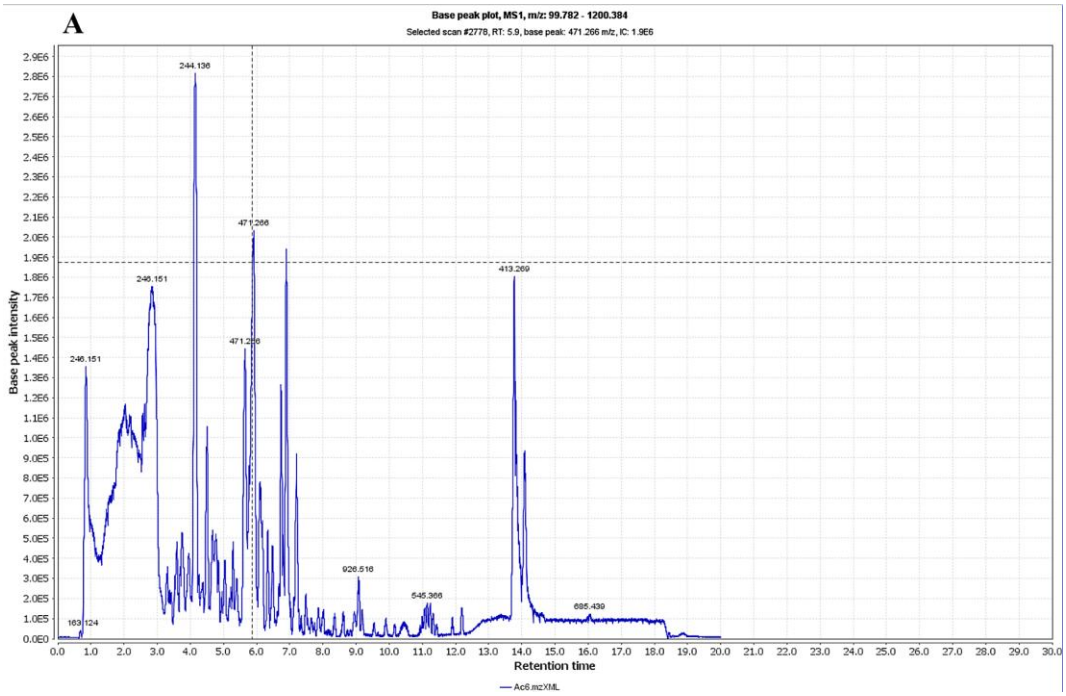
Table S2. Drug-likeness based on Lipinski's rule of five, TPSA and number of rotatable bonds.

Molecule	*HBD*	*HBA*	*MlogP*	*# of violations*	*TPSA*	*# of rot. Bonds*
1	1	2	1.83	0	23.47	3
2	0	3	2.08	0	29.54	2
3	0	3	2.00	0	29.54	2
4	1	3	1.75	0	40.54	1
5	0	3	2.68	0	21.70	3
6	1	3	2.08	0	40.54	4
7	0	3	2.24	0	29.54	5
8	0	3	2.33	0	29.54	5
9	1	4	2.27	0	43.38	6

Table S3. ADME properties and medicinal chemistry parameters.

Molecule	*GI Absorption*	*BBB permeation*	*Pgp substrate*	*Bioavailability score*	*PAINS alerts*	*Synthetic accessibility*
1	High	Yes	No	0.55	0	1.00
2	High	Yes	No	0.55	0	2.66
3	High	Yes	No	0.55	0	3.32
4	High	Yes	No	0.55	0	3.25
5	High	Yes	No	0.55	0	2.99
6	High	Yes	No	0.55	0	1.80
7	High	Yes	No	0.55	0	3.02
8	High	Yes	No	0.55	0	1.95
9	High	Yes	Yes	0.55	0	3.32

Supplementary Figures



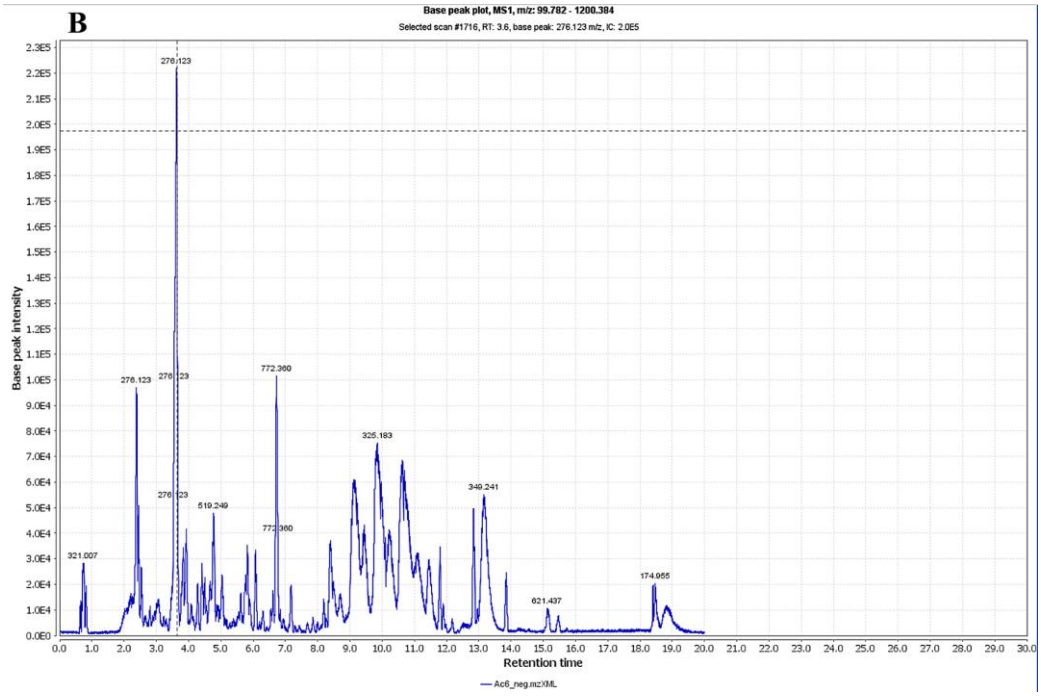


Figure S1. LC/MS total ion chromatogram (A: positive mode, B: negative mode) of the alkaloidal fraction of *M. cordifolium* (*A. cordifolia*) root

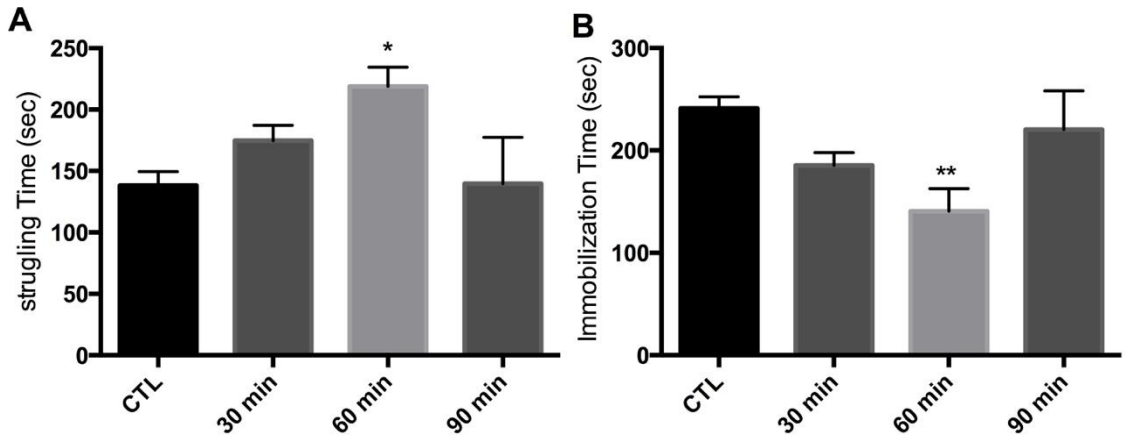


Figure S2. Bar charts showing the effect of different time intervals (30, 60 and 90 minutes) after injection of the alkaloidal fraction of *M. cordifolium* (*A. cordifolia*) root (100 mg/kg, i.p.) on A: struggling time and B: immobilization time in a forced swimming test. One-way analysis of variance (ANOVA) test was used followed by Tukey post hoc test to test the significance between groups. Data are presented as mean ± S.E.M. *, **: significantly different from control (CTL) at $p<0.05$ and 0.01, respectively.

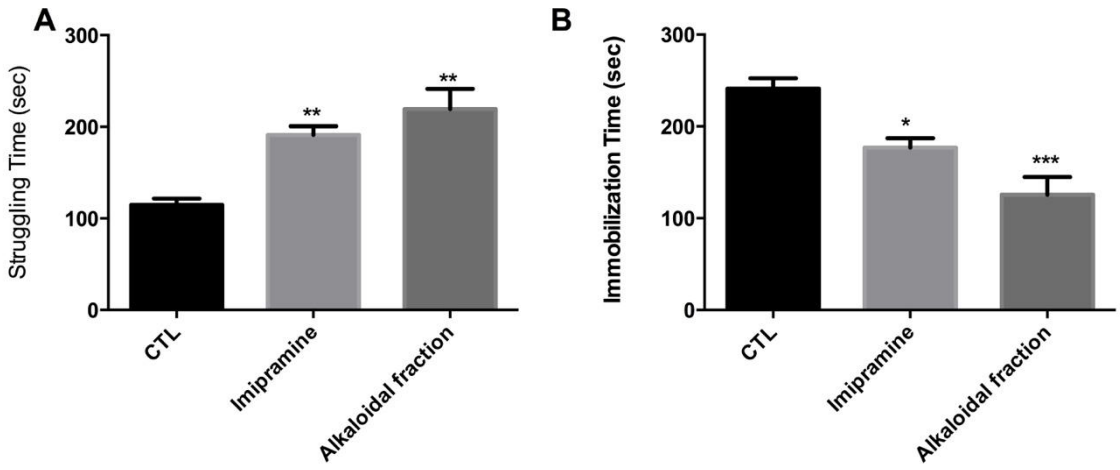


Fig. S3. Bar charts showing the effect of the alkaloidal fraction of *M. cordifolium* (*A. cordifolia*) root (100 mg/kg, i.p.) on A: struggling time and B: immobilization time on forced swimming test. Data are presented as mean ± S.E.M. *, **, ***: significantly different from control (CTL) at $p<0.05$, 0.01 and 0.001, respectively.

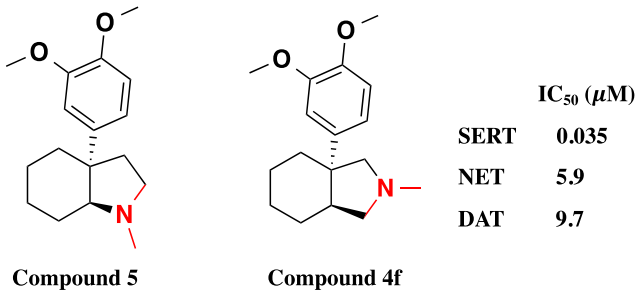
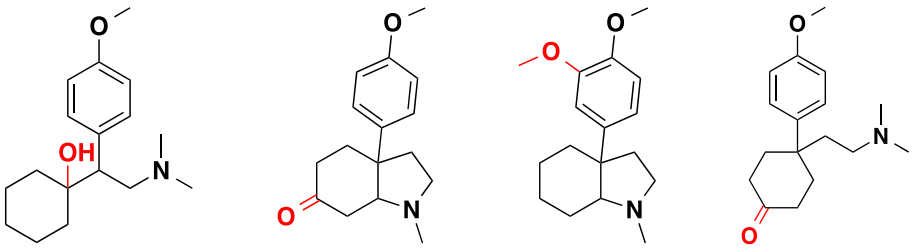


Figure S4: Comparison of compound **5** and the most similar bioactive molecule revealed by virtual screening (the structural differences are highlighted in red color)



	Venlafaxine	Compound 2	Compound 5	Compound 8
2D Similarity score	**(1.00)**	**(0.69)**	**(0.69)**	**(0.66)**

Figure S5. Structural comparison of antidepressant drug Venlafaxine and its most similar compounds by virtual screening (the structural differences are highlighted in red color)

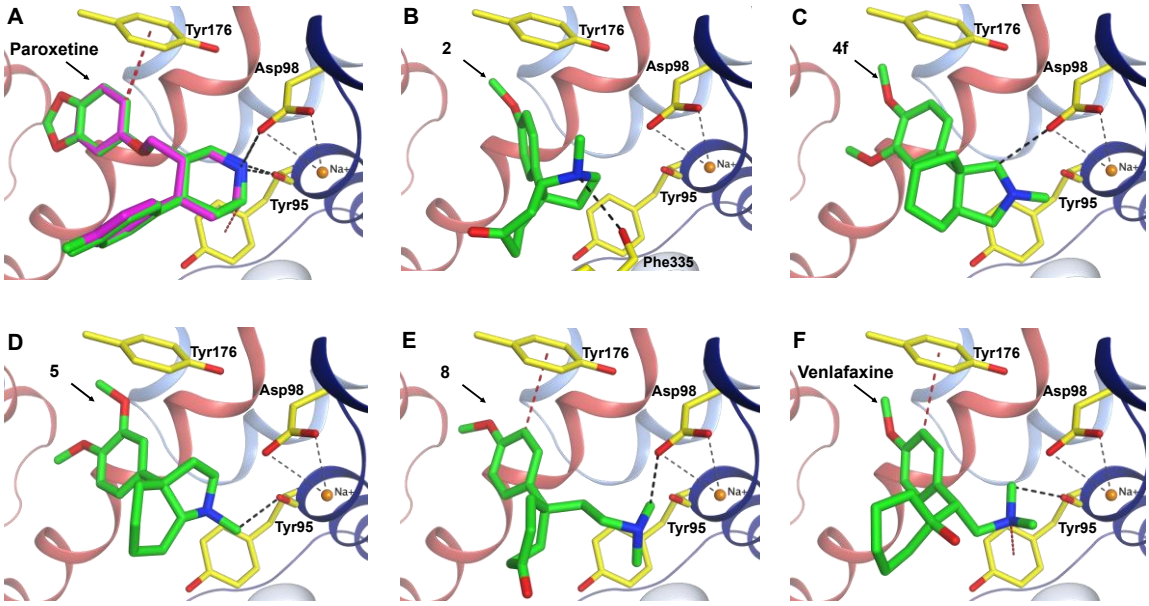


Fig. S6. The top-scoring docked poses of compounds **2**, **4f**, **5** and **8** (green sticks) interacted with the key amino acid residues (yellow sticks) in the binding pocket of SERT (PDB code: 5I6X) compared to the antidepressant drug venlafaxine (green sticks) and the co-crystallized ligand paroxetine (green sticks) and its superposed docking conformation (magenta sticks). The black and red dashed lines indicate a hydrogen bond and a CH-π interaction, respectively.

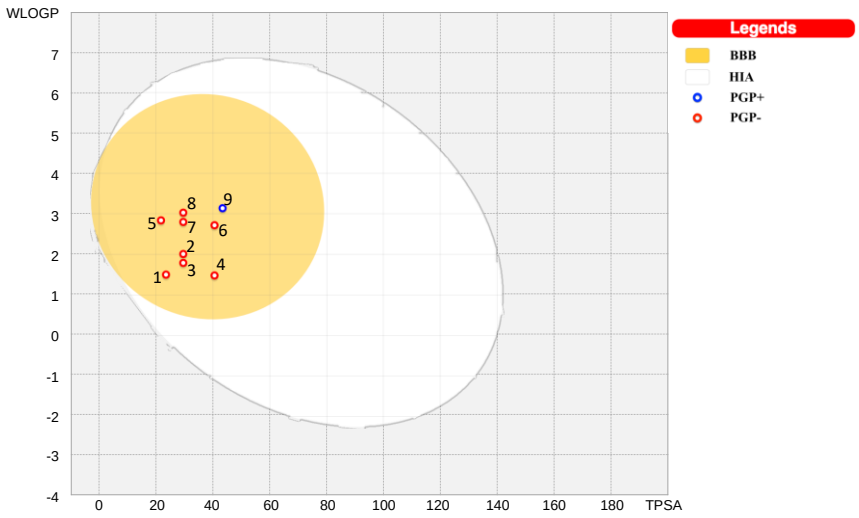
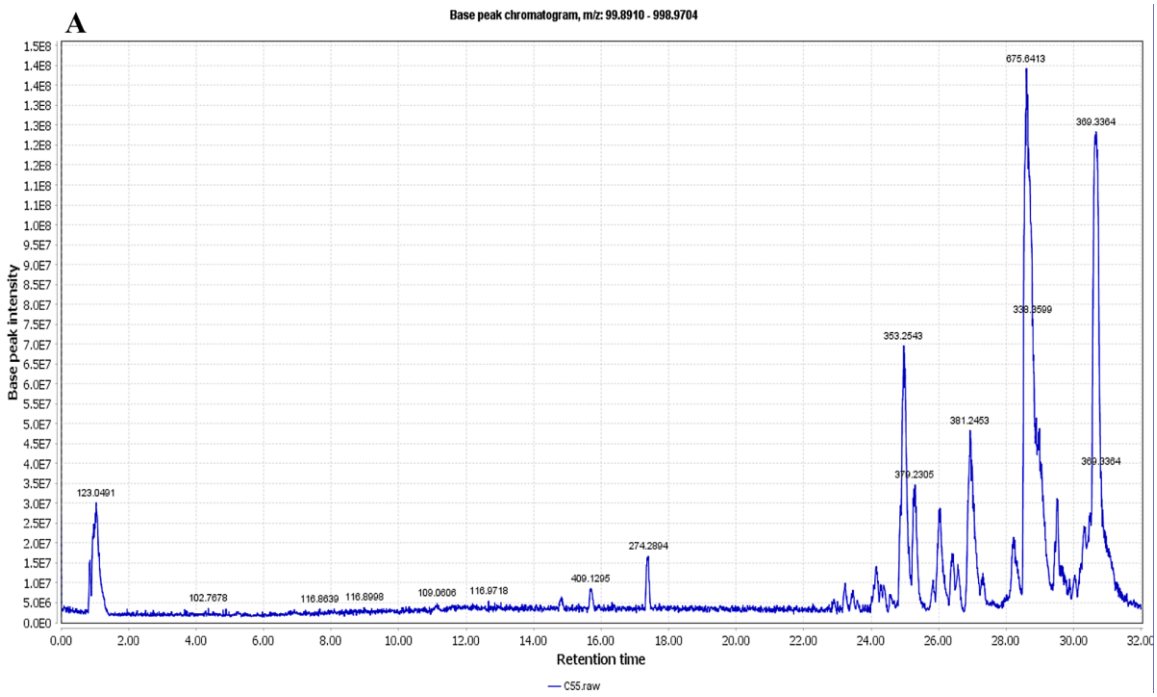
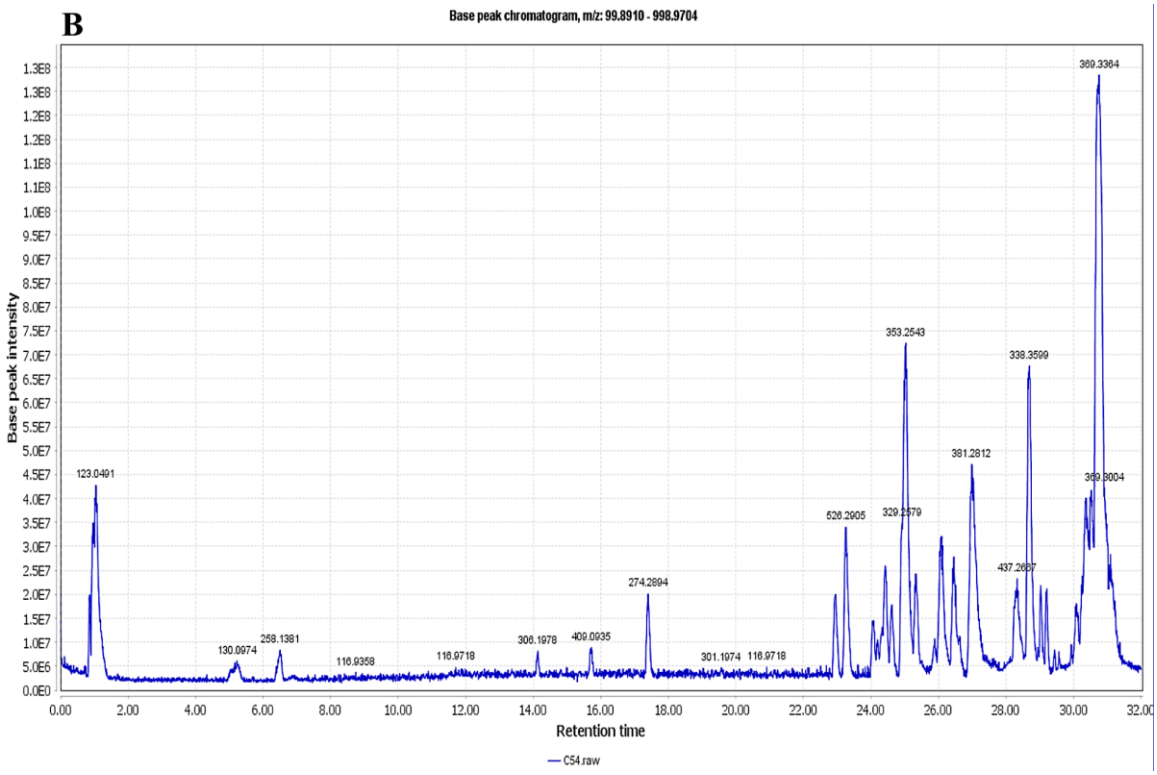


Figure S7. Boiled-egg prediction plot of passive human gastrointestinal absorption (HIA) and brain (BBB) penetration for our compounds. All compounds are located in yellow region which indicates their high probability to permeate BBB.


A
Base peak chromatogram, m/z: 99.8910 - 998.9704
Base peak intensity
Retention time
C55.raw
123.0491
102.7678
116.8839
116.8998
109.0606
116.9718
409.1295
274.2894
379.2305
353.2543
381.2453
338.3599
675.6413
369.3364
369.3364


B
Base peak chromatogram, m/z: 99.8910 - 998.9704
Base peak intensity
Retention time
C54.raw
123.0491
130.0974
258.1381
116.9358
116.9718
306.1978
409.0935
274.2894
301.1974
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381.2812
437.2687
338.3599
369.3364
369.3004

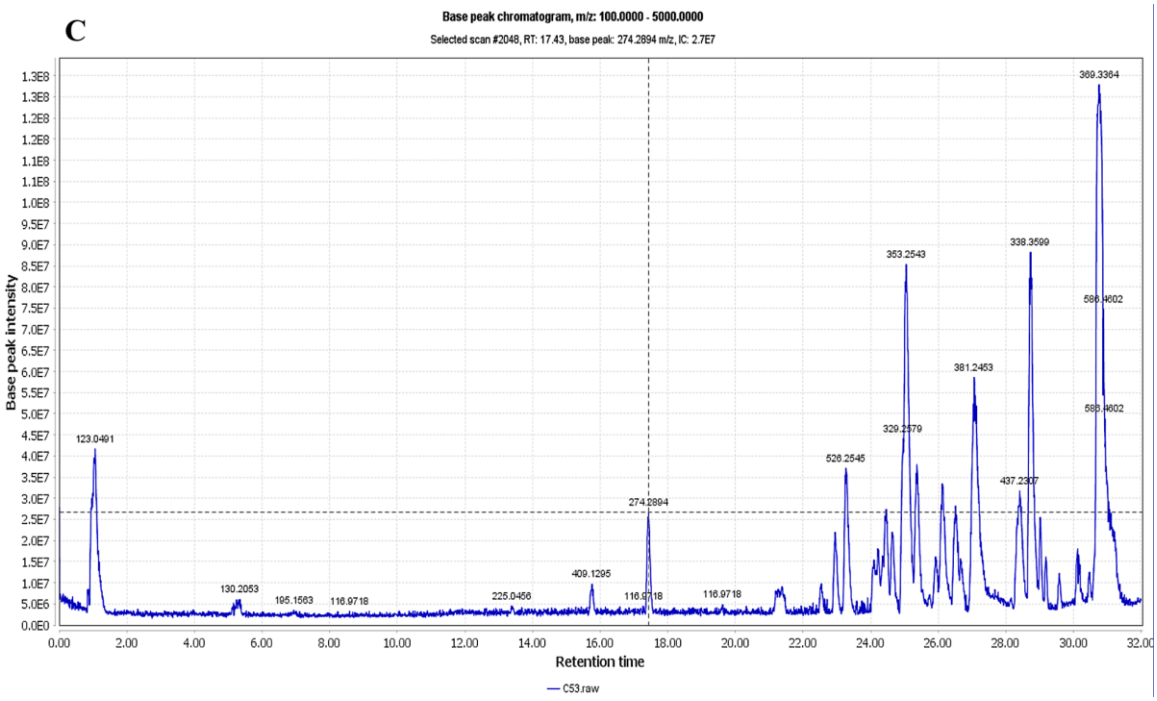
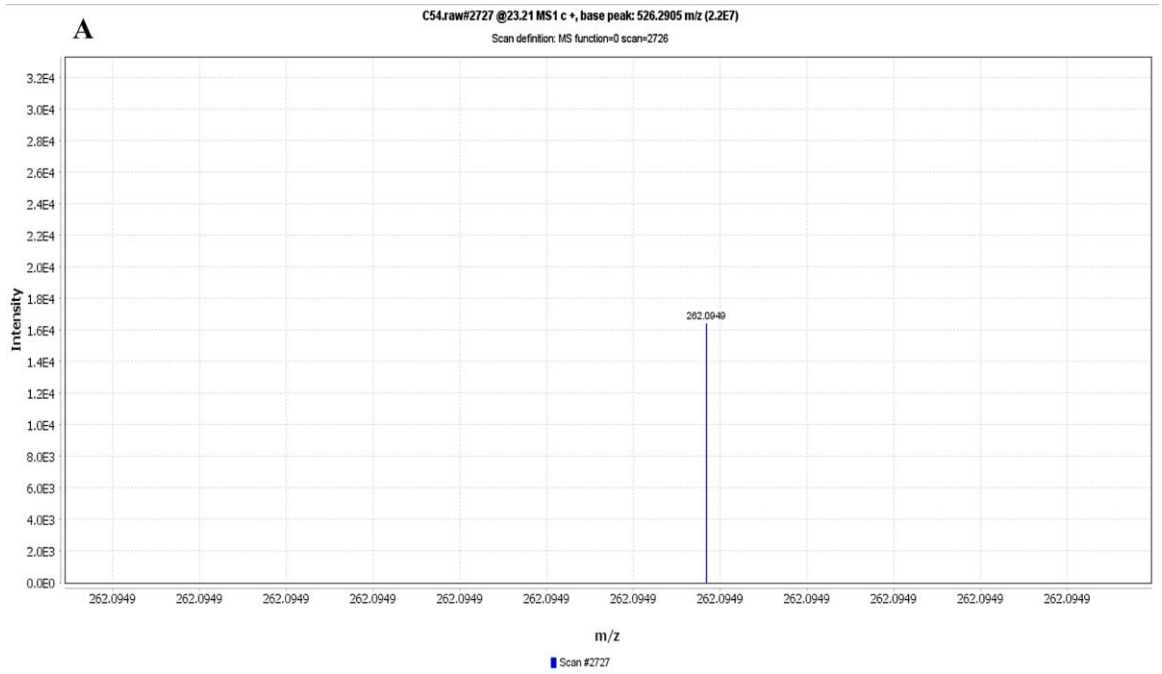


Figure S8. Mass spectra of the brain extracts from four rats A: control (vehicle treated), B: alkaloidal extract treated and C: a brain with exogenously added alkaloidal extract.


A
C54.raw#2727 @23.21 MS1 c +, base peak: 526.2905 m/z (2.2E7)
Scan definition: MS function=0 scan=2726
Intensity
3.2E4
3.0E4
2.8E4
2.6E4
2.4E4
2.2E4
2.0E4
1.8E4
1.6E4
1.4E4
1.2E4
1.0E4
8.0E3
6.0E3
4.0E3
2.0E3
0.0E0
262.0949
262.0949
262.0949
262.0949
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262.0949
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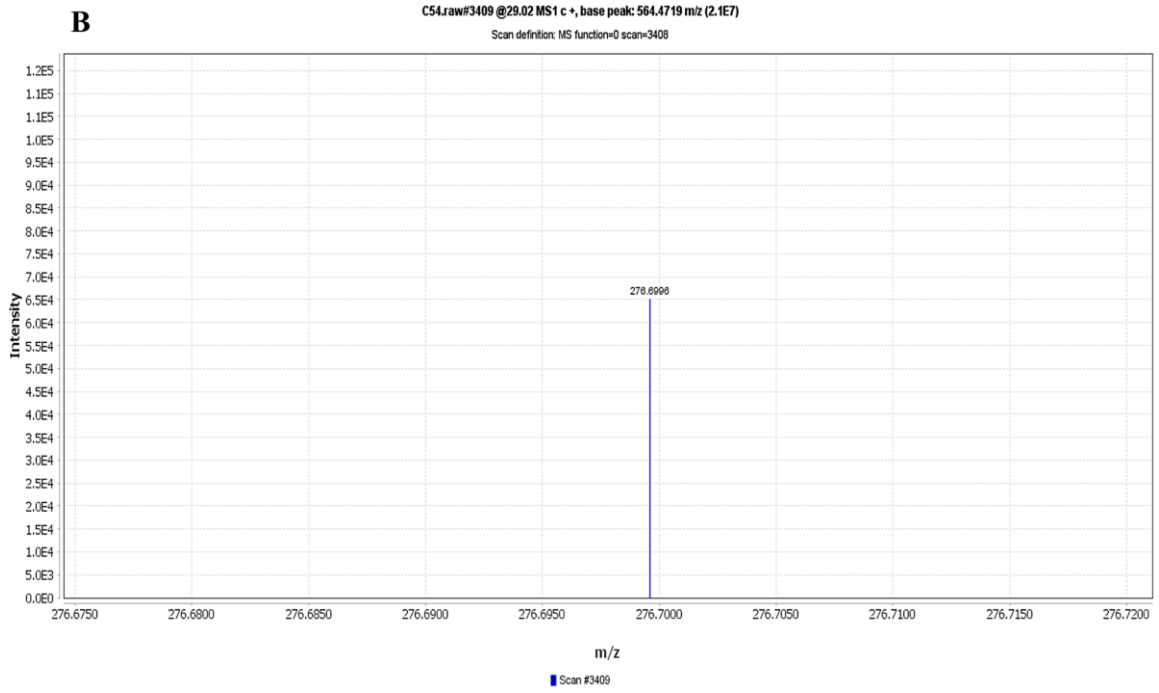
262.0949
m/z
Scan #2727


B
C54.raw#3409 @29.02 MS1 c +, base peak: 564.4719 m/z (2.1E7)
Scan definition: MS function=0 scan=3408
Intensity
1.2E5
1.1E5
1.1E5
1.0E5
9.5E4
9.0E4
8.5E4
8.0E4
7.5E4
7.0E4
6.5E4
6.0E4
5.5E4
5.0E4
4.5E4
4.0E4
3.5E4
3.0E4
2.5E4
2.0E4
1.5E4
1.0E4
5.0E3
0.0E0
276.6996
276.6750
276.6800
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m/z
Scan #3409

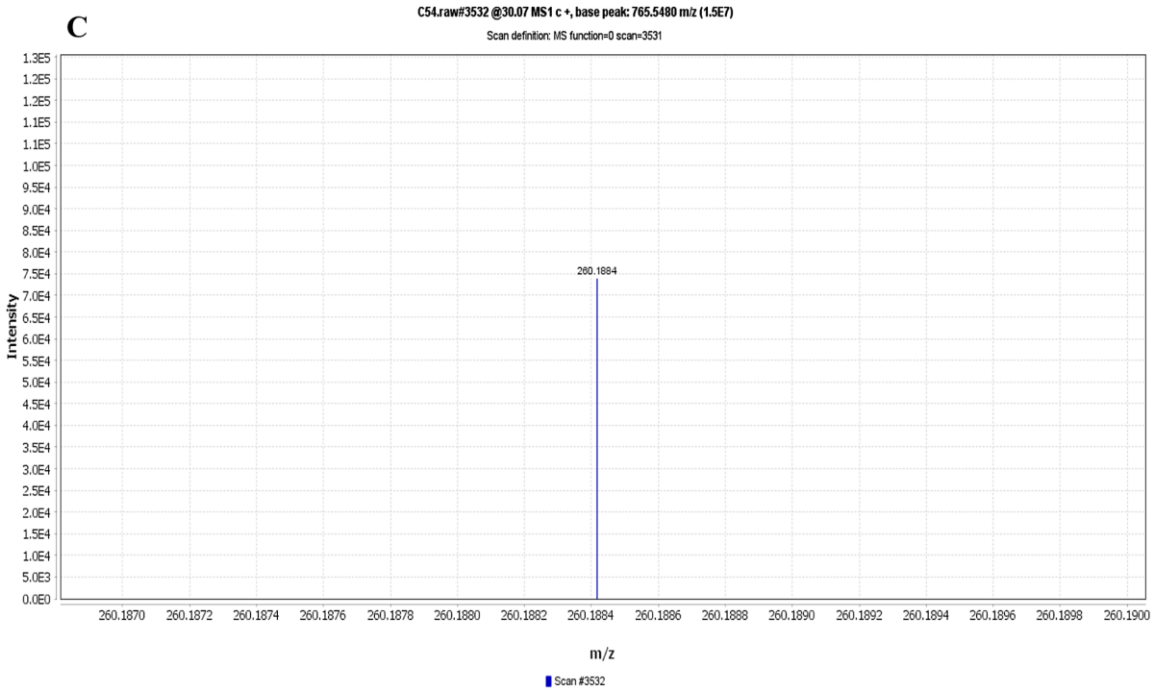

Figure S9. Mass spectra data of A: dihydrojoubertiamine, B: mesembrane, C: 4, 5-Dihydro-4`-*O*-methyl sceletenone.

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