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Original Article

Kaempferia parviflora rhizome extract and *Myristica fragrans* volatile oil increase the levels of monoamine neurotransmitters and impact the proteomic profiles in the rat hippocampus: Mechanistic insights into their neuroprotective effects

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ABSTRACT

Potentially useful in the treatment of neurodegenerative disorders, *Kaempferia parviflora* and *Myristica fragrans* have been shown to possess a wide spectrum of neuropharmacological activities and neuroprotective effects *in vivo* and *in vitro*. In this study, we determined whether and how *K. parviflora* ethanolic extract and *M. fragrans* volatile oil could influence the levels of neurotransmitters and the whole proteomic profile in the hippocampus of Sprague Dawley (SD) rats. The effects of *K. parviflora* and *M. fragrans* on protein changes were analyzed by two-dimensional gel electrophoresis (2D-gel), and proteins were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). The target proteins were then confirmed by Western blot. The levels of neurotransmitters were evaluated by reversed-phase high-performance liquid chromatography (RP-HPLC). The results showed that *K. parviflora*, *M. fragrans* and fluoxetine (the control drug for this study) increased serotonin, norepinephrine and dopamine in the rat hippocampus compared to that of the vehicle-treated group. Our proteomic data showed that 37 proteins in the *K. parviflora* group were up-regulated, while 14 were down-regulated, and 27 proteins in the *M. fragrans* group were up-regulated, while 16 were down-regulated. In the fluoxetine treatment group, we found 29 proteins up-regulated, whereas 14 proteins were down-regulated. In line with the proteomic data, the levels of GFAP, PDIA3, DPYSL2 and p-DPYSL2 were modified in the SD rat groups treated with *K. parviflora*, *M. fragrans* and fluoxetine as confirmed by Western blot. *K. parviflora* and *M. fragrans* mediated not only the levels of monoamine neurotransmitters but also the proteomic profiles in the rat hippocampus, thus shedding light on the mechanisms targeting neurodegenerative diseases.

1. Introduction

The World Health Organization (WHO) predicted that by 2040, neurodegenerative diseases (NDs) will become the second leading cause of death in the world.[1–4] NDs, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington disease (HD), belong to a heterogeneous group of disorders that are characterized by progressive degeneration of the structure and function of the central nervous system or peripheral nervous system. Common NDs include psychiatric disorders such as depression and bipolar

Abbreviations: GFAP, glial fibrillary acidic protein; PDIA3, protein disulfide-isomerase A3; DPYSL2, dihydropyrimidinase-related protein 2; p-DPYSL2, dihydropyrimidinase-related protein 2; NE, norepinephrine; DA, dopamine; 5-HT, serotonin.

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disorder. In addition, depression is the most prevalent symptom in PD and HD, occurring in approximately 40–60% of patients.[5] The hippocampus is considered to be one of the most important brain regions for mood regulation. The monoamine neurotransmitters, including dopamine (DA), norepinephrine (NE), epinephrine (E), and serotonin (5-hydroxytryptamine, 5-HT) are produced from neurons both in the brain and peripheral nervous system.[6,7] The functions of monoamine neurotransmitters are considered to play crucial roles in arousal, emotion, and cognition. Drugs that augment the effects of monoamines on their target tissue are used to treat psychiatric disorders, including depression, anxiety, and schizophrenia.[8,9] For these reasons, the measurement of monoamine neurotransmitters in the rat hippocampus is important for understanding the effects of herbal treatments on the secretion of neurotransmitters. Recently, proteomics is an important tool for identifying the expression of proteins that are important for a comprehensive understanding of the pharmacological role of a drug.[10–12] Many medicinal plants have been identified and used for neuroprotective and neurotrophic agents that promote neuronal survival, differentiation, neuritogenesis and synaptic plasticity, both in *in vitro* and *in vivo* models.[13–15] *Kaempferia parviflora* Wall Ex. Baker, or black ginger with the local name of "Kra-chai-dam", is a plant from the family Zingiberaceae used for health promotion in traditional Thai medicine. The phytochemical studies revealed that the rhizomes of *K. parviflora* contained volatile oil,[16] chalcones,[17] phenolic glycosides[18] and many flavonoids such as 5-hydroxy-7-methoxyflavone, 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone.[19,20] 5,7-Dimethoxyflavone is a major active constituent of *K. parviflora*[20] that showed high potential inhibitory activity toward acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).[21,22] Moreover, polymethoxyflavones from black ginger (*K. parviflora*) were discovered to be potential inhibitors of β-secretase (BACE1).[23] The rhizomes of this plant have been traditionally used for leucorrhea, oral diseases, abdominal pain, and health promotion as well as an aphrodisiac.[24] In addition, the ethanolic extract of *K. parviflora* has been shown to induce relaxation of both the aortic rings and the ileum precontracted with phenylephrine and acetylcholine.[25] It has been reported that the alcoholic extract of *K. parviflora* rhizomes contained numerous flavonoids[20] that were previously reported to possess antioxidant activity and neuroprotective and cognitive enhancing effects.[26] A recent finding showed that the alcoholic extract of *K. parviflora* rhizome could mitigate depression-like behavior in aged rats[27] and displays antidepressant activity in aged rats. We hypothesized that alcoholic extract of *K. parviflora* rhizome might also possess other neuropharmacological activities. *Myristica fragrans* Houtt. (nutmeg, a tropical evergreen dioecious tree with a narrow range of distribution) is a source of high-value medicinal spices, nutmeg (endosperm) and mace (the reddish aril) with immense phytochemical diversity.[28] It contains volatile oils that include myristicin, elemicin, eugenol, isoeugenol, geraniol, pinese, cineole, borneol, and safrole. Its reported antibacterial, antiviral, antidiabetic, and antileukemic effects as well as its other biological activities[28–30] indicate its enormous therapeutic potential. Medicinally, eugenol and isoeugenol from nutmeg are known for their anti-inflammatory and antithrombotic,[31] as well as anti-rheumatic, carminative and stimulant properties.[32] The psychoactive effects of nutmeg have been reported to cause hallucinations, feelings of unreality, euphoria, and delusions. Both myristicin and elemicin have been shown to be metabolized into amphetamine-related compounds that have effects on the serotonergic systems and possibly an antidepressant effect,[33] but this has not yet been verified. These compounds were shown to have an antidepressant effect in the forced swimming test in male rats.[34] The main bioactive compounds of *M. fragrans* seed essential oil were identified as including camphene, elemicin, eugenol, isoelemicin, isoeugenol and methoxyeugenol.[29] Sabinene, α-pinene, β-pinene, terpine-4-ol, limonene, safrole and myristicin were also reported to be included in the essential oil of nutmeg.[35,36] In this study, we aim to investigate the effects of *K. parviflora* and *M. fragrans* on the levels of monoamine neurotransmitters (norepinephrine, serotonin, and dopamine), as well as on the proteomic profiles in the rat hippocampus. Discoveries from this study could help us to better understand the molecular mechanisms of these compounds.

2. Material and methods

2.1. Plant material and extraction

K. parviflora was collected from the Loei province in northeast Thailand, and *M. fragrans* was from the Nakhonpathom province in central Thailand. Plants were identified by an expert Ass. Prof. Dr. Nijsiri Ruangrungsi. *K. parviflora* rhizomes were shade dried and agitated by using a blender, and the powder was extracted with 95% ethanol by maceration for 4 days (1 kg sample: 3 L) with occasional stirring. The extract was dissolved in 2% Tween-80 in water to a final concentration of 200 mg/ml.[17] Crude extracts of *K. parviflora* were standardized by reversed-phase HPLC (Agilent 1260 Infinity, Austria). 5,7-Dimethoxyflavone was used for the standard. For *M. fragrans*, 1 kg of powder from dry seeds was extracted by stream distillation for 12 h. Then, the volatile oil was dissolved in 2% Tween-80 in water to a final concentration 300 mg/ml.[34] The *M. fragrans* nutmeg volatile oil was analyzed by GC-MS on gas chromatograph with a model 7890A GC and 5978C MSD mass selective detector (EIMS, electron energy, 70 eV) and an Agilent ChemStation data system. Fluoxetine (20 mg tablet) was dissolved in 2% Tween-80 in water.[17,34]

2.2. Experimental protocol for animals

Two-month-old healthy male Sprague Dawley rats (300–400 g) were obtained from and approved by the National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), Mahidol University, Salaya, Nakornpathom, Thailand. Rats were housed individually (one per cage) in standard conditions (NLAC-MU; SOP-VM.VCP-01.10.) at 22 ± 1 °C on a standard fluorescent 12:12 h light:dark cycle, with changing of bedding once a week. Rats were given access to a standard diet and water (hyperchorinate 10–12 ppm) ad libitum.

All rats were randomly divided into 4 groups, each group containing 6 rats.

Group 1: The control group or vehicle-treated group was treated with 2% Tween-80, 1 mL/kg BW/day via oral route for 12 days.

Group 2: The fluoxetine group was treated with 20 mg/kg BW via oral route for 12 days.

Group 3: The *K. parviflora* group was treated with ethanolic extract (200 mg/kg BW via oral route once per day for 12 days).

Group 4: The *M. fragrans* group was treated with seed oil (300 mg/kg BW via oral route once per day for 12 days).

2.3. Tissue preparation and protein extraction

Dissected brains were frozen in liquid nitrogen at −80 °C until extraction. Hippocampal tissue was powderized in liquid nitrogen, and proteins were extracted with buffer containing 40 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS, 65 mM DTT, 0.3 mg/ml EDTA, 35 μg/

ml PMSF and 1 tablet of protease inhibitor cocktail. The protein was precipitated with 20% trichloroacetic acid (TCA) in acetone with 20 mM dithiothreitol (DTT). The protein was re-suspended in lysis buffer, and the protein concentration was measured by Bradford assay.

2.4. Determination of the concentration of monoamine neurotransmitters including norepinephrine, serotonin (5-hydroxytryptamine, 5-HT), and dopamine by reversed-phase high-performance liquid chromatography (HPLC)

The hippocampal tissue was extracted with 0.1 M perchloric acid and sonicated for 30 min at an ice-cold temperature. Homogenates were centrifuged at 12,000 g for 15 min at 4 °C, while clear supernatants were decanted and filtered through a 0.45-μm filter. The concentration of monoamine neurotransmitters was determined from this filtrate. The HPLC system consisted of the analytical C18 reversed-phase column (ODS3 C18, 4.6 × 250 mm i.d., 5-micrometer particle size) and UV detector (best condition at 220 nm). The mobile phase consists of 0.02 M sodium acetate, buffered to a pH of 4 with 0.0125 M citric acid, containing 0.042 M methanesulfonic acid and 0.1 mM EDTA. The flow rate was set at 1 mL/min. The working standard solutions were freshly prepared in 0.05 M perchloric acid containing 0.1 mM Na_2EDTA on ice and stored at −20 °C before using. Peaks were identified by comparing the retention time of each peak in the sample solution, where each individual peak was further compared to the standard solution of norepinephrine, serotonin (5-hydroxytryptamine, 5-HT), dopamine and caffeine (Sigma-Aldrich, USA) served as an internal standard.

2.5. Determination of protein changes in the SD rat hippocampus by two-dimensional gel electrophoresis (2-DE)

Here, 400 μg of protein was mixed with 340 μl of rehydration buffer (8 M urea, 4% CHAPS, 0.001% bromphenol blue and 3 mM dithiothreitol) containing 1% IPG buffer, pH gradients 3–10. The sample was loaded onto 13-cm IPG strips with a pH range of 3–10 using an isoelectric focusing system (Ettan IPGphore III, GE Healthcare Biosciences, USA). Samples were run through the steps of strip rehydration (20 °C, 16 h) and isoelectric focusing (500 V for 500 V^{-h}, 1,000 V for 800 V^{-h}, and 10,000 V to reach 27,000 V^{-h}). The maximum current was maintained at 75 μA per strip. After the completion of the process, the strips were equilibrated twice (15 min each) in equilibration buffer supplemented with 65 mM DTT and 135 mM iodoacetamide. Each strip was subjected to a second dimensional separation (PROTEAN II xi cell, Bio-Rad, USA) using an SDS-polyacrylamide gel (12.5%). Separation of protein was executed under the applied voltage of 300 volts with 16 mA to 24 mA per gel at 20 °C until the bromophenol blue dye front reached 0.5 cm from the bottom of the gel. The gels were stained with colloidal Coomassie blue staining according to the standard recommendation. After staining, gel images were acquired using an Image scanner III (GE Healthcare Biosciences). Differential analysis was performed by Image Master 2D Platinum version 7.0 (GE Healthcare Biosciences) software tool, and spots of interest were excised for identification by mass spectrometry.

2.6. Protein identification with liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The spots of interest were carefully cut and destained in a solution containing 50% ACN in 100 μL of 25 mM ammonium bicarbonate ($(NH_4)HCO_3$) and then submitted to an in-gel trypsin digestion overnight at 37 °C in 25 mM ($(NH_4)HCO_3$). Peptides were extracted in 50 μL of 5% formic acid/50% ACN and then put into the ultrasonic bath for 15 min and dried in a speed-vac. Peptide samples were dissolved in 98% H_2O, 2% ACN and 0.1% formic acid. The LC–MS/MS system consists of a liquid chromatography part (Dionex Ultimate 3000, Thermo Scientific) in

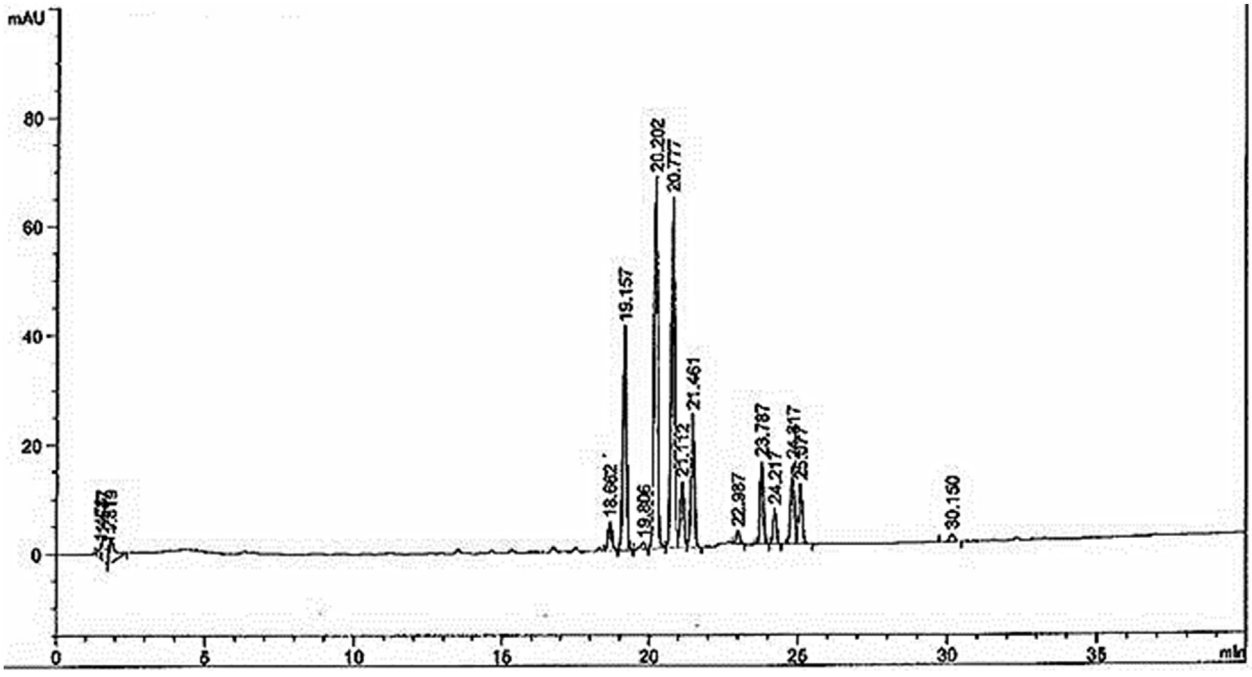

Fig. 1. Representative HPLC fingerprint of *K. parviflora* ethanolic extract.

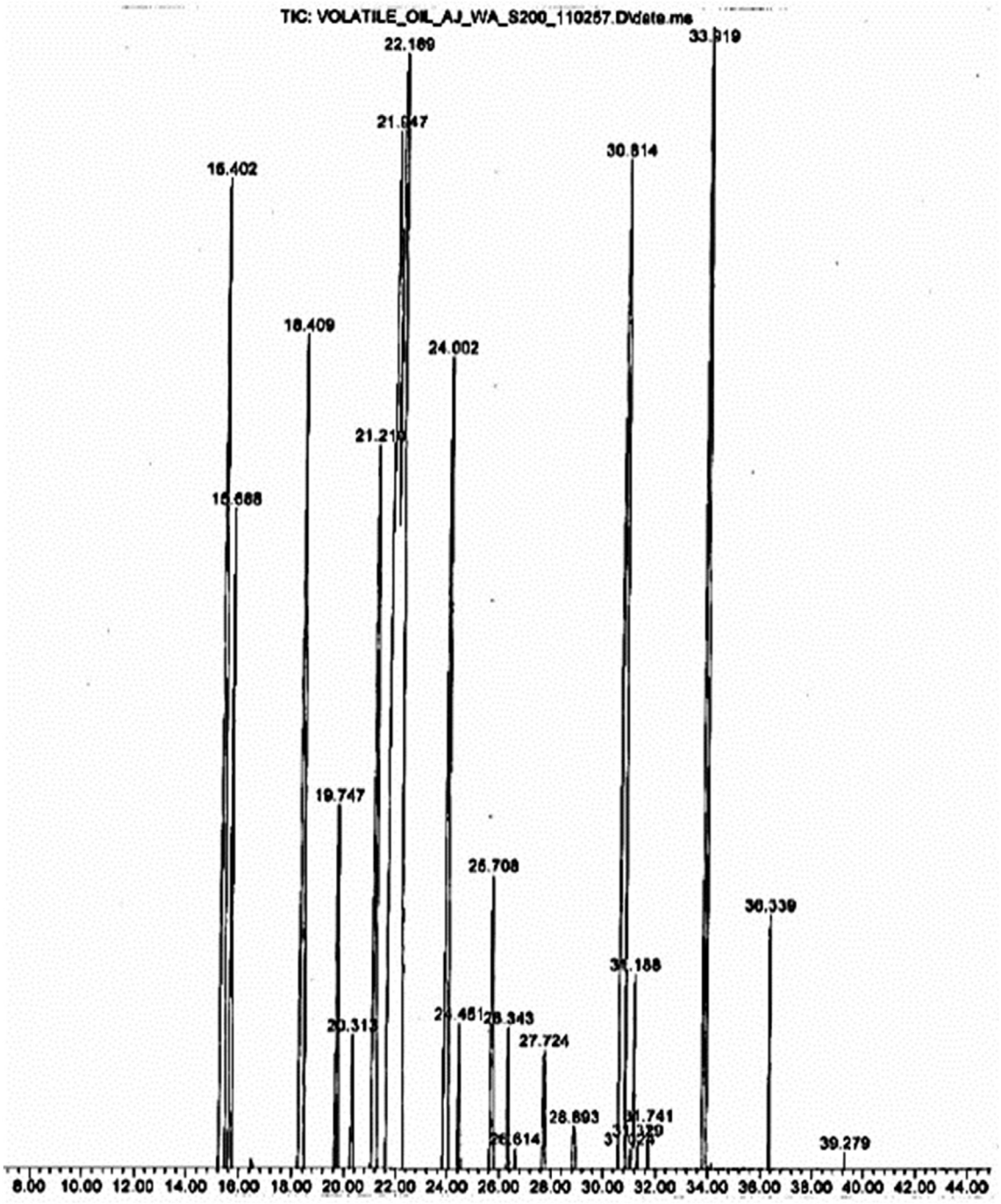

Fig. 2. GC–MS chromatogram of *M. fragrans* nutmeg volatile oil.

combination with an electrospray ionization (ESI)-ion trap mass spectrometer (amaZon SL, Bruker, Germany). The LC separation was performed on a reversed-phase column (Hypersil GoLD 50 × 0.5 mm, 5 μm C18), protected by a guard column, eluted at a flow rate of 100 μl/min under gradient conditions of 5–80% B over 50 min. Mobile phase A consists of water/formic acid (99.9:0.1, v/v), and B consists of acetonitrile (100, v). Mass spectral data from 150 to 1500 m/z was collected in the positive ionization mode. For protein identification, MS/MS data were analyzed by the Mascot search engine against the SwissProt database. The Mascot score was taken into consideration and reported after manual verification of the fragmentation spectrum. Protein–protein interaction and signaling pathway analysis, using the Ingenuity Pathways Analysis (IPA), offered us some additional valuable clues about the complex interactive links between the various identified proteins within their commonly known interactive protein networks also obtained from other cellular metabolic information.

2.7. Determination of protein expression by Western blot analysis

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF). Membranes were blocked with non-fat dried milk in Tris-buffered saline containing 0.01% Tween-20 and incubated in the following primary antibodies at 1:8000: Anti-PIA3 (ERp57), Anti-GFAP, Anti-DPYSL2 (CRMP2), Anti-Phospho-DPYSL2 (Phospho-CRMP2) and Anti-β-actin (cell signaling, USA). After secondary antibody treatment (HRP159 linked antibody), the signal was developed with a chemiluminescence reagent (GE healthcare, USA), and band images were detected with an X-ray film.

2.8. Data analysis and statistical methods

Raw data are presented as the mean and standard deviation, and the data were analyzed to find significant *P*-values <0.05 and 0.01. Differences between the groups were established by using an unpaired Student's *t-test*, while within-group comparisons were performed using the paired Student's *t-test*. The spot densities were further compared to control using an ANOVA.

3. Results

3.1. Standardized crude extract of K. parviflora and M. fragrans nutmeg volatile oil

The content of 5,7-dimethoxyflavone in *K. parviflora* extract was analyzed by reversed-phase HPLC. The sample contained 13.4618 ± 0.0145 mg/g ($R^2 = 0.9997$) of 5,7-dimethoxyflavone in the dry sample, with a representative HPLC fingerprint as shown in Fig. 1. The chemical composition of *M. fragrans* nutmeg volatile oil was analyzed by using the GC–MS technique (Fig. 2). A total of 26 volatile compounds were tentatively identified and reported in the nutmeg oil. The data describing all compounds are shown in Table 1. This research demonstrated that the crude extract of *K. parviflora* and nutmeg volatile oil are of good quality with a high potential.

Table 1
Chemical composition of nutmeg volatile oil obtained by GC–MS.

NO.	RT	Area%	Compound
1	14.428	1.97	z1R-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene
2	15.434	6.17	Bicyclo [3.1.1] hex-2-ene, 2-methyl-5(1-methylethyl)
3	15.729	2.68	1R-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene
4	16.522	0.12	Camphene
5	18.462	5.13	Cyclohexene, 4-methylene-(1-methylethyl)
6	19.066	0.52	Beta-Myrcene
7	20.646	1.59	4-Carene
8	21.266	7.09	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)
9	22.024	14.89	o-cymene
10	22.702	10.04	Limonene
11	23.574	1.95	Gamma-terpinene
12	24.059	5.28	Gamma-terpinene
13	25.396	0.46	Cyclohexene, 1-methyl-4-(1-methylethylidene)
14	28.767	0.03	(+)-2-Bornanone
15	30.696	5.67	Terpinen-4-ol
16	30.885	6.20	3-Cyclohexene-1-ol, 4-methyl-1-(1-methylethyl)
17	31.051	0.16	Benzenemethanol, alpha., 4-trimethyl
18	31.228	0.61	Alpha-terpineol
19	31.217	0.78	Alpha-terpineol
20	31.347	0.15	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl), cis
21	31.761	0.23	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl), cis
22	33.961	8.88	Safrole
23	35.369	0.18	Eugenol
24	36.334	0.78	Methyleugenol
25	36.695	0.01	Caryophyllene
26	38.168	0.05	Benzene, 1,2-dimethoxy-4-(1-propenyl)
27	38.665	0.90	Meristicin
28	41.320	0.03	Ar-turmerone
29	43.024	0.06	Tetradecanoic

Table 2
Concentration of neurotransmitters (μg/g wet weight) in the hippocampal homogenates from control and treated rats.

Group	Neurotransmitter (μg/g wet weight)		
	NE (Mean ± SD)	DA (Mean ± SD)	5-HT (Mean ± SD)
Control ($n = 6$)	0.1789 ± 0.0153	0.2594 ± 0.1077	0.3695 ± 0.0262
K. parviflora ($n = 6$)	0.3316 ± 0.0365	0.3337 ± 0.0674	0.5132 ± 0.0137
M. fragrans ($n = 6$)	0.2825 ± 0.0133	0.4517 ± 0.0259	0.5897 ± 0.0189
Fluoxetine ($n = 5$)*	0.2408 ± 0.0171	0.3463 ± 0.0221	0.5811 ± 0.0208

* On the fifth day, one rat in the fluoxetine treatment group died. Data from the liver and kidney function and histology in the dead rat did not show indications of toxicity, but the data are not shown. NE, norepinephrine; DA, dopamine; 5-HT, serotonin.

3.2. Effects of K. parviflora rhizome ethanolic extract and M. fragrans nutmeg volatile oil on the levels of monoamine neurotransmitters including norepinephrine, serotonin (5-hydroxytryptamine, 5-HT), and dopamine

Reversed-phase high performance liquid chromatography (HPLC) is a commonly used method that can accurately determine the content of neurotransmitters upon treatment with *K. parviflora* rhizome ethanolic extract (200 mg/kg BW), *M. fragrans* nutmeg volatile oil (300 mg/kg BW) or fluoxetine (20 mg/kg BW) for 12 days compared with that of the control group (2% tween 80, 1 mL/kg BW). The concentration of monoamine neurotransmitters is reported in Table 2, and the representative HPLC fingerprints are show in Fig. 3B. Administration of *K. parviflora* group and *M. fragrans* via the oral route once per day for 12 days led to a significantly higher level of serotonin (5-HT), norepinephrine and dopamine than the control treatment, as shown in Fig. 3A ($P < 0.01$). However, *M. fragrans*-treated rats exhibited a non-significant increase in serotonin compared with that of the fluoxetine-treated group, as shown in Fig. 3A. All results were expressed as the mean ± SD, and a student's paired *t-test* was used to compare the different groups. Results were considered statistically significant at a *P*-value less than 0.01.

3.3. Proteomic identification of proteins modulated by K. parviflora, M. fragrans and fluoxetine in the SD rat hippocampus

We have extended our study to identify possible molecular targets for *K. parviflora* rhizome ethanolic extract (200 mg/kg BW), *M. fragrans* nutmeg volatile oil (300 mg/kg BW) and fluoxetine (20 mg/kg BW) treatment on hippocampal proteomic profiles in SD rats. The hippocampal protein extracts of rats from the control and treated groups were analyzed by 2-DE. For each experimental group, gels were made in triplicate (12 gels in total). The representative gels are presented in Fig. 4. The image analysis of the gels demonstrated a similar number and pattern distribution of spots in

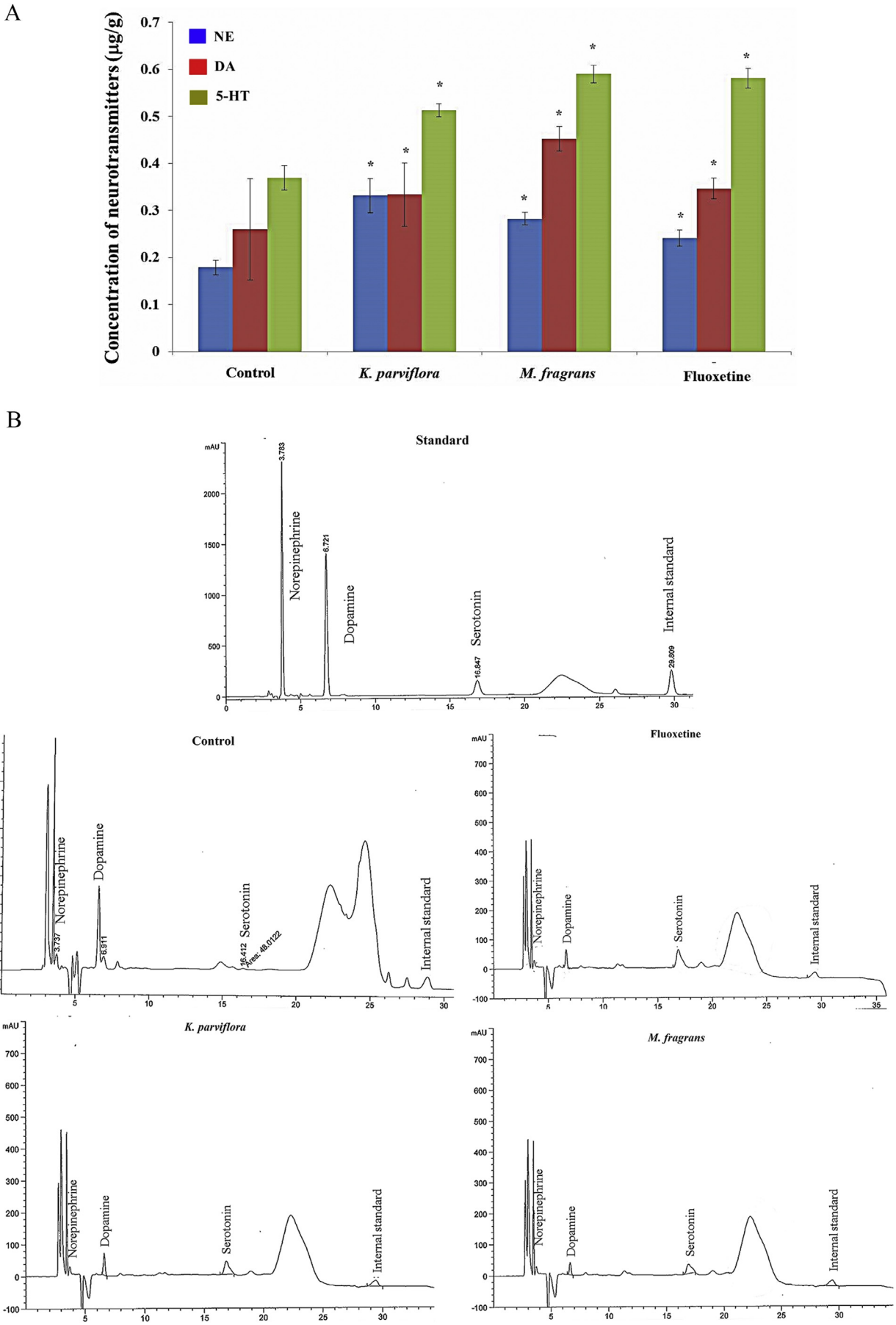

Fig. 3. (A) The increase in the level of monoamine neurotransmitters including norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) in the rat hippocampus when treated with *K. parviflora* rhizome ethanolic extract, *M. fragrans* nutmeg volatile oil and fluoxetine. The statistical analysis showed a significant difference between the control and each of the treatment groups at $P < 0.05$ (control: n = 6, *K. parviflora*: n = 6, *M. fragrans*: n = 6, fluoxetine: n = 5). Data are expressed as the mean ± standard deviation (S.D.). (B) HPLC chromatograms of monoamine neurotransmitters.

the twelve 2D gels by using Image Master 2D Platinum version 7. Approximately 1050 spots were detected on each gel (as shown in Fig. 4A).

We focused our analysis on the proteins in the three replicates of each treatment group that showed a fold change ≥1.5 compared with that in the control group ($P < 0.05$). Table 3 presents the functional classification and regulation of the differentially expressed proteins in each group. Ninety proteins were accepted in total as significantly different and were analyzed with LC-MS/MS. Mass spectrometry gave identified 79 of the 90 spots (87.78% success rate). However, we found the presence of the same proteins in different spots, which our data does not display. After that each group was compared with the control group, the analysis indicated that 37 proteins were up-regulated and 14 were down-regulated in the *K. parviflora* group. The *M. fragrans* group had 27 proteins up-regulated and 16 down-regulated. In the fluoxetine-treated group, we found 29 proteins that were up-regulated, and 14 that were down-regulated.

Bioinformatics analysis (SwisProt, UniProtKB, IPA and STRING-10.0) has been used for pathway analysis, the functional classification and regulation of the differentially expressed proteins (see Table 3 and Supplementary data). Considering their biological function, the proteins were classified by their functional process into nine different cellular or subcellular localizations. This analysis revealed that most of the entries were localized in the cytoplasm (47% of total), and others were localized to the nucleus (14%), cytoskeleton (12%), cell membrane (8%), mitochondrial matrix (7%), mitochondrion (5%), and mitochondrial membrane (4%) (see Fig. 6). Moreover, the IPA analysis showed that the numerous proteins participate in many pathways important to nervous system development and function and neurological disease (see Tables 1–5 in Supplementary data). The protein-protein interaction data (STRING and IPA) integrates the information and provides an understanding of the cellular physical and functional interactions. The IPA analysis demonstrated the involvement of the identified proteins and their interactive pathways within a neuronal network especially important for neuro-regenerative processes such as neural plasticity (Supplementary data). In addition, proteins that have a potential antioxidative role, which is important for neuronal protection in the brain, were up-regulated, while proteins that are known for their inhibitory effects on neuro-regenerative processes were down-regulated as shown in Table 3 (see Fig. 7).

3.4. Validation of the change in protein expression from the 2D-GE results by Western blot analysis

We validated the expression level of proteins by choosing representative proteins from those that were down- and up-regulated in the 2D gels. Western blot analysis was performed with specific antibodies that detect bands of PDIA3 at 57 kDa, GFAP at 50 kDa, and DPYSL2 and p-DPYSL2 at 55–65 kDa. The intensity of the bands was normalized by β-actin, and the values were obtained in the experimental groups, which were normalized to the corresponding value in the control group. Semi-quantitative analysis of the Western blot confirmed the tendencies observed in the 2D gel image analysis. Representative Western blots of hippocampal samples are presented in Fig. 5A and B. In detail, the GFAP protein level was significantly higher in the hippocampus of the *K. parviflora*- and *M. fragrans*-treated groups but non-significantly higher in the fluoxetine-treated group than in the control group (Fig. 5C). The PDIA3 level was significantly increased in the *M. fragrans* and fluoxetine groups but not changed in the *K. parviflora* group (see Fig. 5D). The statistical analysis demonstrated significant differences in the levels of DPYSL2 and p-DPYSL2 between the experimental and control groups (see Fig. 5E and F). The expression level of DPYSL2 was higher in the two herbal groups than in the control group (Fig. 5D). However, no difference was observed in the fluoxetine-treated group. In this study, we are interested in the role of p-DPYSL2 in neuronal development. Western blotting revealed that p-DPYSL2 was significantly lower in the experimental groups than in the control group (Fig. 5E). The expression levels of the proteins were in accordance with the data from the proteomics analysis.

4. Discussion

The level of monoamine neurotransmitters in the hippocampus of the brain is involved in the regulation of cognitive processes such as mood, attention, sleep, arousal, and certain types of memory.[37] Monoamine neurotransmitters have been found to play an important role in neurological disorders. Drugs or herbal remedies that augment the effects of monoamines on their target tissue are used to treat psychiatric disorders, including depression, anxiety, and schizophrenia.[38] This study demonstrates, for the first time, the effect of *K. parviflora* and *M. fragrans* on the levels of NE, DA and 5-HT. Our results showed that *K. parviflora*, *M. fragrans* and fluoxetine increased the levels of serotonin (5-HT), norepinephrine and dopamine in the hippocampus of SD rats when compared with that of the control group. Disturbances in the DA, 5-HT and NA neurotransmitter systems have been suggested to be involved in the pathogenesis of mood disorders.[39,40] Moreover, we found an up-regulation of proteins that are involved in synaptic function and endocytosis, including BIN1 and SYT1, in the *K. parviflora* and *M. fragrans* group and SNAP25 only in the *K. parviflora*-treated group. Interestingly, a previous study demonstrated that norepinephrine triggers the release of glial ATP to increase postsynaptic efficacy,[41] which is related to our proteomic results. Our results showed that *K. parviflora*, *M. fragrans* and fluoxetine regulate seven proteins involved in ATP synthesis: ATP5H, APRT, URCRFS1, CKB, ENO1, NDUFS and NSF. Several findings have been discovered in the rat model: selective serotonin reuptake inhibitors (SSRI), such as fluoxetine, increase the extracellular 5-HT levels in the rat hippocampus.[37,42–44] Our observations indicate that *K. parviflora* and *M. fragrans* increase the level of 5-HT through similar mechanisms as fluoxetine.

In the present work, we focused on the expression of GFAP, PDIA3, DPYSL2 and p-DPYSL2. Glial fibrillary acidic protein (GFAP) is the intermediate filament protein in mature astrocytes, in addition to vimentin, nestin and synemin.[45–48] There is mounting evidence suggesting aberrant astrocytic function in neurodegenerative diseases such as dementia, Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD).[46] Moreover, the expression of both GFAP mRNA and protein was reduced in the hippocampus in major depressive disorder (MDD). Astrocyte pathology in depression is less well known in the human hippocampus, but the literature suggests the involvement of hippocampal astrocytes in the blood of depressed subjects at the

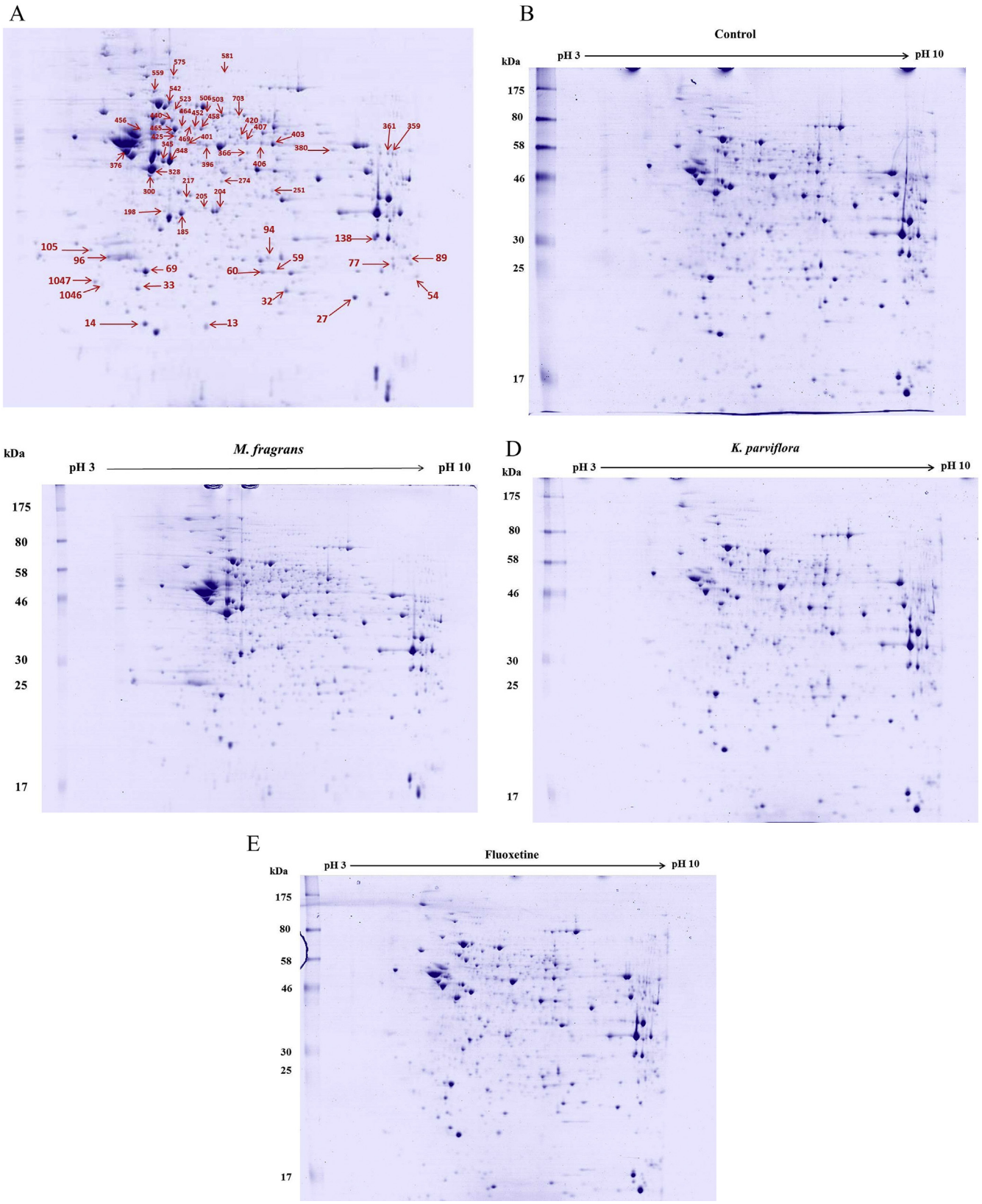

Fig. 4. Representative gels showing the two-dimensional gel analysis profile of the hippocampal protein from control (B), fluoxetine (E), *K. parviflora* (D) and *M. fragrans* (C) -treated rats. Gels were made in triplicate (12 gels in total). The gels were stained with Coomassie blue and analyzed by HPLC-ESI-IT-MS/MS. The spots were identified by MASCOT, and the names of the proteins are presented in Table 3 (Not all listed proteins are shown in Fig. 4).

Table 3
Functional classification and regulation of differentially expressed proteins in treated rat hippocampal tissue.

Spot No.	Gene symbol	Proteins name	*pI*	MW (Da)	localization	Molecular function	Regulated		
							KP	MP	Flu
12	Tagln3	Transgelin-3	8.96	24887	Mt (matrix)	Development of central nervous system.	↑	↓	↑
13	Atp5H	ATP synthase subunit d	6.79	18491	Mt	Enzyme, produces ATP from ADP.	↑	↓	↑
14	Prdx2	Peroxiredoxin-2	5.34	21941	Cp	Enzyme, redox regulation of the cell.	↑	↑	↑
15	Aprt	Adenine phospho-ribosyltransferase	6.17	19761	Cp	Enzyme, catalyzes a salvage reaction in the formation of AMP.	↓	–	↑
27	Sod2	Superoxide dismutase [Mn]	8.96	24887	Mt (matrix)	Enzyme, destroys superoxide anion radicals.	↑	–	–
32	Uqcrfs1	Cytochrome b-c1 complex subunit Rieske	9.04	29712	Mt (Inner membrane)	Enzyme, generates an electrochemical potential coupled to ATP synthesis.	↑	↓	↑
33	Ddir1	Rho GDP-dissociation inhibitor 1	5.12	23450	Cp	Controls the homeostasis of Rho proteins.	–	↓	–
59	Tpi1	Triosephosphate isomerase	6.89	27345	Cp	Enzyme, involved in the gluconeogenesis pathway and carbohydrate biosynthesis.	↑	↓	↑
69	Uchl1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	5.14	25165	Cp, ER (membrane)	Enzyme, involved both in the processing of ubiquitin precursors and of ubiquitinated proteins.	–	↑	↑
77	Gstm	Glutathione S-transferase	8.78	25360	Cp	Enzyme, conjugation of reduced glutathione to exogenous and endogenous hydrophobic electrophiles.	↑	↑	↑
89	Map	Microtubule-associated protein	4.87	300831	Cp, Csk	Bind to the tubulin subunits that regulate microtubule stability.	↑	–	–
94	Hagh	Hydroxyacyl glutathione hydrolase	7.64	34544	Cp	Enzyme, catalyzes the hydrolysis of S-D-lactoyl-glutathione to form glutathione and D-lactic acid.	↑	–	–
96	Ywhag	Tryptophan 5-monooxygenase activation protein, gamma (14-3-3 protein gamma)	4.80	28456	Cp	Intracellular signaling and cell cycle.	↓	↑	–
105	Ank3	Ankyrin-3	7.94	285692	Cp, Csk, CM	Maintenance/targeting of ion channels and cell adhesion molecules at axonal initial segments and the nodes of Ranvier.	↑	–	↑
138	Vdac1	Voltage-dependent anion-selective channel protein 1	8.62	30851	Mt (outer membrane)	Forms a channel through the mitochondrial outer membrane and the plasma membrane.	↑	↓	↓
169	Hnrnpa1	Heterogeneous nuclear ribonucleoprotein A1	9.20	34362	Cp, Nu	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm.	↑	↑	↑
185	Ldhb	L-lactate dehydrogenase B chain	5.70	36874	Cp	Involved in the subpathway that synthesizes (S)-lactate from pyruvate.	↓	–	↓
194	Ppp1B	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	5.84	37961	Cp, Nu	Cell division, role in the control of chromatin structure and cell cycle progression during the transition from mitosis into interphase.	↑	↑	↑
204	Syt1	Synaptotagmin-1	9.43	43385	Cp (vesicle)	A regulatory role in the membrane interactions of synaptic vesicles at the active synapse zone.	↑	↑	–
205	Akt	Serine/threonine-protein phosphatase	5.94	38229	Cp, Nu	Regulates many processes including metabolism, proliferation, cell survival, growth and angiogenesis.	↑	↓	↑
217	Ddah1	N(G)-dimethylarginine dimethylaminohydrolase	5.75	31805	Cp	A role in the regulation of nitric oxide generation, inhibits NOS	–	↑	–
227	Ark72	Acetyl-CoA carboxylase 1	5.97	266678	Cp	Role in controlling fatty acid metabolism.	↑	↑	↑
245	Ak1A1	Alcohol dehydrogenase 1	6.84	36711	Cp, Nu	Catalyzes the oxidation of long-chain primary alcohols and the oxidation of S-(hydroxymethyl) glutathione.	↑	↓	↑
251	LOC102548564	Peptidyl-prolyl cis-trans isomerase D	6.73	41139	Cp, Nu	Catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.	↑	–	↑
262	Tmod2	Tropomodulin 2	5.34	39468	Cp, Csk	Blocks the elongation and depolymerization of the actin filaments at the pointed end.	–	↑	↑
274	Pura	Transcriptional activator protein Pur-alpha (Fragments)	4.69	15370	Nu	Role in the initiation of DNA replication and in recombination.	↑	↓	–
300	Sh3gl2	Endophilin-A1	5.26	40045	Cp, CM	Implicated in synaptic vesicle endocytosis.	↓	–	↓
317	Pgk1	Phosphoglycerate kinase 1	8.02	44909	Cp	Role as a glycolytic enzyme.	↑	↑	↑
331	Gfap	Glial fibrillary acidic protein	5.35	49984	Cp	Role in astrocyte-neuron and many important processes in CNS such as development of CNS.	↑	↑	↑
345	Ckb	Creatine kinase B-type	5.39	42983	Cp	Catalyzes the transfer of phosphate between ATP and various phosphagens.	↓	↑	↓
354	Eftu	Elongation factor Tu, mitochondrial	7.23	49890	Mt	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes in protein synthesis.	–	↓	↓
359	Sept7	Septin-7	8.82	50818	Nu, Cp	Normalizes the organization of the actin cytoskeleton, role in ciliogenesis and collective cell movements.	↑	–	↑

361	Sept11	Septin-11	6.24	50005	Cp, Csk	Plays important role in cytokinesis, cytoarchitecture of neurons (dendritic arborization and dendritic spines), and in GABAergic synaptic connectivity.	↑	↓	↑
366	Aldh5a1	Succinate-semialdehyde dehydrogenase	8.35	56723	Mt	Catalyzes the degradation of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA).	↓	↓	↓
396	Eno1	Alpha-enolase	6.16	47440	Cp, CM	Plays role in glycolysis.	↑	–	↑
401	Dlst	dihydrolipoyllysine-residue succinyltransferase	8.76	67637	Mt (matrix)	Converts 2-oxoglutarate to succinyl-CoA and CO2.	↑	↑	↑
403	Glud1	Glutamate dehydrogenase 1	8.05	61719	Mt (matrix)	Converts L-glutamate into alpha-ketoglutarate and increases the turnover of the excitatory neurotransmitter glutamate	↑	–	↑
442	Tba4a	Tubulin alpha-4A chain	4.95	50634	Cp, Csk	A major constituent of microtubules	↓	↑	↓
450	Cap1	Adenylyl cyclase-associated protein 1	7.61	51899	CM	Regulates filament dynamics and plays role in a number of complex developmental and morphological processes, (mRNA localization and cell polarity).	↑	↑	↑
452	Pdia3	Protein disulfide-isomerase A3	4.74	64400	ER	Plays a role in protein folding by promoting the formation of disulfide bonds.	↑	↑	↑
456	Vim	Vimentin	5.06	53757	Nu (matrix)	The major IF protein in mesenchymal cells.	–	↑	↓
464	Hspd1	60-kDa heat-shock protein	5.91	61088	Mt (matrix)	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides.	–	↑	↑
465	Dpysl2	dihydropyrimidinase-related protein 2	5.95	62638	Cp, Csk, CM	Involved in neuronal differentiation and axonal guidance.	↑	↑	↑
490	Cct5	T-complex protein 1 subunit epsilon	5.51	59955	Cp, Csk, MT	Molecular chaperone, helps the folding of proteins upon ATP hydrolysis.	↓	↑	↓
559	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit	8.51	51499	Mt (Inner membrane)	Role in the transfer of electrons from NADH to the respiratory chain.	↓	–	↓
574	Gsn	Gelsolin	5.76	86413	Cp, Csk	Promotes the assembly of monomers into filaments.	↓	↓	↓
581	Pdcd6ip	Programmed cell death 6-interacting protein	6.15	97141	MT	Plays a role in the regulation of both apoptosis and cell proliferation.	↓	–	–
641	Pura	Transcriptomal activator protein Pur-alpha	4.69	15370	Nu	Plays important role in the initiation of DNA replication and recombination.	↑	↓	↓
687	Sept8	Septin 8	5.74	51562	Cp, Csk	Role in cytokinesis	↑	↑	↑
703	Me1	NADP-dependent malic enzyme	6.49	64589	Cp	Generates NADPH for fatty acid biosynthesis and reverses malate to pyruvate.	–	–	↓
819	Stag3	Cohesin subunit SA-3	5.81	143628	Nu	A component of cohesin complex.	↑	↑	↑
846	Hyou1	Hypoxia up-regulated protein 1	8.73	63702	Cp, Nu	Role in cytoprotective, mechanisms triggered by oxygen deprivation.	–	–	↓
848	Nsf	Vesicle-fusing ATPase	6.55	83170	Cp	Catalyzes the fusion of transport vesicles within the Golgi cisternae.	↓	↓	↓
849	Dldh	Dihydrolipoyl dehydrogenase	7.96	54574	Mt (matrix)	A component of the glycine cleavage system and alpha-ketoacid dehydrogenase complexes.	↑	↑	↓
873	Hnrpm	Heterogeneous nuclear ribonucleoprotein M	8.90	74076	Nu (matrix)	Important role in p53/TP53 response to DNA damage.	↓	–	↓
882	Tkt	Transketolase	7.23	68342	Ly	Catalyzes the transfer of a two-carbon ketol group from a ketose donor to an aldose acceptor.	↑	↑	–
890	Arhgef28	Rho guanine nucleotide exchange factor 28	5.47	192748	Cp, CM	Functions in axonal branching, synapse formation and dendritic morphogenesis. Regulates numerous cellular responses such as proliferation, differentiation and movement.	↑	↑	↓
918	Bin1	Myc box-dependent interacting protein 1	4.95	64721	Cp, Nu	Role in the regulation of synaptic vesicle endocytosis.	↑	↑	–
960	Sptn1	Spectrin alpha chain	5.20	285261	Cp, Csk	Role in secretion and interaction of calmodulin in a calcium-dependent manner, candidate for the calcium-dependent movement of the cytoskeleton.	↓	↓	↓
1046	Snap25	Synaptosomal-associated protein 25	4.66	23528	Cp, CM	Important role in synaptic function including molecular regulation of neurotransmitter release, vesicle docking and membrane fusion.	↑	–	–

The table reports the list of spots, SwissProt protein, full name, theoretical *pI* and MW (Da), molecular function and protein regulation. The main localization: Cp, Cytoplasm; Nu, Nucleus; Csk, Cytoskeleton; CM, Cell Membrane; Mt, Mitochondrion; Ly, Lysosome; ER, Endoplasmic Reticulum.

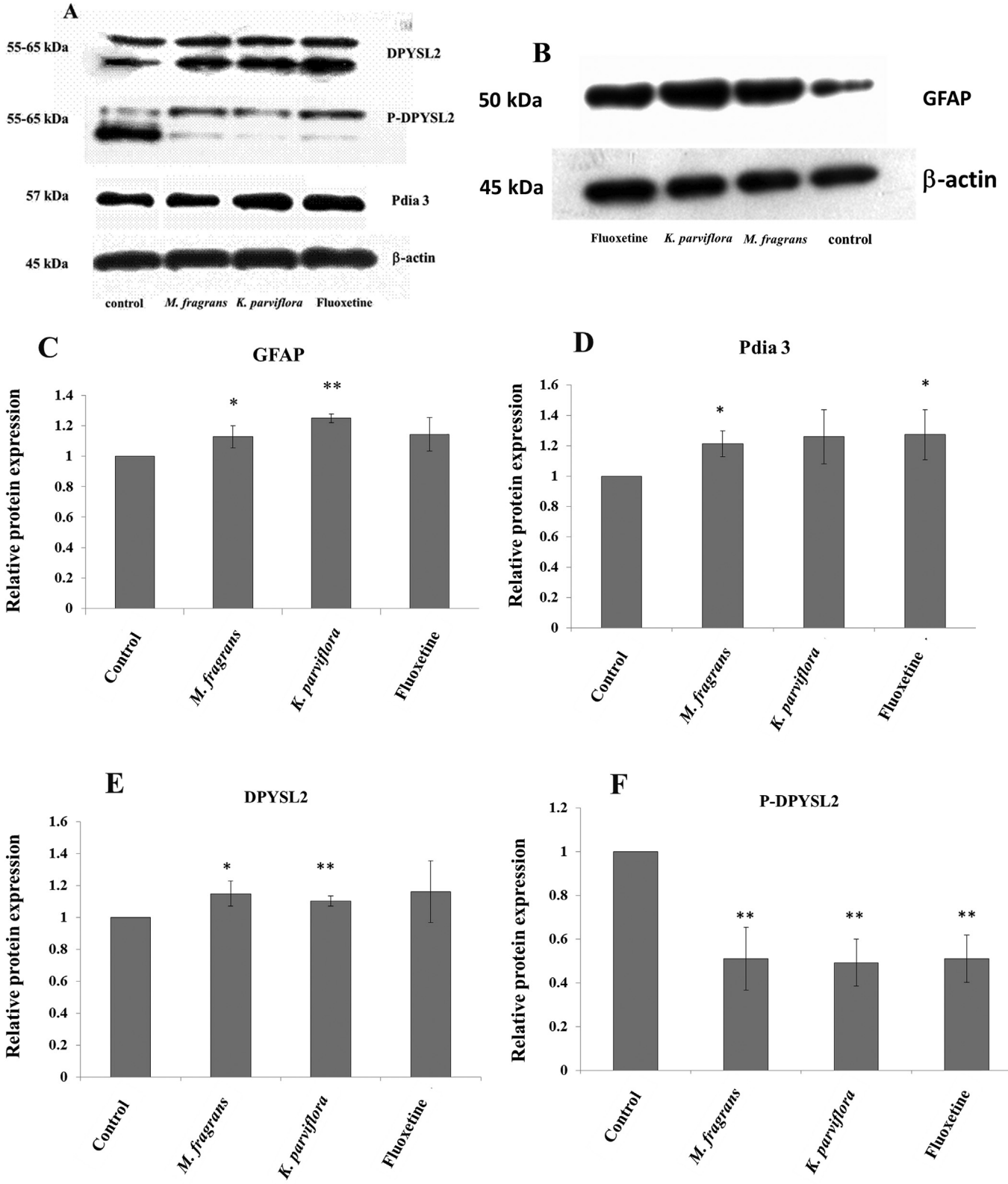

Fig. 5. (A–F) Representative image of Western blots evaluating the expression of GFAP, PDIA3, DPYSL2 and p-DPYSL2 from the rat hippocampus. Data are expressed as the mean ± standard deviation (S.D.); statistically significant differences were analyzed by *t-test* ($P < 0.05$).

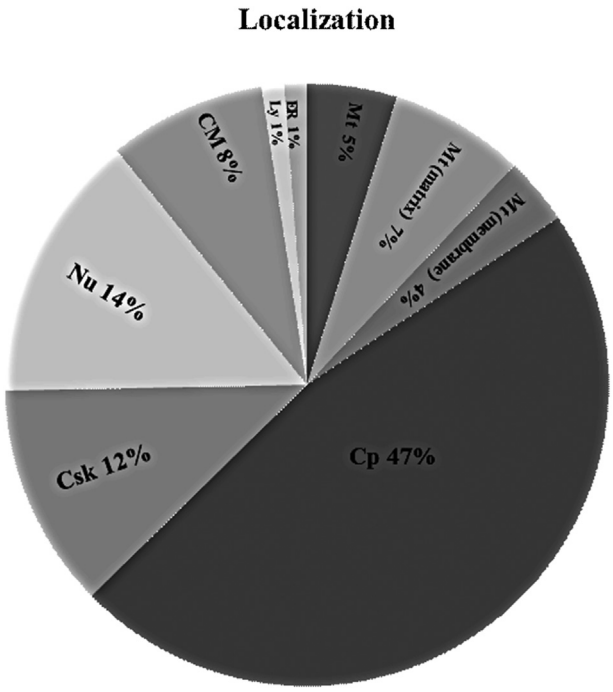

Fig. 6. Pie chart represents the percentage of nine different cellular/subcellular localizations by using the Protein Knowledgebase UniProtKB.

time of death without antidepressant medication.[48] Recently, GFAP antibodies demonstrated a protective effect on oxidative-stressed neuroretinal cells via an interaction with PDIA3.[49] Our study presents an up-regulation of GFAP and PDIA3 in the rat hippocampus treated with *K. parviflora* and *M. fragrans*. Furthermore, we show that *M. fragrans* increases the level of vimentin (VIM), a type III intermediate filament (IF). Thus, *M. fragrans* may be effective in promoting neuroregeneration in plasticity. In addition, we found that the effected proteins play important roles in neuronal differentiation and morphology of neuronal cells, including microtubule-associated protein (MAP), which binds to the tubulin subunits regulating microtubule stability, tubulin alpha-4A chain (Tba4a), a major constituent of microtubules, and dihydropyrimidinase-related protein 2 (Dpysl2), which is involved in neuronal differentiation and axonal guidance.

In addition to the role of Dpysl2 in cytoskeleton organization, Dpysl2 is also a component of the dynein complex, acting on the intracellular retrograde motility of vesicles and organelles along with microtubules at the synaptic level.[50] DPYSL2 down-regulation might be associated with neuronal and synaptic loss, as the protein is involved in axon guidance and growth, neurite development, and neuronal polarization.[51] The expression of DPYSL2 was significantly increased in the *K. parviflora* and *M. fragrans* groups, although in the fluoxetine group, there was no significant difference in its expression. A previous study identified a crucial function of DPYSL2 in axon formation of hippocampal neurons, thereby establishing and maintaining neuronal polarity.[52] DPYSL2 interacts with tubulin heterodimers and promotes microtubule assembly *in vitro*. Thus, DPYSL2 seems to promote neurite elongation and axon specification by regulating microtubule assembly, endocytosis of adhesion molecules and reorganization of actin filaments.[53] In addition, we found that *K. parviflora* up-regulated microtubule-associated protein (MAP), which plays an important role in microtubule stability. A more direct link to changes in synaptic activity suggested by our results is the up-regulation of SNAP25, which plays a key role in synaptic functioning and synaptic formation. We also found that the *M. fragrans* group showed an increase in the expression of tubulin alpha-4A chain (TBA4A), which is a major constituent of microtubules. Phosphorylated DPYSL1 and DPYSL2 are localized in the dendrites of cortical neurons where they regulate dendritic branch trajectories.[54] A previous study in mice revealed that the abnormal phosphorylation of DPYSL1 and DPYSL2 resulted in a curling dendrite phenotype, suggesting that these two molecules are critical for normal dendrite patterning in cortical neurons.[55] The most important mechanism of action of non-phosphorylated DPYSL2 is its strong binding to tubulin, which leads to microtubule formation. Phosphorylation of DPYSL2 by Rho kinase suppresses its binding to tubulin and Numb. Surprisingly, our study found that p-DPYSL2 was significantly decreased in the rat hippocampus treated with *K. parviflora*, *M. fragrans* and fluoxetine. This is the first time that the effects of these compounds on DPYSL2 and p-DPYSL2 expression have been reported.

Moreover, we found that the PDIA3 level was increased in the *M. fragrans* and fluoxetine group but not changed in the *K. parviflora* group. ERp57, also known as Pdia3, Grp58, ER60 and 1,25D_3-MARRS, is a member of the protein disulfide isomerase (PDI) family and a lumenal protein of the endoplasmic reticulum (ER). To enter the secretory pathway, proteins are cotranslationally translocated across the membrane of the endoplasmic reticulum (ER) as extended polypeptide chains.[56,57] ERp57 can interact with calreticulin and calnexin to ensure the correct folding of newly synthesized glycoproteins as a molecular chaperone and is associated with a number of diseases including cystic fibrosis, prion diseases, Huntington's disease and Alzheimer's disease.[57]

In fact, impaired mitochondrial function is commonly observed in many types of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, alcoholic dementia, brain ischemia-reperfusion related injury, and others. Although many of these neurological disorders contain unique etiological factors, may share common factors of increased nitroxidative stress and mitochondrial dysfunction, likely resulting from protein post-translational modifications (PTMs) including oxidation, nitration, hyperphosphorylation, acetylation, glycosylation and formation of various protein adducts.[58,59] Interestingly, *K. parviflora* increased the level of superoxide dismutase [Mn] or SOD2, which is an enzyme that destroys superoxide anion radicals. In addition, *K. parviflora*, *M. fragrans* and fluoxetine increased the level of glutathione S-transferase. We expect that an increase in some of these beneficial agents in the treatment of mitochondrial dysfunction-related organ damage may prevent some neurodegenerative conditions because these mitochondria-targeted antioxidants were effective in preventing mitochondrial dysfunction and nitroxidative tissue injuries in various disease models.[37,60,61] Lastly, these findings may lead to the development of new herbal medications for neurological disorders, including neurodegenerative disease and psychiatric disorders.

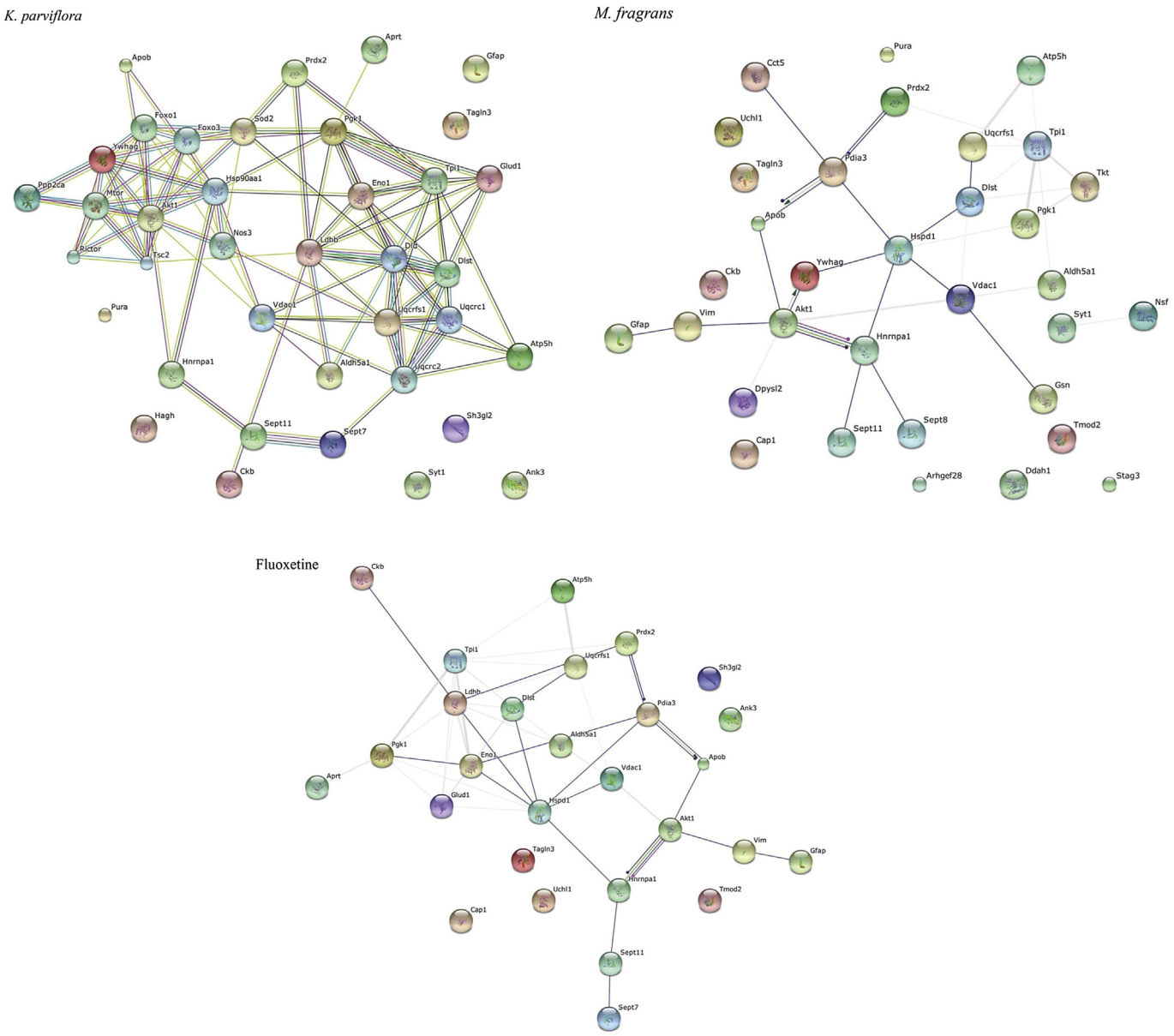

Fig. 7. STRING analysis of fluoxetine-, *K. parviflora*- and *M. fragrans*-modulated proteins in the rat hippocampus. Different colored lines represent the types of evidence for the association.

5. Conclusion

In summary, we revealed that *K. parviflora*, *M. fragrans* and fluoxetine increase the level of serotonin (5-HT), norepinephrine, and dopamine in the rat hippocampus. The proteomic analysis data revealed that 37 proteins were up-regulated, while 14 were down-regulated in the *K. parviflora* group. In the *M. fragrans* group, 27 proteins were up-regulated, while 16 were down-regulated. In the fluoxetine group, we found that 29 proteins were up-regulated, and 14 were down-regulated. The levels of GFAP, PDIA3, DPYSL2 and p-DPYSL2 were up- and down-regulated in the hippocampus of *K. parviflora*-, *M. fragrans*- and fluoxetine-treated SD rats. The data suggest that *K. parviflora* and *M. fragrans* can target and regulate multiple pathways involved in the underlying molecular therapeutic mechanisms; specifically, they can change the expression levels of GFAP, PDIA3, DPYSL2 and p-DPYSL2. In this study, *K. parviflora* and *M. fragrans* are shown to exert a synergistic therapeutic effect on the prevention and treatment of neurodegenerative diseases.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jtcme.2017.01.002.

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