SUPPLIMENTARY MATERIAL

Moringa oleifera (Lam.): A natural remedy for ageing?

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Abstract

disorderss. Effect of *Moringa oleifera,* is an age old ingredient of Indian aurvedic and traditional medicine was tested for its effect on age related antioxidant activity in Wistar albino rats of three age groups (6 and 18 months old) Aqueous extract of. *M. oleifera* leaves (MOAE) was administered orally at a dosage of 200mg/kg body wt. for a period of 30 days. MOAE treatment showed significant reduction in lipid peroxidation and lipofuscin pigmentation along with elevated serotonin and antioxidant enzymes in the brains of treated group of aged rats. D LC-MS-MS analysis revealed blood brain barrier permeable secondary metabolites *viz.*,9,9-bianthracene, 4-Methoxycinnamic acid, Cinnamic acid, (E)-p-coumaric acid pyrogallol and ostruthin from the extract. 9,9-bianthracene and ostruthin showed better binding affinity to Keap-1 and SERT i*n silico* The present result suggests the protective efficacy of *M oleifera* against age related oxidative stress in brain.

Key Words: Ageing, *Moringa oleifera*, lipid peroxidation, antioxidant enzymes, serotonin, lipofuscin

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Experimental

Plant Material Collection, Identification and Authentication

Moringa oleifera leaves were collected during the months of April - May 2018 from homestead garden (10° 01'53.5"N 76° 29'15.2"E), Ernakulam, Kerala, India. For identification of the plant, bar coding of the leaf was carried out at Regional Facility for DNA Fingerprinting, Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India. The plant material authentication was done at CSIR-NISCAIR RHMD (Raw Material Herbarium and Museum, Delhi). (Authentication no. NISCAIR/RHMD/Consult/2019/3403-04) The identification was done on the basis of macroscopic studies of the sample followed by scrutiny of literature and matching the sample with authentic samples deposited in the Raw Material Herbarium and Museum, Delhi). Voucher specimen and herbarium (Voucher no.487) was deposited at St. Albert's College Herbarium (SAC), Ernakulam, India.

Leaf Extract Preparation

M.oleifera leaves collected was washed thoroughly in distilled water and shade dried for 7 days and powdered in an electric blender. The powder was subjected to continuous hot extraction process with water and concentrated in rotary vacuum evaporator (Hahnvapor HS2005V, Hahnshin Scientific) and lyophilised (Operon FDU 7003).

***In vitro* cytotoxicity assay**

Moringa oleifera leaf aqueous extract (MOAE) was studied for its short term *in vitro* cytotoxicity in rat spleen cells (Strober 2001).

Animal Study

For the study, 36 male Wistar rats belonging to the three age groups (6 months old, 12 months old and 18 months old) were procured from College of Veterinary and Animal Sciences, Mannuthy, Kerala, India. All the study protocols were done as per CPCSEA guidelines, Government of India and the IAEC approval for the study was obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India (No. Acad(3)/6554/04, dated 27/09/18). Free access to normal pelleted feed and water were given *ad libitum* during the acclimatization period then the animals of each age group were divided randomly in to control (6C,12C &18C) and treatment groups (6E,12E &18E) of 6 animals each. Body weight of the animals were recorded on a daily basis.

Test Protocol

The animals belonging to the treatment groups were administered orally with *M. oleifera* leaf aqueous extract (MOAE) *(vide supra)* at a dosage of 200mg/kg body weight for 30 days and the corresponding control animals received distilled water. On the 31st day, the animals were sacrificed by CO_2 euthanasia and the brain was removed immediately, washed in normal saline, weighed, homogenized in ice-cold 0.1 M Tris HCl buffer and centrifuged.

Estimation of Lipid Peroxidation and Antioxidant Enzymes

The supernatant was used for the analysis of lipid peroxidation (Fraga et al., 1988), Superoxide dismutase (Madesh and Balasubramanian (1998)), catalase (Takahara et al., (1960)) and glutathione peroxidase (Paglia and Valentine (1967)).

Gene Expression Analysis of *sod1, sod 2, cat* and *gpx 1* by Real Time PCR Analysis

Gene expression analysis was carried out using kits by carefully following the manufacturer's instructions. GenElute Mammalian RNA Miniprep Kit (Sigma Aldrich) was used for RNA isolation, Dnase 1 (Thermoscientific USA) was used to make the sample DNA free, Revert Aid First Strand Cdna Synthesis Kit (Thermoscientific USA) was used for cDNA synthesis and Maxima SYBR Green/rox Qpcr Master Mix (Thermoscientific USA) for PCR amplification. The primer sequences used were; *sod 1*- F: GCAGAAGGCAAGCGGTGAAC and R: TGGACCGCCATGTTTCTTAG, *sod 2*- F: CTGAGGAGAGCAGCGGTCGT and R: CATGATCTGCGCGTTAATGT, cat - F: GCGAATGGAGAGGCAGTGTAC and R: AGGATGGGTAATTGCCACTG, *gpx 1*- F: CTCTCCGCGGTGGCACAGT and R: CGCTTCTGCAGATCATTCAT, and *gapdh* - F: CAACTCCCTCAAGATTGTCAGCAA and R: GGCATGGACTGTGGTCATGA.

Dopamine and serotonin Analysis

Basal ganglia from all the rats were separated carefully and the preparation of tissue extracts and the assay of dopamine and serotonin were done by following the method described by Schlumpf et al. (1974) using fluorospectrometer (Nanodrop USA, ND3300).

Autofluorescence of Lipofuscin

Deparaffinized unstained sections were observed under trinocular research microscope (Leica trinocular research microscope) for autofluorescence of lipofuscin..

LCMS Orbitrap Analysis

LCMS Orbitrap analysis (Q-Exactive Plus Biopharma, Thermo Scientific) was carried out at Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, India. The sample was prepared by redissolving the lyophilized leaf extract (*vide supra*) in methanol and the solvent system used were Solvent A was 0.1% formic acid in milli-Q water and Solvent B was Methanol. Data Acquisition Software was Thermo Scientific Xcalibur, Version 4.2.28.14 and the data processing software was Compound Discoverer 2.1 SP1. Column used was Hypersil Gold 3micron 100 x 2.1 MM (Thermo Scientific). Flow rate was 3.000 µL/min and the duration were 30 minutes.

ADME (**Absorption Distribution Metabolism and Excretion) Prediction**

ADME prediction was done for all the secondary metabolites obtained from LCMS orbitrap analysis of *M.oleifera* aqueous extract (MOAE). The analysis was done using Swiss ADME (Daina et al., 2017) developed and maintained by Swiss Institute of Bioinformatics (SIB).

Molecular Docking Analysis

From the ADME Prediction, the compounds that satisfied all the criteria for a lead compound along with blood brain barrier permeability were selected for Molecular Docking Analysis to analyse their binding affinity with Keap-1 and SERT. PDB structure of proteins Keap-1 (PDB ID: 4L7B) and SERT (PDB ID: 6AWO) were downloaded from RCSB- PDB website and the structures of 9,9-bianthracene, cinnamic acid, 4-methoxycinnamic acid and (E)-p-coumaric acid, pyrogallol and ostruthin were downloaded from PubChem database. Docking analysis were done using AutoDock Vina (Trott and Olson, 2009) and the docking poses were visualized using Discovery Studio Visualizer. After docking analysis, the compounds showing lowest binding energy was considered to possess better interaction with Keap-1 and SERT. Widely used anti-depressant drugs like Fluoxetine, citalopram and sertraline were taken as the standard compounds for the analysis of anti-depressant activity.

Statistical Analysis

Statistical analysis was done with One-way ANOVA with repeated measures and Tukey's multiple comparison test using Graphpad Prism (Ver 5.0).

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Results

***In vitro* cytotoxicity**

Table: S1 showing the results of *in vitro* cytotoxicity assay

Concentration of MOAE (μ g/ml)	**Cytotoxicity (% death of normal cells)**
200	8
100	2
50	0
20	0
10	0

Lipid Peroxidation

Table: S2. Showing the results of levels of TBARS in the brain tissue of Wistar rats treated with MOAE at a dosage of 200 mg/kg body wt. (Mean ± SEM) (n=6)

Age (months)	**Lipid Peroxidation** nM / mg tissue)		**% inhibition of lipid peroxidation in experimental groups**
	control group (6C)	**Experimental Group (6E)**	
6	3196.66±209[a]	3001±159[a]	6%
12	4442.66±113[b]	2431.66±119[a]	47%
18	5336.66±55[c]	2641.66±202[a]	50%

One-way ANOVA with repeated measures Tukey's multiple comparison test using Graphpad Prism (Ver 5.0). Groups with same superscript did not vary significantly at $p<0.05$

Antioxidant Enzyme Analysis

Table: S3. superoxide dismutase, catalase, and glutathione peroxidase activity in the brain tissue of Wistar rats treated with MOAE at a dosage of 200 mg/kg body wt. (n=6)

Age	**Superoxide Dismutase** (units/mg	**Catalase** (units/mg protein)	**Glutathione peroxidase** (µmol GSH

(months)	protein)				oxidized/min/mg protein)	
	Control group (6C)	**Experimental Group (6E)**	**Control group (6C)**	**Experimental Group (6E)**	**Control group (6C)**	**Experimental Group (6E)**
6	68.56667±2.39[a]	151.05±4.34[c]	835±15.44[a]	967.1±20.2[d]	0.0182±0.00013[a]	0.0315±0.032[b]
12	272.3±12.85[b]	289.3167±10.99[b]	501±10.48[b]	672.716±7.96[e]	0.018±0.00074[a]	0.018±0.0004[a]
18	149.6±8.41[cd]	164.4±4.69[d]	199.33±6.9[c]	167.31±5.16[c]	0.0057±0.00016[c]	0.0096±0.00015[d]

One-way ANOVA with repeated measures Tukey's multiple comparison test using Graphpad Prism (Ver 5.0). Groups with same superscript did not vary significantly (P<0.05), where; 6C- 6months old control; 6E-6months old experimental; 12C-12months old control; 12E-12 months old experimental; 18C-18 months old control; and 18E- 18 months old Experimental

Gene expression Analysis

Table: S4. *sod1, sod2, cat* and *gpx1* gene expression in the brain tissue of Wistar rats treated with MOAE at a dosage of 200 mg/kg body wt. (n=6)

	6E	12E	18E
sod1 △△ct (Mean±SE)	2.45±0.228[1]	-0.1±0.52[2,3]	0.15±0.34[3]
sod2 △△ct (Mean±SE)	-0.34±0.13[1]	-2.03±0.66[1]	-2.0±0.88[1]
cat △△ct (Mean±SE)	0.22±0.56[1]	1.14±0.52[1]	-0.90±0.35[1]
gpx1 △△ct (Mean±SE)	1.89±0.51[1]	1.87±0.44[1]	1.41±0.12[1]
sod1 fold change (Mean±SE)	0.1874±0.03	1.22±0.41	0.94±0.20
sod2 fold change (Mean±SE)	1.27±0.11	4.98±2.0	5.89±3.5
cat fold change (Mean±SE)	0.97±0.31	0.50±0.15	1.99±0.53
gpx1 fold change (Mean±SE)	0.30±0.10	0.50±0.17	0.37±0.03

One-way ANOVA with repeated measures Tukey's multiple comparison test using Graphpad Prism (Ver 5.0). Groups with same superscript did not vary significantly (P<0.05), where; 6C- 6months old control; 6E-6months old experimental; 12C-12months old control; 12E-12 months old experimental; 18C-18 months old control; and 18E- 18 months old Experimental

Dopamine and Serotonin in basal ganglia of rat brain.

Table: S5. Dopamine and serotonin levels in the basal ganglia of Wistar rats treated with MOAE at a dosage of 200 mg/kg body wt. (Mean ± SEM) (n=6)

Age (months)	**Dopamine (µg / mg tissue)**		**Serotonin (µg / mg tissue)**	
	Control group (C)	Experimental group (E)	Control group (C)	Experimental group (E)
6	1.156±0.022[a]	2.155±0.13[c]	23.145±1.6[ab]	18.06±2.09[bc]
12	2.4±0.093[bc]	2.628±0.106[b]	17.00±1.08[ac]	25.07±0.309[a]
18	1.89±0.022[b]	2.545±0.104[b]	16.185±0.72[c]	24.96±1.29[a]

One-way ANOVA with repeated measures Tukey's multiple comparison test using Graphpad Prism (Ver 5.0). Groups with same superscript did not vary significantly (P<0.05) where; 6C- 6months old control; 6E-6months old experimental; 12C-12months old control; 12E-12 months old experimental; 18C-18 months old control; and 18E- 18 months old Experimental

LCMS Orbitrap Analysis

Table: S6. Amino acids & other essential nutrients detected from *M. oleifera* leaf aqueous extract (MOAE)

Sl. no	Name & Molecular Formula	Molecular Mass	Ionization mode	Retention time (min)	Relative Abundance (%)
1.	Isoleucine, $C_6H_{13}NO_2$	131.09467	+	1.593	2.797
2.	L-Phenylalanine, $C_9H_{11}NO_2$	165.07898	+	2.588	1.021
3.	Valine, $C_5H_{11}NO_2$	117.07896	+	1.04	0.849
4.	Proline, $C_5H_9NO_2$	115.06329	+	1.01	0.736
5.	DL-Tryptophan, $C_{11}H_{12}N_2O_2$	204.08994	+	4.167	0.170
6.	L-(-)-Threonine, $C_4H_9NO_3$	119.05825	+ & -	1.066	0.133
7.	DL-Glutamine, $C_5H_{10}N_2O_3$	146.06914	+ & -	1.094	0.118
8.	DL-Arginine, $C_6H_{14}N_4O_2$	174.11162	+	1.024	0.0923
9.	L-Tyrosine, $C_9H_{11}NO_3$	181.07389	+	1.374	0.0900
10.	L-Glutamic acid, $C_5H_9NO_4$	147.05316	+	1.093	0.0241
11.	L-(+)-Aspartic acid, $C_4H_7NO_4$	133.03751	+ &-	1.088	0.0214
12.	L-(-)-Asparagine, $C_4H_8N_2O_3$	132.05346	+ & -	1.051	0.0639
13.	DL-Histidine, $C_6H_9N_3O_2$	155.06954	+	1.034	0.0356
14.	L-(-)-Serine, $C_3H_7NO_3$	105.04261	+	1.062	0.0245
15.	L-(+)-Leucine, $C_6H_{13}NO_2$	131.09467	+	2.36	0.0007
16.	DL-Lysine, $C_6H_{14}N_2O_2$	146.10547	+	1.026	0.0171
17.	Choline, $C_5H_{13}NO$	103.09959	+	0.945	3.015
18.	Nicotinamide, $C_6H_6N_2O$	122.04798	+	1.151	0.195
19.	Coenzyme Q1, $C_{14}H_{18}O_4$	250.12051	+	19.082	0.0712
20.	Niacin, $C_6H_5NO_2$	123.03203	+	1.164	0.0341
21.	DL-Carnitine, $C_7H_{15}NO_3$	161.10513	+	0.957	0.0135
22.	Vitamin K3, $C_{11}H_8O_2$	172.05242	+	1.006	0.00481

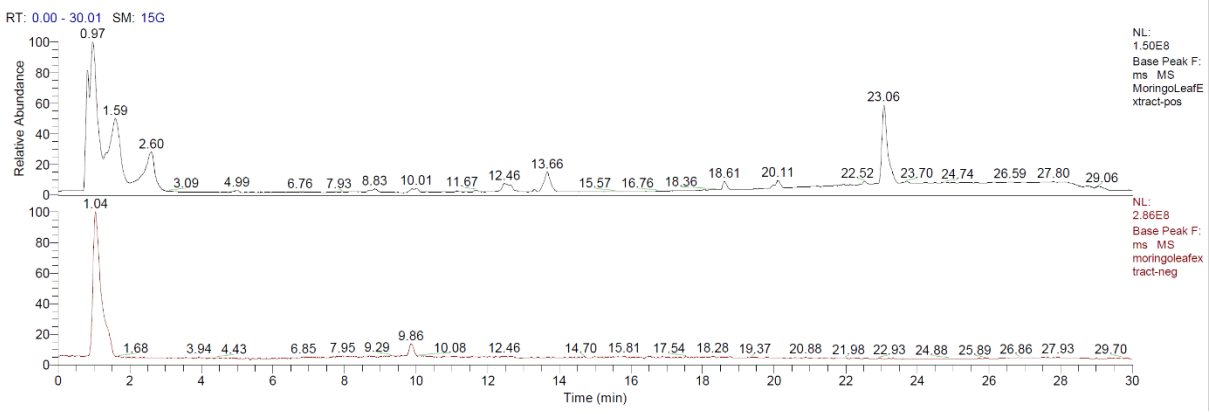

Fig.S1: chromatogram

Table: S7. Secondary metabolites detected and ADME Prediction

Sl. no	Name & Molecular Formula	Molecular Mass	Ionization mode	Retention time (min)	Relative Abundance (%)	Lipophilicity (Log P)	Water solubility	GI Absorption	BBB permeability	P-gp substrate	Drug likeliness (Lipinski's Rule)	Bioavailability score
1	Trigonelline, $C_7 H_7 N O_2$	137.047	+	1.061	1.34	-0.61	Yes	High	No	No	Yes	0.55
2	Quercetin-3β-D glucoside $C_21 H_{20} O_{12}$	464.095	+ & -	12.44	0.351	-0.48	Yes	Low	No	No	No	0.16
3	Chlorogenic acid, $C_{16} H_{18} O_9$	354.095	+ & -	8.84	0.224	-0.39	Yes	Low	No	No	Yes	0.11
4	Asperulosidic acid, $C_{18} H_{24} O_{12}$	432.1267	+	1.017	0.205	-2.2	Yes	Low	No	No	No	0.11
5	Cynaroside , $C_{21} H_{20} O_{11}$	448.100	+	13.3	0.129	0.15	Yes	Low	No	Yes	No	0.17
6	Vitexin, $C_{21} H_{20} O_{10}$	432.105	+	11.75	0.113	11.75	Yes	Low	No	No	Yes	0.55
7	Arecoline, $C_8 H_{13} N O_2$	155.094	+	1.066	0.0212	0.80	Yes	High	No	No	Yes	0.55
8	Rhusflavanone, $C_{30} H_{22} O_{10}$	542.121	+	0.983	0.0791	3.20	No	Low	No	No	No	0.17
9	Cryptolepine, $C_{16} H_{12} N_2$	232.099	+	11.214	0.0447	3.29	Yes	High	Yes	Yes	Yes	0.55
10	Quercitrin, $C_{21} H_{20} O_{11}$	448.100	+	13.106	0.0260	0.22	Yes	Low	No	No	No	0.17
11	Lariciresinol 4-O-glucoside $C_{26} H_{34} O_{11}$	544.192	+	12.693	0.0254	0.68	Yes	Low	No	Yes	No	0.17
12	Linustatin, $C_{16} H_{27} N O_{11}$	409.158	+	9.479	0.0221	-2.84	Yes	Low	No	Yes	No	0.17
13	Daucosterol, $C_{35} H_{60} O_6$	576.436	+	27.109	0.0205	5.55	Yes	Low	No	No	Yes	0.55
14	4-Methoxycinnamic acid $C_{10} H_{10} O_3$	160.052	+	1.334	0.0204	1.87	Yes	High	Yes	No	Yes	0.56
15	Pyrogallol, $C_6 H_6 O_3$	126.0317	+	1.297	0.0196	0.58	Yes	High	Yes	No	Yes	0.55
16	Cinnamic acid, $C_9 H_8 O_2$	148.052	+	1.011	0.0181	1.79	Yes	High	Yes	No	Yes	0.56

17	Naringin, $C_{27}H_{32}O_{14}$	580.179	+	9.95	0.0127	-0.87	Yes	Low	No	Yes	No	0.17
18	(E)-p-coumaric acid, $C_9H_8O_3$	164.047	+	1.37	0.0121	1.26	Yes	High	Yes	No	Yes	0.56
19	Osajin, $C_{25}H_{24}O_5$	404.165	+	11.062	0.0102	4.70	No	High	No	No	Yes	0.55
20	Nictoflorin, $C_{27}H_{30}O_{15}$	594.158	+	10.574	0.00970	4.70	No	High	No	No	Yes	0.55
21	9,9'-Bianthracene, $C_{28}H_{18}$	354.142	+	1.027	0.00816	5.15	No	High	Yes	No	Yes	0.55
22	Cinnamaldehyde, C_9H_8O	132.057	+	1.018	0.00701	1.97	Yes	High	Yes	No	Yes	0.55
23	D-(-)Quinic acid, $C_7H_{12}O_6$	192.063	-	0.986	0.959	-1.66	Yes	Low	No	Yes	Yes	0.56
24	Neochlorogenic acid, $C_{16}H_{18}O_9$	354.095	-	8.646	0.666	-0.46	Yes	Low	No	No	Yes	0.11
25	Malonic acid, $C_3H_4O_4$	104.010	-	1.084	0.5103	-0.46	Yes	High	No	No	Yes	0.56
26	Gluconic acid, $C_6H_{12}O_7$	196.058	-	0.996	0.320	-2.42	Yes	Low	No	No	Yes	0.56
27	Astragalin, $C_{21}H_{20}O_{11}$	448.100	-	13.24	0.251	-0.09	Yes	Low	No	No	No	0.17
28	Avenein, $C_{14}H_{18}O_8$	314.100	-	9.732	0.1661	-0.78	Yes	High	No	No	Yes	0.55
29	1,3,7-Trimethyluric acid $C_8H_{10}N_4O_3$	210.073	-	0.999	0.1173	-2.4	Yes	High	No	No	Yes	0.55
30	Corchorifatty acid F, $C_{18}H_{32}O_5$	328.224	-	17.06	0.106	2.68	Yes	High	No	No	Yes	0.56
31	Azelaic acid, $C_9H_{16}O_4$	188.104	-	13.492	0.0935	1.49	Yes	High	Yes	No	Yes	0.55
32	Dihydrolipoic acid, $C_8H_{16}O_2S_2$	208.058	-	1.071	0.0532	2.11	Yes	High	No	No	Yes	0.56
33	Ostruthin, $C_{19}H_{22}O_3$	298.156	-	23.532	0.046	4.46	Yes	High	Yes	No	Yes	0.55
34	Isorhamnetin 3-glucoside $C_{22}H_{22}O_{12}$	478.110	-	13.421	0.0461	0.12	Yes	Low	No	Yes	No	0.17
35	Lactobionic acid, $C_{12}H_{22}O_{12}$	358.110	-	1.008	0.0332	-3.94	Yes	Low	No	Yes	No	0.17

Molecular Docking Analysis

Table: S8. Showing the binding affinity and amino acid interactions of the compounds with Keap-1 and SERT

Compounds	Affinity (kcal/ mol)	Amino acid Interactions
Molecular docking analysis with Keap1		
9,9-Bianthracene	-9.8	Vander -Waal's Interaction, Conventional Hydrogen Bonds, Pi-alkyl.
Ostruthin	-8.3	Vander -Waal's Interaction, alkyl, pi-alkyl
(E) p-coumaric acid	-7.6	Vander -Waal's Interaction, Conventional Hydrogen Bonds, carbon hydrogen bond, alkyl, Pi-alkyl.
4-methoxycinnamic acid	-6.6	Vander -Waal's Interaction, Conventional Hydrogen Bonds, carbon hydrogen bond, Pi-sigma
Cinnamic acid	-5.9	Vander -Waal's Interaction, Conventional Hydrogen Bonds, Pi-sigma
Pyrogallol	-5.7	Vander -Waal's Interaction, Conventional Hydrogen Bonds, carbon hydrogen bond, pi-sigma, Pi-alkyl, covalent bond
Molecular docking analysis with SERT		
9,9-Bianthracene	-11	Vander -Waal's Interaction, Pi-anion, pi-sigma, alkyl, Pi-Pi T shaped, Pi-alkyl
Ostruthin	-9.3	Vander -Waal's Interaction, Conventional Hydrogen Bonds, pi-sigma, alkyl, Pi-Pi T shaped, amide pi-stacked, pi alkyl
(E) p-coumaric acid	-8.8	Vander -Waal's Interaction, Conventional Hydrogen Bonds, carbon hydrogen bond, Halogen, pi-sigma, alkyl, Pi-Pi T shaped, Pi-pi stacked
Cinnamic acid	7	Vander -Waal's Interaction, Conventional Hydrogen Bonds, carbon hydrogen bond, Pi-Pi T shaped, Pi alkyl
4-methoxycinnamic acid	-6.8	Vander -Waal's Interaction, Conventional Hydrogen Bonds, pi-pi stacked
Pyrogallol	-5.6	Vander -Waal's Interaction, Conventional Hydrogen Bonds, Pi-Pi T shaped, Pi-pi stacked
Sertraline	-8.9	Vander -Waal's Interaction, Conventional Hydrogen Bonds, carbon hydrogen bond, pi-anion, Pi-Pi T shaped, pi-sigma, pi-alkyl.
Fluoxetine	-8.6	Vander -Waal's Interaction, Halogen, Pi-Pi stacked, Pi alkyl
Citalopram	-8.3	Vander -Waal's Interaction, Halogen, Pi-Pi stacked, Pi alkyl

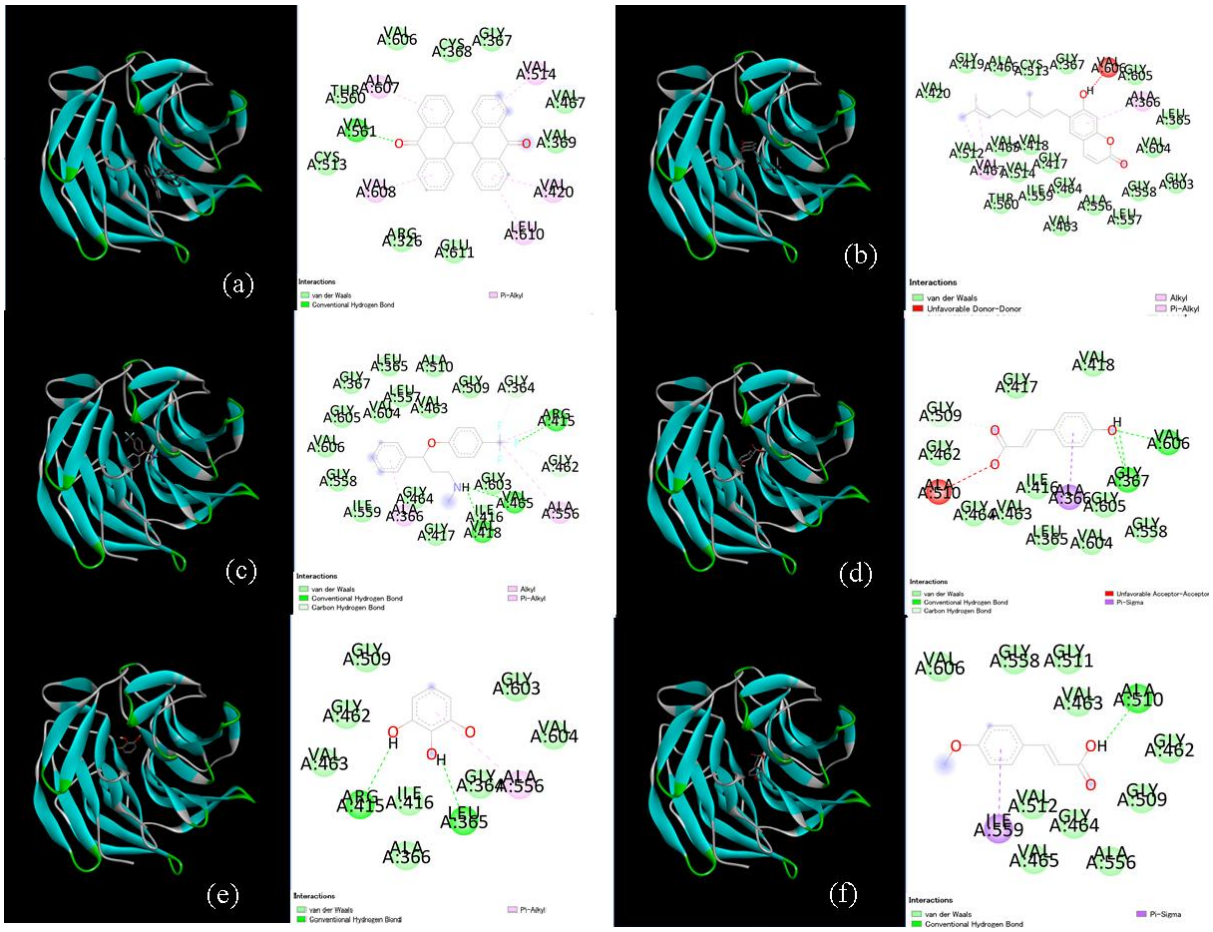

Fig.S2: Two-dimensional binding site interaction: (a) 9,9-bianthracene with Keap1, (b) ostruthin with Keap-1, (c) (E) p-coumaric acid with Keap-1, (d) 4-methoxycinnamic acid with Keap-1, (e) pyrogallol with Keap-1, (f) cinnamic acid with Keap-1.

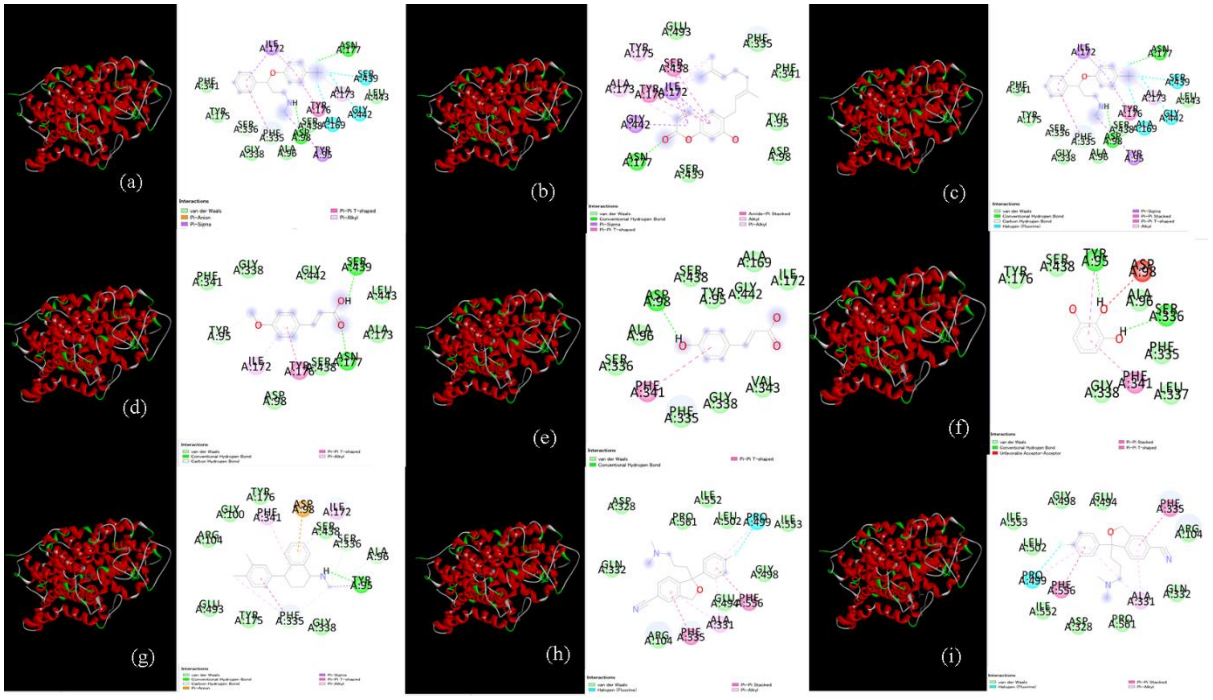

Fig.S3: Two-dimensional binding site interaction: (a) 9,9-bianthracene with SERT, (b) ostruthin with SERT, (c) (E) p-coumaric acid with SERT, (d) cinnamic acid with SERT, (e) 4-methoxycinnamic acid with SERT, (f) pyrogallol with SERT, (g) sertraline with SERT, (h) fluoxetine with SERT, (i) citalopram with SERT.