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Natural Products as an Emerging Therapeutic Alternative in the Treatment of Neurological Disorders

Lead Guest Editor: Nasiara Karim
Guest Editors: Heba Abdelhalim, Navnath Gavande, Imran Khan, and Haroon Khan

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Editorial

Natural Products as an Emerging Therapeutic Alternative in the Treatment of Neurological Disorders

Nasiara Karim,[1] **Heba Abdelhalim**,[2] **Navnath Gavande**,[3] **Imran Khan**,[4] **and Haroon Khan**[5]

[1]*Department of Pharmacy, University of Malakand, Khyber Pakhtunkhwa, Pakistan*
[2]*Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan*
[3]*School of Medicine, Indiana University, Bloomington, IN, USA*
[4]*Department of Pharmacy, University of Swabi, Khyber Pakhtunkhwa, Pakistan*
[5]*Department of Pharmacy, Abdul Wali Khan University, Mardan, Khyber Pakhtunkhwa, Pakistan*

Correspondence should be addressed to Nasiara Karim; nasiara.karim@hotmail.com

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Neurological disorders are common and represent a major public health problem. Neurological disorders include dementia, epilepsy, headache disorders, multiple sclerosis, and neuroinfections, neurological disorders associated with malnutrition, pain associated with neurological disorders, Parkinson's disease, stroke, and traumatic brain injuries. There are approximately 450 million of world population suffering from these mental disorders [1]. For example, 50 million people have epilepsy and this number is increasing day by day [2]. The number of people suffering from dementia and memory disorders is projected to be doubled every 20 years. Currently, 322 million people suffer from major depression [3] and this number is on the rise. Neurological disorders constitute over 6% of the global burden of disease [4]. This burden is especially high in many low- and middle-income countries.

Considerable efforts have been made in recent decades to discover substances which can help prevent these serious neurological disorders. Natural products are small molecules found in divergent natural sources. They possess a coveted position in the treatment of all human illnesses including neurological disorders. They are believed to be the single most important source of drug leads [5]. The importance of plant derived natural products for the treatment of neurological disorders is evident by the fact that most of the earlier drugs used for the treatment of neurological disorders were derived from plants including opioids alkaloids [6], galantamine [7], and anticholinesterases like physostigmine and neostigmine [8]. During the last decade out of total 26 natural drugs approved, 7 were for the treatment of neurological disorders, out of which 3 were for Parkinson's disease [9].

This special issue focuses on original contributions for natural products being useful in various neurological disorders including anxiety, depression, stroke, epilepsy, and other CNS disorders and their possible mechanisms of action.

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Nasiara Karim
Heba Abdelhalim
Navnath Gavande
Imran Khan
Haroon Khan

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Research Article

Cannabis Essential Oil: A Preliminary Study for the Evaluation of the Brain Effects

Nadia Gulluni,[1] **Tania Re**,[1,2] **Idalba Loiacono**,[1] **Giovanni Lanzo**,[3] **Luigi Gori**,[1,4] **Claudio Macchi**,[5] **Francesco Epifani**,[6] **Nicola Bragazzi**,[2] **and Fabio Firenzuoli**[1,3]

[1]*Referring Center for Phytotherapy (CERFIT), Region of Tuscany, Florence, Italy*
[2]*Unesco Professorship "Antropologia della Salute-Biosfera e Sistemi di Cura", University of Genoa, Genoa, Italy*
[3]*Center for Integrative Medicine, Careggi University Hospital, Florence, Italy*
[4]*Azienda USL 11, Empoli, Italy*
[5]*Center IRCCS Don Carlo Gnocchi, Florence, Italy*
[6]*Synlab, via della Querciola 12, Sesto Fiorentino, Italy*

Correspondence should be addressed to Tania Re; tania.re77@gmail.com

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We examined the effects of essential oil from legal (THC <0.2% w/v) hemp variety on the nervous system in 5 healthy volunteers. GC/EIMS and GC/FID analysis of the EO showed that the main components were myrcene and β-caryophyllene. The experiment consisted of measuring autonomic nervous system (ANS) parameters; evaluations of the mood state; and electroencephalography (EEG) recording before treatment, during treatment, and after hemp inhalation periods as compared with control conditions. The results revealed decreased diastolic blood pressure, increased heart rate, and significant increased skin temperature. The subjects described themselves as more energetic, relaxed, and calm. The analysis EEG showed a significant increase in the mean frequency of alpha (8–13 Hz) and significant decreased mean frequency and relative power of beta 2 (18,5–30 Hz) waves. Moreover, an increased power, relative power, and amplitude of theta (4–8 Hz) and alpha brain waves activities and an increment in the delta wave (0,5–4 Hz) power and relative power was recorded in the posterior region of the brain. These results suggest that the brain wave activity and ANS are affected by the inhalation of the EO of *Cannabis sativa* suggesting a neuromodular activity in cases of stress, depression, and anxiety.

1. Introduction

The European Pharmacopoeia, sixth Edition (2007), lists 28 essential oils (EOs) [1]. These oils are employed by inhalation and dermal (percutaneous absorption) and oral ingestion in the form of capsules. Industrial hemp (*Cannabis sativa* L. cultivars) is cultivated for fiber and seed production, but has an incredible number of possible applications as ingredient in the cosmetics industry, as flavouring for beverages (food industry), and in medicine. Several studies have been carried out on the cannabinoid content, resin, and seed oil of *Cannabis sativa* L. cultivars, but few studies have focused on the chemical composition and pharmacology of the essential oil extracted from fresh inflorescences and even fewer studies are concerned with its possible uses [2–6].

The hemp essential oil is a complex mixture of many volatile compounds, mainly monoterpenes, sesquiterpenes, and other terpenoid-like substances [7]. The main chemical components are myrcene, β-caryophyllene, limonene, α-pinene, β-pinene, terpinolene, and α-humulene. The general properties of these substances include antidepressant, relaxant, anxiolytic, sedative, antimicrobial, and antioxidants [8]. Some researchers studied the antibacterial properties of this oil. These findings show that industrial hemp EOs exhibited good antimicrobial activities, with respect to Gram (+) bacteria such as *Enterococcus hirae, Enterococcus faecium,* and *S. salivarius* subsp. *thermophilus* and against clostridia (in this case only *C. sativa* L. varieties Futura) [9]. The study of Russo describes pharmacological properties of main terpenoids present in industrial hemp EOs [8].

In the research by Bahia et al., they reported that β-caryophyllene may be useful in treating anxiety and depression. Moreover they demonstrated the effect of β-caryophyllene and its underlying mechanism in a CB2 receptor-dependent manner in mice [10]. These β-caryophyllene's properties may explain why Cannabis users often cite relief of anxiety and depression as reason for their use. But, presently, the effects of hemp EOs inhalation on the brain in humans have not been studied and there are no studies on the possible therapeutic use. However, these studies support that hemp EOs inhalation can have a relaxing effect on the nervous system. Thus, this study is the first to focus on aspects such as brain wave activity and ANS parameters such as heart rate, blood pressure, respiratory rate, and skin temperature, as well as the assessment of mood states through comparative measures. Moreover gas chromatography characterization of hemp EOs was conducted.

2. Materials and Methods

2.1. Essential Oil Analysis. Hexane used for preparing working solution was purchased from Carlo Erba (Rodano, MI, Italy), while the linear n-hydrocarbons (C9–C40) were purchased from Sigma-Aldrich.

The EO used in this study is an extract of *Cannabis sativa L.* (Cannabaceae; *hemp*) purchased from Assocanapa Association (Carmagnola, TO, Italy). The EO was obtained from fresh leaves and inflorescences of *Cannabis sativa L.* were collected and steam distilled by Assocanapa Association, as given by the Italian Official Pharmacopoeia (2,5 L water distilled, 2 h in Clevenger-type apparatus). The Cannabis EO (CEO) yield was 0,11% v/w. CEO and EOs dilutions were stored at 4°C before use. Gas chromatography/electronic ionization mass spectrometry (GC/EIMS) and gas chromatography/Flame Ionization Detector (GC/FID) analyses were accomplished using an HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 μm × 0.25 mm, 0.25 μm film thickness), detector dual FID, working with the following temperature programme: 60°C for 10 min, ramp of 5°C/min to 220°C; injector and detector temperatures 250°C; carrier gas nitrogen (2 ml/min); detector dual FID; split ratio 1 : 30; injection of 0.5 μl. For both columns, components were identified by comparing their retention times with those of pure authentic samples and by means of their linear retention indices (l.r.i.) [11, 12] relative to the series of n-hydrocarbons. The relative proportions of the EO constituents were percentages obtained by FID peak-area normalization, all relative response factors being taken as one. GC/EIMS analyses were performed with Varian CP-3800 gas chromatograph (Variant, Inc. Palo Alto CA) equipped with a DB-5 capillary column (Agilent Technologies Hewlett-Packard, Waldbronn, Germany, 30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperatures 220 and 240°C, respectively; oven temperature programmed from 60 to 240°C at 3°C/min; carrier gas helium at 1 ml/min; injection of 0.2 μl (10% hexane solution); split ratio 1 : 30. Identification of constituents was based on comparison of the retention times with those of authentic samples, comparing their l.r.i. relative to the series of n-hydrocarbons (C9–C40), and on computer matching against commercial (NIST 98 and ADAMS 95) and home-made library mass spectra built up from pure substances and components of known oils and MS literature data [13, 14].

The percentage compositions were computed from the GC peak areas. Moreover, the molecular weights of the all identified substances were confirmed by GC/CIMS, using MeOH as CI ionizing gas. Analysis of the essential oils identified 35 constituents (Table 1), accounting for 97.6% of the total oils (only compounds >0,1% are reported in Table 1). Monoterpene hydrocarbons represented the 57.2% of total volatiles and myrcene was the main constituent (22.9%). Sesquiterpenes hydrocarbons represented the second class of volatiles (34.3%) with the most abundant being β-caryophyllene (18.7%).

2.2. Subjects. Five healthy volunteers (3 males and 2 females) aged 30 to 57 years (mean age 40,8 ± 12,19 years) and with body mass index (BMI) between 19,05 and 34,60 kg/m^2 (mean BMI 26,986 ± 7,18 kg/m^2) participated in this study. Demographic data of the participants is presented in Table 2. Only five volunteers were available for the experimental session preprogrammed; other recording experimental sessions were not made because environmental parameters would not be reproducible and comparable. None of the subjects had cardiovascular disease, they did not exhibit any symptoms of upper respiratory infection, and women were not to be in their menstrual period on the day of the tests. Two subjects were smokers and one of the male subjects had a slight headache at the time of the experiment. All experimental procedures were followed with the strict ethical standards formulated in the Helsinki Declaration of 1964 that was revised in 2000 and all the subjects have participated in the study after signing the informed consent. The study was conducted in private healthcare facilities outside the network of the Regional Health System. Therefore, any ethical and managerial assumption is rooted in the direct relationship between the patient, who has released the relative consent, and the host structure.

2.3. Methods. One milliliter of sweet almond oil (SAO, base oil, purchased from Carlo Erba) was used for control condition as placebo and one milliliter of CEO was administered according to the protocol described in Figure 1. The sweet almond oil was administered with gauze and participants were asked to inhale simultaneously with both nostrils. The same procedure was also adopted for the CEO. In accordance with previous studies, it has been found that the pleasantness of the aroma of the oil could alter autonomic activity [15, 16]. As a result of these facts, the subjects were asked to inhale SAO and CEO to assess the pleasantness or less pleasantness of the aroma.

2.4. ANS and Mood State Measurement. The mood state and ANS parameters of the subjects such as blood pressure, heart rate, skin temperature, and respiratory rate were recorded simultaneously. The ANS parameters were measured manually. While the evaluation of the changes of moods was made

Table 1: GC-MS results of the essential oil extracted from hemp inflorescences (*Cannabis sativa* L. var. monoica).

Constituents	l.r.i.^	Percentage
α-Pinene	941	7,7
Camphene	955	0,2
Sabinene	978	0,2
β-Pinene	982	3,7
Myrcene	993	22,9
α-Phellandrene	1007	0,3
δ-3-Carene	1010	0,6
α-Terpinene	1020	0,3
p-Cymene	1028	0,5
Limonene	1033	3,9
1,8-Cineole	1035	0,2
(Z)-β-Ocimene	1042	0,7
(E)-β-Ocimene	1053	3,9
γ-Terpinene	1063	0,3
Terpinolene	1090	12,0
Linalool	1101	0,3
p-Cymen-8-ol	1184	0,5
α-Terpineol	1192	0,2
Carvacrol	1301	0,2
(Z)-Caryophyllene	1406	0,7
β-Caryophyllene	1419	18,7
trans-α-Bergamotene	1438	1,5
α-Humulene	1455	6,2
9-*epi*-Caryophyllene	1468	2,3
γ-Muurolene	1478	0,2
β-Selinene	1487	1,6
α-Selinene	1495	1,5
β-Bisabolene	1508	0,4
trans-γ-Cadinene	1514	0,2
δ-Cadinene	1524	0,2
Selina-3,7(11)-diene	1544	0,6
Germacrene B	1557	0,2
Caryophyllene oxide	1582	3,7
Humulene oxide II	1607	1,0
Monoterpene hydrocarbons		57,2
Oxygenated monoterpenes		1,4
Sesquiterpene hydrocarbons		34,3
Oxygenated sesquiterpenes		4,7
Total identified		*97,6*

Percentages are obtained by FID peak-area normalization, all relative response factors being take as one (HP-5) column. Mean of three analyses. ^Linear retention indices (HP-5 column) relative to the series of n-hydrocarbons.

through a subjective evaluation of the participants using a list of 8 terms selected for their relevance to describe affective feelings induced by odors and mood state after inhalation of CEO: anxiety, calm, hungry, hilarity, fatigue, apathy, energy, and heavy eye [17–19].

2.5. EEG Recording. A set of 21 electrodes with an additional ground electrode and a reference electrode were placed onto the subject's head with electrocap in accordance with the international 10–20 system at F1, F2, F7, F3, Fz, F4, F8, T3, C3, Cz, C4, T4, T5, P3, Pz, P4, T6, O1, and O2. Mizar 40 EBNeuro-Firenze was used as the recording system. Sampling rate was set at 512 Hz; the HF filter was set at 70 Hz; time constant 0,3; range −/+4,1 mV. The relative power spectrum of the respective frequency bands was expressed as follows: delta (0,5–4 Hz), theta (4–8 Hz), alpha (8–14 Hz), and beta (14–30 Hz). Furthermore, the beta wave was further categorized as beta 1 or low frequency beta (14–18 Hz) and beta 2 or high frequency beta (18,5–30 Hz) activities.

2.6. Experimental Protocol/Procedure. All the steps in this experiment were similarly conducted as the previous study recorded on the effects of rosemary oil inhalation [20]. All activities were conducted in a quiet room and the subjects were seated in a comfortable chair. The ANS electrodes were attached to the appropriate positions. The ANS parameters: heart rate, skin temperature, respiratory rate, and systolic and diastolic blood pressure were recorded at the beginning of the experiment before resting (baseline) EEG measurement and after CEO inhalation.

The experiment consisted of three trials: first session served as a baseline (resting period) and took ten minutes. The second and the third sessions took five minutes each.

The first session baseline EEG recording was done with both eyes opened and eyes closed, respectively. In the second session, SAO was inhaled by the subjects. In the third session, CEO was applied and mood state was measured after inhalation. EEG was recorded for five minutes during SAO inhalation and after five minutes of rest it was recorded again for five minutes during CEO inhalation. After the recordings, the subjects were asked to give their preference and impression of the odors presented and of their moods (Figure 1).

2.7. Statistical Analyses. The MedCalc statistics for biomedical research software version 16.2.1 was done for data analysis of the effects of CEO on physiological reactions and mood states, before and after the hemp inhalation. A nonparametric Kruskal Wallis signed rank test was used for EEG data analysis and Friedman test was performed to determine whether the activity changed significantly in any of frequency bands in P4-O2 and P3-O1 brain region. A paired *t*-test was carried out on data concerning the subjects' blood pressures, heart rates, skin temperatures, and respiratory rates. A p value $< 0,05$ was considered significant. A percentage evaluation was done for the mood states.

3. Results and Discussion

In the present research, CEO was administered by inhalation to healthy subjects and we examined the effects of the oil on the human nervous system. Brain wave activity and ANS parameters (blood pressure, heart rate, respiratory rate, and skin temperature) were recorded as the indicators of the arousal level of the nervous system. In addition, we studied the effects of CEO on moods by performing subjective self-evaluation in order to assess the arousal levels.

Table 2: Demographic data for subjects.

Parameters	Subjects (*M, *F)					Minimum	Maximum	Mean	SD
	M1	M2	M3	F1	F2				
Age	57	30	50	30	37	30	57	40,8	12,19
Weight (Kg)	90	100	95	55	50	50	100	78	24,04
Height (cm)	176	170	172	165	162	162	176	169	5,56
BMI (Kg/m^2)	29,05	34,6	32,11	20,2	19,05	19,05	34,6	26,996	7,18

*M: Male; *F: Female.

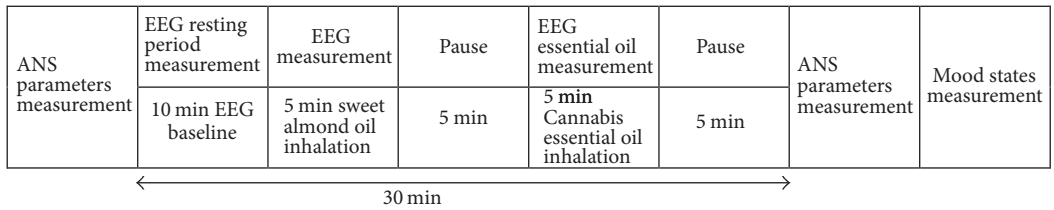

Figure 1: Experimental protocol of EEG, ANS, and mood states measurements divided into 8 blocks: ANS parameters recording; EEG recording in baseline condition (10 min), EEG in placebo condition (1 ml sweet almond oil inhalation, 5 min); pause (5 min); EEG *Cannabis* EO inhalation (1 ml, 5 min); pause (5 min); ANS parameters recording; and finally mood states measurement.

3.1. Autonomics Nervous System Parameters. Inhalation of CEO has been correlated with changes in ANS parameters and skin temperature significantly increased ($p < 0,05$). The data of various ANS parameters were compared during rest (control) and CEO inhalation as shown in Table 3. In 60% of subjects heart rate had increased during CEO exposure. In contrast, diastolic blood pressure had decreased in 80% of subjects. But these data did not reach statistical change. These changes of the ANS parameters indicated an involvement of the autonomic nervous system and parasympathetic nervous system (PNS). The stimulatory effects on the ANS and PNS may be explained through the presence of monoterpenes (limonene and α-pinene) that are present in CEO.

The α-pinene inhibits acetylcholinesterase [21], which results in the activation of the PNS, and this might be responsible for the reduction in diastolic blood pressure. While the stimulatory effects on the sympathetic system determined by limonene might be responsible for the increase in heart rate and skin temperature [22].

3.2. Emotional Parameters. All subjects found the CEO pleasant. The alterations of mood states after exposure to CEO are shown in Table 4. The subjects felt more calm, relaxed, and energetic, were in a good mood, and had increased feeling of hunger and the subject with headache had no more pain. These results indicate that CEO inhalation increases the level of relaxation and general well-being as assessed through our test subjects' self-evaluation. This relaxing and anxiolytic effect on ANS could be explained by the abundance of limonene, myrcene, and β-caryophyllene, main components of the EO. Several studies in animals and humans suggest that the limonene can be a powerful anxiolytic agent via 5-HT. Also, limonene demonstrated antistress effects on the brain of rats. Bahia and colleagues found that b-caryophyllene has an anxiolytic and antidepressant activity in a CB2 receptor-dependent manner [10, 22].

Myrcene, the main component of CEO, has a sedative, analgesic, and relaxing activity [23, 24]. Thus, these results confirm that CEO contains mood-elevating bioactive components that prove to be beneficial to its users.

3.3. EEG Parameters. EEG spectral analysis was done with quantitative evaluation of windows on 2 seconds with Interpolation Algorithm Rectangular.

Five frequency bands were evaluated (delta, theta, alpha, beta 1, and beta 2) and values of power (μV^2), amplitude (μV), relative power (μV^2), and mean frequency were calculated during rest, during SAO inhalation, and during CEO inhalation states. The studied areas were divided into the right posterior area (P4-O2) and left posterior area (P3-O1) brain regions. The data recording shows an alteration of EEG during exposure to the CEO. There were noticeable changes of band power, amplitude, and relative power in alpha, theta, delta, and beta waves as reported in Tables 5, 6, 7, and 8 (mean and median value).

During the CEO inhalation the power, the relative power, and amplitude of the alpha waves in both brain regions were increased and mean frequency for alpha significantly increased (compared with SAO) in P4-O2 brain area ($p < 0,05$). The power and relative power changes in the theta and delta waves in the left posterior brain region were also increased. In contrast, the power in the delta wave in the right posterior brain region was decreased. A significant decrease was observed in the case of the beta 2 wave's relative power (compared with resting condition) and mean frequency (compared with SAO) in P4-O2 ($p < 0,05$), Figures 2 and 3. The present research shows the effects of CEO inhalation on brain waves. This research showed that alpha (8–13 Hz) and theta (4–8 Hz) activity increased during CEO exposure in the posterior regions, and mostly left posterior area P3-O1 brain regions. Moreover, alpha mean frequency increased significantly in P4-O2 region. These results show

Table 3: In detail the values, mean, and standard deviation of ANS parameters for resting condition and after Cannabis oil inhalation.

Subjects (*M, *F)	Age	Demographic data			ANS parameters									
					Resting					Cannabis essential oil				
		Weight (kg)	Height (cm)	BMI (kg/m)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Heart rate (bmp)	Respiratory rate (bpm)	Skin temperature (°C)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Heart rate (bmp)	Respiratory rate (bmp)	Skin temperature (°C)
M1	57	90	176	29,5	140	90	88	14	36,5	150	95	88	13	36,5
M2	30	100	170	34,6	120	80	66	18	36,3	115	75	74	14	36,7
F1	30	55	165	20,2	88	65	72	14	36,6	85	60	72	15	36,8
M3	50	95	172	32,11	125	95	62	15	36,2	120	80	75	15	36,4
F2	37	50	162	19,0	110	70	88	18	36	108	65	94	20	36,4
MEAN	40,8	78	169	26,9	116,6	80	75,2	15,8	36,3	115,6	75	80,6	15,4	*35,6*
SD (±)	12,2	24,0	5,6	7,2	19,3	12,7	12,2	2,1	0,24	23,4	13,7	9,8	2,7	*0,18**

* $p < 0,05$ significance when compared to resting condition; *M: Male; *F: Female.

Table 4: Percentages of emotional states scores after cannabis essential oil inhalation.

Subjects (*M, *F)	Demographic date				Emotional states (self-evaluation)							
	Age	Weight (kg)	Height (cm)	BMI (kg/m)	Anxiety	Calm	Hunger	Hilarity	Heaviness eye	Tiredness	Apathy	Energy
M1	57	90	176	29,5	Decreased	-	Increased	Increased	-	-	Increased	*Increased*
M2	30	100	170	34,6	Decreased	Increased	Increased	-	Increased	-	-	Decreased
F1	30	55	165	20,2	-	Increased	Increased	-	Increased	-	Increased	Decreased
M3	50	95	172	32,1	-	Increased	Increased	-	Increased	-	-	Decreased
F2	37	50	162	19,0	-	Increased	-	Increased	-	-	-	*Increased*
%					40%	80%	80%	40%	60%	-	40%	60%/*40%*

*M: Male; *F: Female.

Table 5: Mean and median power values and p value for resting condition, sweet almond oil inhalation, and cannabis essential oil inhalation.

Brain area	Resting	Sweet almond oil	Cannabis essential oil	p value
Theta power (μV^2)				
P4-O2	3,286 (2,77)	3,704 (2,75)	3,366 (3,36)	0,268
P3-O1	3,166 (3,19)	3,522 (3,16)	7,776 (5,21)	0,497
Alpha power (μV^2)				
P4-O2	41,266 (38,95)	48,592 (39,41)	58,788 (40,17)	0,599
P3-O1	46,698 (48,45)	36,8 (30,88)	51,036 (51,26)	0,268
Delta power (μV^2)				
P4-O2	10,734 (4,68)	18,96 (12,23)	15,36 (15,72)	0,167
P3-O1	8,768 (8,4)	13,756 (8,14)	19,542 (17,09)	0,268
Beta 1 power (μV^2)				
P4-O2	5,816 (4,61)	8,778 (5,53)	9,05 (7,12)	0,849
P3-O1	6,402 (8,19)	8,01 (7,61)	8,268 (8,4)	0,849
Beta 2 power (μV^2)				
P4-O2	3,086 (4,05)	2,47 (3,08)	2,83 (3,1)	0,268
P3-O1	2,714 (2,64)	2,918 (2,3)	3,734 (4,14)	0,849

SAO: sweet almond oil; CEO: cannabis essential oil.

Table 6: Mean and median relative power values and p value for resting condition, sweet almond oil inhalation, and cannabis essential oil inhalation.

Brain area	Resting	Sweet almond oil	Cannabis essential oil	p value
Theta PotR				
P4-O2	5,082 (4,06)	5,048 (4,61)	4,642 (3,78)	0,268
P3-O1	5,01 (5,53)	5,386 (5,67)	7,248 (6,32)	0,497
Alpha PotR				
P4-O2	65,098 (69,39)	58,608 (60,74)	60,042 (63,23)	0,497
P3-O1	67,488 (68,22)	55,212 (56,46)	55,746 (50)	0,268
Delta PotR				
P4-O2	15,64 (14,32)	22,254 (24,25)	21,284 (25,16)	0,497
P3-O1	13,234 (13,36)	21,606 (20,48)	23,34 (25,39)	0,497
Beta 1 PotR				
P4-O2	8,224 (8,4)	9,444 (7,98)	9,09 (9,89)	1,00
P3-O1	9,106 (7,57)	12,054 (8,59)	8,318 (9,4)	0,849
Beta 2 PotR				
P4-O2	4,482 (2,97)	3,028 (2,82)	3,432 (2,48)	*0,05308**
P3-O1	3,986 (2,84)	4,226 (3,89)	3,862 (3,54)	0,849

SAO: sweet almond oil; CEO: cannabis essential oil. *CEO differs from resting with p value < 0,05.

concordance with the past EEG studies on the effects of odors which demonstrated increased alpha activity by administration of several EOs such as lavender, chamomile, α-pinene, and limonene oil [25–28]. Instead alpha activity is attenuated under emotional tension and stress condition [29].

The EEG evidence of relaxation can be seen in various practices such as meditation, yoga, Qigong, and mindfulness [30, 31]. The study among people meditating can demonstrate similar EEG changes with CEO inhalation, which presented as increase in theta and alpha activities in the brain during meditation [32, 33]. In addition, the studies of Aftanas [34–37] show that during meditation there is also release of hormones such as melatonin, serotonin, and cortisol. These results lend support that increases in theta and alpha waves activity cause a range of general relaxation and anxiolytic effects on the brain and also some possible decreases of pain. Thus, the data recorded after CEO inhalation shows relaxation and anxiolytic effects on the brain at level of the ANS, CNS, and mood states. At level of mood states a feeling of calm, relaxation, and decreased anxiety was recorded indicating the involvement of the limbic system.

The changes in ANS parameters (heart frequency, skin temperature, and diastolic blood pressure) can be explained by the α-pinene activity on the parasympathetic system

Table 7: Mean and median amplitude power values and p value for resting condition, sweet almond oil inhalation, and cannabis essential oil inhalation.

Brain area	Resting	Sweet almond oil	Cannabis essential oil	p value
Theta Amp				
P4-O2	3,11 (2,89)	3,336 (3,06)	3,142 (3,12)	0,268
P3-O1	3,082 (3,13)	3,234 (3,18)	4,066 (3,99)	0,497
Alpha Amp				
P4-O2	10,574 (10,06)	10,104 (9,63)	11,204 (10,07)	0,599
P3-O1	10,614 (10,37)	8,88 (8,4)	10,872 (11,65)	0,073
Delta Amp				
P4-O2	4,312 (3,63)	5,564 (5,25)	5,45 (5,55)	0,073
P3-O1	4,148 (4,4)	5,246 (4,43)	6,06 (5,53)	0,167
Beta 1 Amp				
P4-O2	4,946 (4,76)	5,32 (5,2)	5,548 (5,87)	0,599
P3-O1	5,06 (5,85)	5,37 (6,07)	5,592 (6,36)	0,849
Beta 2 Amp				
P4-O2	3,526 (4,14)	3,296 (3,76)	3,458 (3,72)	0,268
P3-O1	3,408 (3,56)	3,432 (3,56)	3,838 (4,28)	0,958

SAO: sweet almond oil; CEO: cannabis essential oil.

Table 8: Mean and median mean frequency values and p value for resting condition, sweet almond oil inhalation, and cannabis essential oil inhalation.

Brain area	Resting	Sweet almond oil	Cannabis essential oil	p value
Theta Mean$F$				
P4-O2	5,854 (5,83)	5,772 (5,78)	5,732 (5,84)	0,497
P3-O1	5,906 (5,930)	5,848 (5,89)	5,82 (5,94)	0,849
Alpha Mean$F$				
P4-O2	9,982 (9,99)	10,274 (10,28)	10,094 (10,04)	*0,00066**
P3-O1	9,984 (9,96)	10,178 (10,13)	10,026 (9,84)	0,268
Delta Mean$F$				
P4-O2	1,572 (1,61)	1,464 (1,41)	1,474 (1,3)	0,849
P3-O1	1,452 (1,46)	1,428 (1,48)	1,508 (1,38)	0,958
Beta 1 Mean$F$				
P4-O2	14,182 (14,3)	13,89 (14,16)	14,092 (14,58)	0,167
P3-O1	14,026 (14,19)	14,082 (14,34)	14,256 (14,52)	0,599
Beta 2 Mean$F$				
P4-O2	20,026 (20,07)	20,592 (20,57)	20,266 (20,31)	*0,00332**
P3-O1	20,34 (20,55)	20,348 (20,54)	19,982 (19,91)	0,0731

SAO: sweet almond oil; CEO: cannabis essential oil. * CEO differs from SAO with p value < 0,05.

and limonene activity on the sympathetic system action. Komiya et al. [22] found that limonene increases serotonin in the prefrontal cortex, and dopamine (DA) in hippocampus mediated via 5-HT1A. This determines the direct activation of the sympathetic system. At levels of CNS activity, alpha and theta waves increased indicating a relaxing effect and antidepressant and antianxiety effect due to the β-caryophyllene and limonene. The analgesic action of CEO on the subject with headache may be explained by increase of alpha and theta waves and abundance of terpenes such as myrcene, limonene, and β- caryophyllene.

4. Conclusions

The small study population is a limitation of this study but it is however a preliminary study. Further studies of the effect of CEO on the brain are needed with a wider sample in order to have a greater number of significant data. However, the

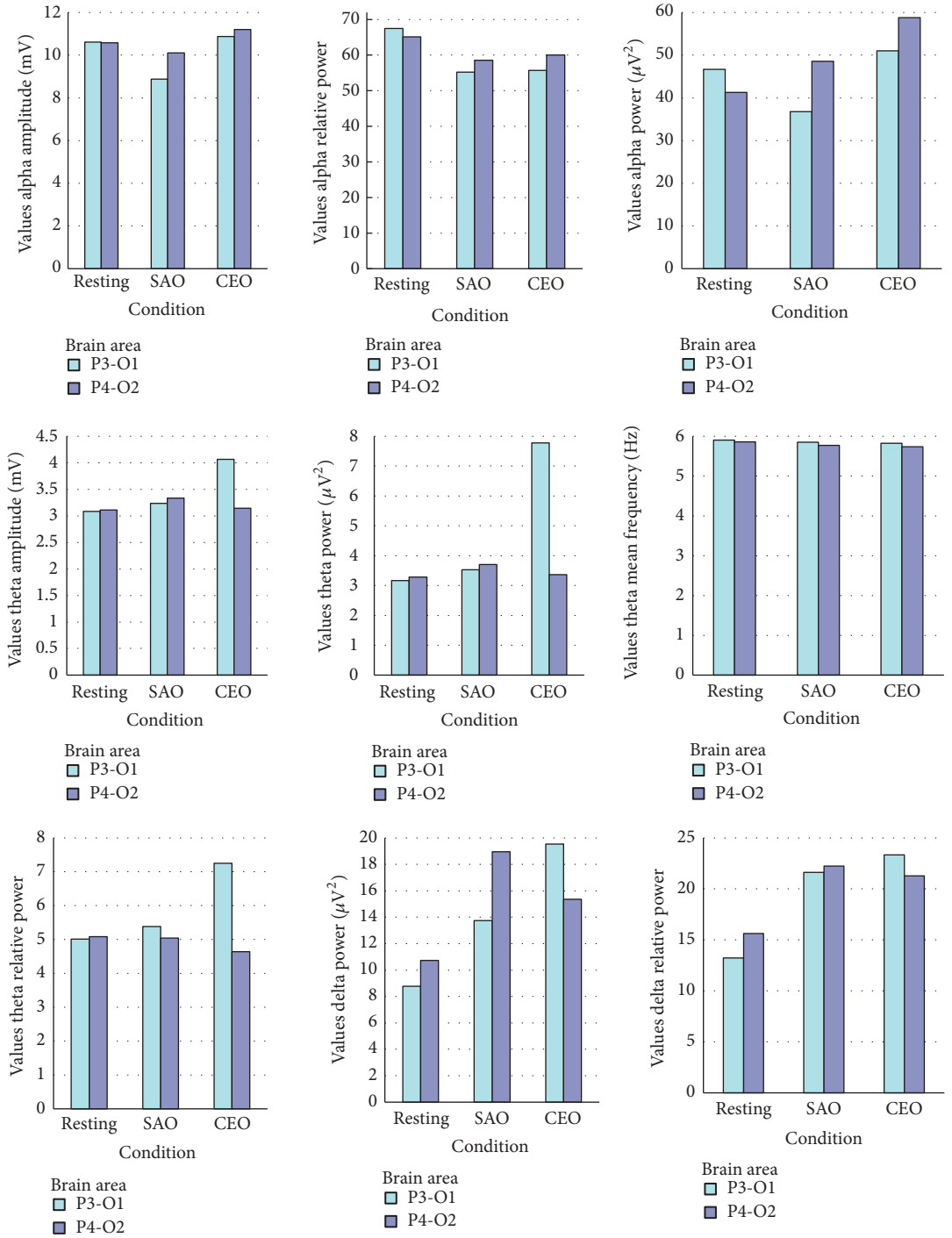

Figure 2: Each panel shows the mean of power, relative power, mean frequency, and amplitude values of alpha and theta activities for the resting and sweet almond oil (SAO) and cannabis essential oil (CEO) inhalation states. The theta and delta waves increased mostly in P3-O1.

results lend some support for including CEO in a perspective integrated therapy aimed at relieving stress or depression.

The results suggest the occurrence of the positive relaxation and anxiolytic effects of CEO. These findings provide evidence that brain wave activity autonomic nervous system response and mood states were affected by CEO.

Abbreviations

ANS: Autonomic nervous system
CNS: Central nervous system
CB2: Cannabinoid receptor type 2
EEG: Electroencephalography
EO: Essential oil

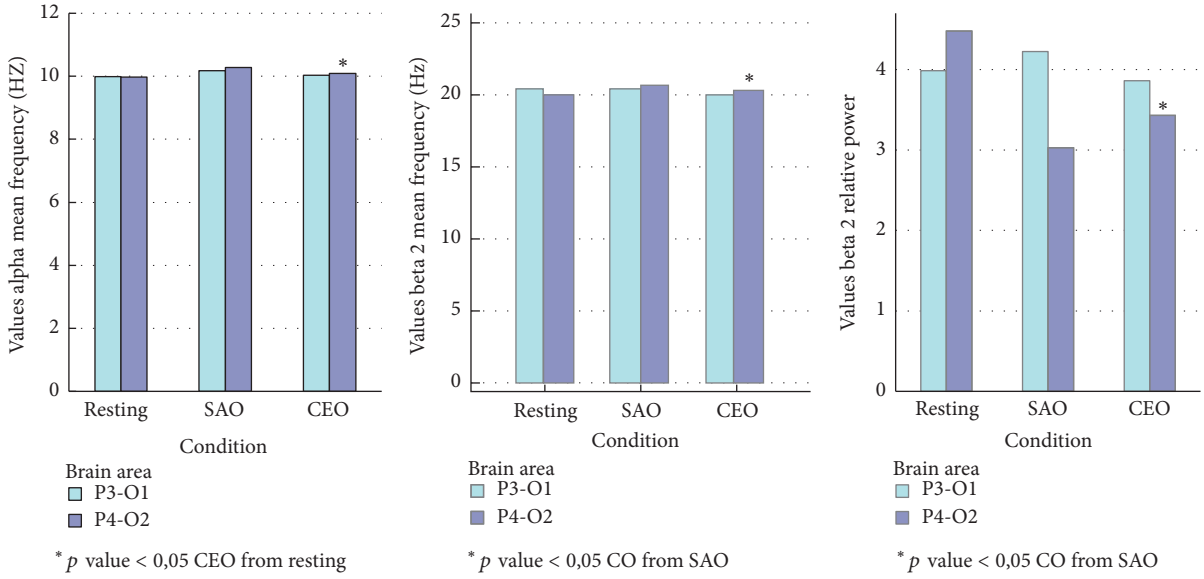

Figure 3: Values of mean frequency and power relative to alpha and beta 2 with significant difference in P4-O2 area of brain.

CEO: Cannabis essential oil
SAO: Sweet almond oil
GC/FID: Flame Ionization Detector
GC/EIMS: Gas chromatography electronic ionization mass spectrometry
PNS: Parasympathetic nervous system
THC: Tetrahydrocannabinol.

Conflicts of Interest

All authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Antidepressant-Like Effect of Selected Egyptian Cultivars of Flaxseed Oil on a Rodent Model of Postpartum Depression

Nebal El Tanbouly,[1] Abeer Mohamed El Sayed,[1] Zeinab Y. Ali,[2] Samia Abdel Wahab,[1] Sabah H. El Gayed,[1] Shahira M. Ezzat,[1,3] Amira Safwat El Senousy,[1] Mouchira A. Choucry,[1] and Essam Abdel-Sattar[1]

[1]*Department of Pharmacognosy, College of Pharmacy, Cairo University, Cairo 11562, Egypt*
[2]*Department of Biochemistry, National Organization for Drug Control and Research (NODCAR), Giza 12553, Egypt*
[3]*Department of Pharmacognosy, College of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th of October 12566, Egypt*

Correspondence should be addressed to Essam Abdel-Sattar; abdelsattar@yahoo.com

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Flaxseed (*Linum usitatissimum* L.) is a multipurpose crop with health promoting potential. This study was undertaken to investigate the fatty acid profile and yield of fixed oil of six Egyptian flaxseed cultivars. The selected cultivars with the highest content of polyunsaturated fatty acids (PUFAs) (G9 and G10) were assessed for their antidepressant-like effect in rat model of postpartum depression (PPD) induced by hormone-simulated pregnancy followed by hormone withdrawal and compared to fluoxetine. As compared to control group, administration of G9 and G10 (270 mg/kg/day, p.o) for two weeks during the postpartum period can alleviate anxiety and depressive-like behaviors and biochemical changes in PPD-induced rats. This was confirmed by evaluation of anxiety-like behaviors (elevated plus maze, open field test, and forced swim test tests), in addition to biochemical analysis (brain monoamine oxidase-A, corticosterone level, proinflammatory cytokines, and hippocampal redox state). In conclusion, flaxseed oil of Egyptian cultivars G9 and G10 exhibited significant antidepressant-like effect in rat model of PPD without affecting locomotor activity. At the treatment doses, the antidepressant-like activity of Giza 9 oil is comparable to fluoxetine.

1. Introduction

According to World Health Organization [1], the postnatal period is a very difficult transition phase for a mother, where she has to face a lot of changes, physically and emotionally. It is a severe condition which may has serious consequences for the whole family and not only the mother or her child. WHO recommended guidelines focused on the six weeks after delivery care including hemorrhage, infections, anemia, and depression [2]. WHO are updating their guidelines on regular basis, and the next update will be in 2018. Postpartum depression (PPD) is one of the major depressive disorders that occurs within the first month after childbirth [2].

Postpartum depression (PPD) occurs in 10–15% of childbearing women [3]. It causes significant morbidity in postpartum women and their newborns. PPD is accompanied by headache, exhaustion, mood swings, irritability, anxiety, and anhedonia [4], and in severe cases the mother can harm herself or her baby [5, 6].

Weigelt et al. (2013) reported a hypothesis based on both experimental and clinical evidences that the immune dysregulation is involved in the development of postpartum depression [7]. It was observed that the level of anti-inflammatory cytokines responsible for suppression of immune function was elevated, where the proinflammatory cytokines such as tumor necrosis factor- (TNF-) α and interleukin- (IL-) 6 are downregulated. Upon delivery the state of the immune system was reversed into a higher level of proinflammatory cytokines, which lasts for several weeks. It is commonly known that higher levels of tumor necrosis factor- (TNF-) α and interleukin- (IL-) 6 were reported in patients with depression [8]. Inconsistent results and controversial studies

failed to confirm the relationship between immune dysregulation and PPD and a better understanding of the pathophysiology of PPD is needed [9, 10]; thus, the role of immune function in PPD needs further studies [11].

Nutritional status also plays a role in mental health and nutritional intervention may improve the postpartum depression. The supplementation with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) rich products in such conditions is of great value, because ω-3 PUFAs aids in membrane integrity and fluidity, which is pivotal for neurotransmitter binding and signaling within the cell, which are dysfunctional in depressive patients [12]. The proinflammatory markers such as IL-1β, IL-2, IL-6, interferon-γ, and TNF-α are expressed in the PPD model of rats. A number of studies have shown a relationship between these cytokines and the development of depression through alteration of the metabolism of neurotransmitters and neurotransmitter transporter mRNAj or their precursors availability [13, 14].

Linum usitatissimum L. (Linaceae), commonly known as flaxseed or linseed, is known to be the richest plant source of α-linolenic acid (ALA), attaining up to 55% of the total fatty acids [15]. A portion of ALA converted in the body to eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), though the actual converted percentage may differ between men and women [16]. The human body requires a variety of ω-3 PUFAs. Since ALA is a precursor to EPA and DHA, so flaxseed oil can be considered as an alternative cheap omega-3 source compared to fish oil, especially for vegetarians.

Among the biological activities of omega-3 PUFAs is their well-documented anti-inflammatory properties, which should be considered to understand the mechanism by which they treat or prevent depression [17, 18]. Despite the belief of importance of omega-3 PUFAs for the nervous system and neuronal dysfunction, the underlying mechanism remains unclear. Numerous studies have shown that deficiency in omega-3 leads to neuronal dysfunction and change in the inflammatory status but results are still inconclusive regarding the association between omega-3 PUFAs and PPD. Su et al. (2015) reported the role of omega-3 polyunsaturated fatty acids in prevention of mood and anxiety disorders such as major depression, bipolar disorders, stress disorder, and other depression related diseases [19].

Therefore, the current study aimed (i) to study the comparative composition of the fatty acid profiles of six selected Egyptian flaxseed cultivars and (ii) to test the hypothesis that the highest cultivars in omega-3 fatty acids G9 and G10 could attenuate the biochemical changes and depression symptoms in rat model of PPD compared to the antidepressant drug fluoxetine (FLX).

2. Materials and Methods

2.1. Plant Material. Samples of *L. usitatissimum* seeds were collected in winter of 2012-2013 from different localities in Egypt, namely, Qalyubia, Sharqia, Gharbia, and Kafr El-Sheikh Governorates, and identified by staff members of Fiber Crops Research Department, Faculty of Agriculture, Giza. Six flax cultivars, namely, Sakha 1 (S1), Sakha 2 (S2), Sakha 3 (S3), Sakha 4 (S4), Giza 9 (G9), and Giza 10 (G10), were used in this study. Specimens of the seeds were deposited in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University (9-11-2015, 1-6).

2.2. Chemical Assessment

2.2.1. Investigation of the Lipoidal Content. One hundred grams of the powdered seeds of each flaxseed cultivar (S1, S2, S3, S4, G9, and G10) was exhaustively extracted by *n*-hexane with the aid of sonication three times each for 30 min with 500 ml *n*-hexane. The extract was evaporated under reduced pressure at a temperature not exceeding 50°C, to yield an oily liquid. An oil sample (2 g) of each cultivar was saponified with 20% ethanolic KOH for four hours and the saponifiable and unsaponifiable fractions were separated [20]. The saponifiable fraction as well as the available authentic samples of fatty acids were methylated with diazomethane [20] and subjected to GC analysis.

2.2.2. Investigation of the Saponifiable Matter (SM). GC-MS of fatty acids: GC/MS analysis was carried out on Hewlett Packard HP 6890 Series Gas Chromatograph System with a HP 5973 Mass Selective Detector, operating with the following parameters: Column TR-FAME (Thermo 260 M142 P) (30 m, 0.25 mm ID, and 0.25 mυ Film) (70% cyanopropyl polysilphphenylene siloxane) capillary column. The carrier gas was helium, flow rate (1.5 ml/min). Split injection was conducted with a split ratio of 10 : 1. Injector temperature was 200°C. Detector temperature was 280°C, with temperature programming as follows: initial temperature 80°C, hold for 2 min, ramp to 230°C at 3°C/min, and hold time for 5 min. The amount of sample injected was about 1 μ l (conc 5 μl sample per 1 ml solvent). The mass spectrometer was operated in electron-impact (EI) mode. Precolumn pressure was 70 kPa. Injection temperature was 250°C. Ion source was EI (200°C). Interface temperature was 280°C. Electron energy was 70 eV. Solvent delay was 5.5 min. For qualitative analysis, the full scan mode was used and the scan range was 40–400 m/z. Qualitative identification of the different constituents was performed by comparison of their relative retention times and mass spectra with those of authentic reference compounds (fatty acid methyl esters, purity 98% by GC). Also, probability merge search software and the NIST MS spectra search program were used. The results of oil yield and fatty acid profile of the investigated oil sample are presented in Table 1.

2.3. In Vivo Assessment of Antidepressant Potential of Selected Cultivars of Flaxseed Oil in a Rat Model of Postpartum Depression

2.3.1. Animals. A total of 40 adult Sprague-Dawley female rats (3 months old) weighing 160 ± 10 g were provided from the animal house of National Organization for Drug Control and Research (NODCAR), Giza, Egypt. They were kept under standard conditions with temperature at 23 ± 2°C and 12/12 hours light/dark cycle and allowed free access to normal food and water throughout the experiment. The experimental

Table 1: Results of GC analysis of fatty acid methyl esters of different oil samples of flaxseeds and their percentage of oil yields.

Peak	Rt*	Comp name	Relative percentage (area%)					
			S1	S2	S3	S4	G9	G10
1	23.71	Palmitic acid methyl ester	5.98	6.47	1.97	6.16	6.44	6.16
2	24.72	Hexadecanoic acid, ethyl ester	1.1	—	5.56	—	—	—
3	28.47	Stearic methyl ester	5.19	4.95	1.76	5.64	4.65	4.95
4	29.07	Oleic acid methyl ester (9*Z*) *ω*-9	20.84	15.93	4.72	13.74	14.72	18.54
5	29.25	9-Octadecanoic acid methyl ester (9*E*)	—	1.01	3.47	—	0.97	—
6	29.90	Ethyl oleate (9*Z*) *ω*-9	1.28	0.76	9.38	1.57	0.48	—
7	30.36	10,13-Octadecadienoic acid methyl ester (10*E*, 13*E*)	11.19	12.70	3.8	13.45	12.96	11.87
8	31.17	Linoleic ethyl ester (9*Z*, 12*Z*) *ω*-6	1.11	0.96	10.52	1.63	0.74	—
9	32.04	*α*-Linolenic acid methyl ester (9*Z*, 12*Z*, 15*Z*) *ω*-3	46.70	51.31	13.47	50.85	53.88	51.74
10	32.69	Linolenic acid ethyl ester *ω*-3	6.61	5.92	45.35	6.96	5.16	6.74
Oil yield (%)			35.8	42.50	33.2	35.1	40.74	34.8

Rt*, retention time in min. S1: Sahka 1, S2: Sakha 2, S3: Sakha 3, S4: Sakha 4, G9: Giza 9, and G10: Giza 10.

design was conducted in accordance with the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication number 85-23, revised 1985). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC, number 9-031), Faculty of Pharmacy, Cairo University.

2.3.2. Induction of PPD in Rats

(1) Surgery. At the beginning of the experiment, rats were subjected to bilaterally ovariectomized (OVX) by using an aseptic technique under anesthesia with thiopental (50 mg/kg i.p.). The OVX rats were allowed to recover for one week following surgery [21].

(2) Hormone-Simulated Pregnancy Regimen. Hormonal withdrawal model represents a valid approach for the study of certain aspects of PPD, as it captures the possible negative impacts of hormonal fluctuations associated with pregnancy and lactation on emotion regulation [22]. In the present study, one week after recovery from surgery, OVX rats were subjected to hormone-simulated pregnancy (HSP) regimen. OVX rats were s.c injected with a low dose of estradiol benzoate (Sigma, St. Louis, MO) (2.5 μg/rat) and a high dose of progesterone (Sigma, St. Louis, MO) (4.0 mg/rat) daily for 16 consecutive days. On days 17–22, a higher dose of estradiol benzoate (50 μg/rat) only was given to mimic the levels observed in the actual rat's pregnancy. Hormone and vehicle treatments ceased after day 22, initiating the hormone withdrawal period. The days after hormone termination, mimicking the postpartum drop in gonadal hormones is considered as the postpartum period [3, 21].

2.3.3. Experimental Design. A total number of 40 adult female rats were divided randomly into 5 equal groups each containing eight rats (Figure 1). *Group 1:* sham-operated animals received vehicle (safflower oil) on the same schedule and served as control. *Group 2:* PPD- induced rats were untreated and served as positive control for two weeks. *Groups 3-4:* PPD-induced rats received flaxseed oil (G9 and G10) at a fixed dose of 270 mg/Kg b.wt/day (equivalent to human dose of 3000 mg/day) for two weeks. *Group 5:* PPD-induced rats received a reference antidepressant drug FLX at a dose of 1.8 mg/Kg b.wt/day (equivalent to human recommended dose of 20 mg/day) for the same period [23].

At the end of the treatment period, all animals were subjected to behavior tests. Blood samples were collected from retro-orbital plexus after overnight fasting. Sera were separated by centrifugation at 640g and 4°C for 15 minutes and stored at −20°C for different biochemical analysis. All rats were sacrificed and the brains were dissected out rapidly and rinsed in ice-cold saline. Hippocampi were quickly dissected on an ice surface homogenized in saline, centrifuged at 640g and 4°C for 15 minutes, and the obtained supernatants were stored at −80°C. Furthermore, spleen and thymus were harvested and weighed in relation to body weight to determine immune organ index.

2.3.4. Behavioral Tests. Anxiety-like behaviors were evaluated by the elevated plus maze (EPM) test and open field test (OFT), whereas depression-like behaviors were assessed by the forced swim test (FST).

(1) Elevated Plus Maze Test (EPM). Elevated plus maze (EPM) is commonly used to assess anxiety-like behavior and to evaluate learning and memory in laboratory animals [24]. The apparatus consists of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) extended from a central platform (10 × 10 cm). The maze is elevated to a height of 40 cm from the floor. Two separated experiments were conducted in EPM. The first one was performed in two stages (learning and acquisition). On the first day (training session), each rat was placed at the end of an open arm facing away from the center. The time in seconds taken to enter any one of the closed arms with its four legs was recorded as transfer latency (TL). Retention testing was conducted 24 h after the first trial and recorded in a similar manner as mentioned before. Significant decrease in TL value of retention was considered as an index of improvement in memory. In the second experiment, each animal was placed in the center of

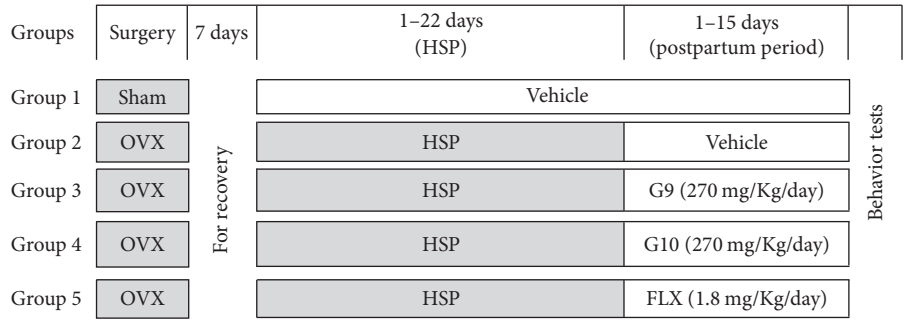

Figure 1: Diagram showing the experimental design and different treatments. OVX: ovariectomized; HSP: hormone-simulated pregnancy; G9 and G10: flaxseed oils from cultivars Giza 9 and Giza 10; and FLX: fluoxetine.

the maze facing a closed arm and the cumulative time spent in the open/closed arms was recorded through a 5 min session. An arm entry was defined when four paws of the rats were inside the arm [25].

(2) Open Field Test. Locomotor activity was assessed by open field test (OFT) according to the method of Stout and Weiss (1994) [26]. Rats were individually housed in a rectangular container made of dark polyethylene ($60 \times 60 \times 30$ cm) to provide best contrast to the white rats in a dimly lit room equipped with a video camera above the center of the room, and their locomotion and exploratory behaviors were then measured. The open field maze was divided into two zones, central and peripheral zone, using the square drawn on the maze. The area was divided into 16 squares of 15×15 cm by painted white lines. Each rat placed in one corner of the open filed and its activity during the subsequent 5 min was recorded. The behavioral parameters assessed were (1) ambulation (number of squares traversed by rat); (2) rearing frequency (number of times the animal stood on its hind legs); and (3) grooming (duration of time the animal spent licking or scratching itself while stationary).

(3) Forced Swim Test (FST). Forced swim test (FST) was performed according to a standard method of Lucki (1997) to determine the antidepressant-like behavior [27]. The test apparatus consisted of a vertical cylindrical glass container (46 cm high, 21 cm in diameter) filled to a depth of 30 cm with tap water at $25 \pm 0.5^{\circ}$C. This depth was sufficient to ensure that animals could not touch the bottom of the container with their hind paws or their tails. The rats had a swimming-stress session for 15 min and then were removed. The water was changed for each rat. The next day, all animals were subjected to 5 min of forced swimming and the three behaviors recorded were (1) climbing behavior, which is defined as upward-directed movements of the forepaws along the side of the swim chamber; (2) swimming behavior, the movement (usually horizontal) throughout the swim chamber that also includes crossing into another quadrant; and (3) immobility, which was assigned when no additional activity was observed other than that required to keep the rat's head above the water.

2.3.5. The Immune Organ Index. The initial and final body weight and thymus and spleen weights were recorded. Thymus and spleen indexes were calculated according to the following formula: thymus or spleen index (mg/g) = weight of thymus or spleen (mg)/body weight of rat (g) [28].

2.3.6. Biochemical Analysis. The clinical diagnostics were determined in accordance with the manufacturers' instructions of the corresponding kit.

(1) Assay of Monoamine Oxidase and Corticosterone. Brain monoamine oxidase-A (MAO-A) was investigated according to the spectrophotometric method of Charles et al. (1977) [29], while serum corticosterone (CORT) level was determined quantitatively using rat ELISA kits (DRG International, USA) [30].

(2) Assay of Proinflammatory Cytokines. The serum levels of proinflammatory cytokines were determined by using rat ELISA kits for TNF-α (IBL International, Hamburg, Germany) and IL-1β and IL-6 (R&D Systems, Minneapolis, MN, USA).

(3) Quantitative Determination of Antioxidant Defense System Markers. Superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) were determined by the method of S. Marklund and G. Marklund (1974) [31] and Aebi (1984) [32], respectively. Reduced glutathione (GSH) and vitamin C content were determined by the method of Ellman (1959) [33] and Omaye et al. (1979) [34], respectively. Protein content of hippocampal supernatant was determined calorimetrically using method developed by Lowry et al. (1951) [35] using bovine serum albumin as standard.

(4) Quantitative Determination of Oxidative Stress Markers. Malondialdehyde (MDA), a marker of lipid peroxidation, was assayed using thiobarbituric acid reacting substance (TBARS) [36]. As a hallmark of protein oxidation, total protein carbonyl (PC) content was determined by the method described by [37]. The level of the nitric oxide (NO) was estimated as nitrate/nitrite by Griess reaction after conversion of nitrate to nitrite according to the method of Montgomery and Dymock (1961) [38].

2.4. Statistical Analysis. The obtained biochemical data are expressed as means ± SE and subjected to statistical analysis by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS Inc., Chicago,

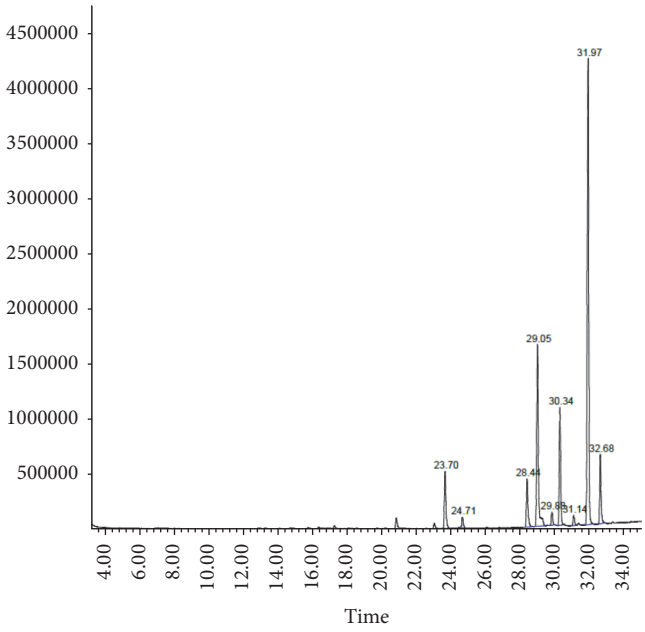

Figure 2: GC chromatogram of fatty acid profile of flaxseed oil cultivar G9.

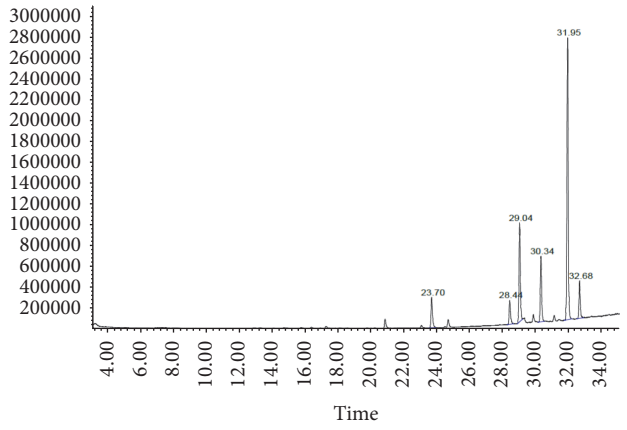

Figure 3: GC chromatogram of fatty acid profile of flaxseed oil cultivar G10.

USA, version 22 software) followed by Duncan's multiple-comparison test. Values of $P < 0.05$ are considered to indicate statistical significance.

3. Results

3.1. Chemical Assessment (Fatty Acids Profile). The fixed oil yield of six different Egyptian cultivars of flaxseed (Table 1) ranged from 33.2 to 42.5%. Fatty acid profiles of investigated samples were similar qualitatively and vary quantitatively among the examined flaxseed oil cultivars. Comparative GC/MS analysis of the fatty acid profile of the tested oil cultivars (Figures 2 and 3 and Table 1) revealed the detection of ten fatty acids, among which the unsaturated fatty acid constituted about 87.73–90.71% of the total composition. GC/MS analysis prevailed that all the tested samples of flaxseed oil cultivars contain comparable amounts of the ω-3, ω-6, and ω-9 PUFAs. The unsaturated fatty acids were dominated by α-linolenic acid (53.31–59.04%) which represented the ω-3 fatty acid followed by oleic acid (14.13–22.12%) which represented ω-9 fatty acid, while ω-6 unsaturated fatty acids were represented by linoleic acid (0.74–10.52%). The major identified saturated fatty acids were palmitic and stearic acids. Fixed oil derived from Giza 9 (G9) and Giza 10 (G10) contains the highest percentage of ω-3 (59.04 and 53.88%, resp.), while the lowest percentage was detected in Sakha 1 (S1).

Table 2: The behavioral parameters recorded in elevated plus maze.

Groups	TL on 15th day (sec.)	TL on 24 hrs (sec.)	ΔTL (sec.)
Sham	41.1 ± 1.39^{A}	34.9 ± 1.49^{A}	6.25 ± 0.41^{C}
PPD	52.1 ± 1.53^{C}	49.9 ± 1.57^{C}	2.25 ± 0.16^{A}
PPD + G9	45.0 ± 1.17^{AB}	39.4 ± 1.08^{B}	5.63 ± 0.38^{BC}
PPD + G10	47.3 ± 1.80^{B}	42.4 ± 1.74^{B}	4.88 ± 0.13^{B}
PPD + FLX	44.5 ± 1.35^{AB}	38.8 ± 1.10^{AB}	5.75 ± 0.37^{BC}

Each value represents the mean of 8 rats ± SE. In the same column, the different letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by Duncan's multiple-comparison test at $P < 0.05$. PPD: postpartum depression; G9 and G10: flaxseed oils from cultivars Giza 9 and Giza 10; and FLX: fluoxetine.

3.2. Evaluation of the Antidepressant Potential of Selected Cultivars of Flaxseed Oil in a Rat Model of Postpartum Depression

3.2.1. Elevated Plus Maze Model (EPM). The results in Table 2 demonstrate a behavioral phenotype that is relevant to PPD symptoms, including increased anxiety and learning and memory diminishing properties. The obtained results showed that the prolongation of transfer latency (TL) in the PPD rats was reversed by treatment with flaxseed oil (G9 and G10) and FLX for 2 weeks during postpartum period.

During the elevated plus maze (Figure 4), PPD rats spent significantly ($P < 0.05$) more time in the closed arms (221.3 ± 3.68 second) in comparison to control rats (176.1 ± 2.70 second). Both of flaxseed oil (G9 and G10) and FLX significantly ($P < 0.05$) decreased the time spent in the closed arms, reflecting a reduction in anxious behavior. Conversely, the time spent of PPD rats in the open arms (58.8 ± 2.14 seconds) were significantly ($P < 0.05$) increased by flaxseed oil (G9 and G10) and FLX treatment as compared to control animals.

3.2.2. Open Field Test (OFT). During the open field test (Figure 5), untreated PPD significantly ($P > 0.05$) decreased the numbers of rearing and increased the time of self-grooming by 24.7% and 91.7%, respectively, versus control group. It is important to notice that no significant differences ($P > 0.05$) were observed in ambulation between all animal groups. However, a significant ($P < 0.05$) increase in numbers of rearing and decrease in grooming time were observed in PPD-treated groups with either FLX or flaxseed oil (G9 and G10) versus sham group.

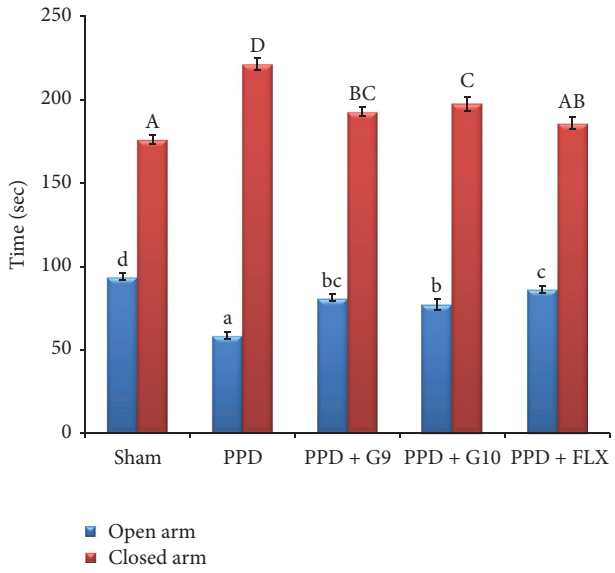

Figure 4: Time spent in open and closed arms on the elevated plus maze test. Each value represents the mean of 8 rats ± SE. The different superscripts (a–d for open arm and A–D for closed arm) indicate a significant difference between groups using one-way ANOVA using SPSS software (version 22) followed by Duncan's multiple-comparison test at $P < 0.05$. PPD: postpartum depression; G9 and G10: flaxseed oil from cultivars Giza 9 and Giza 10; and FLX: fluoxetine. PPD exhibited significant decrease in the time spent in open arm and increase in the time spent in closed arm versus sham-operated group. However, treatment with either flaxseed oil cultivars (G9 and G10) or fluoxetine significantly increased the time spent in open arm in order of FLX ≥ G9 > G10 and decreased the time spent in closed arm in order of FLX > G9 ≥ G10 versus PPD group.

3.2.3. Forced Swim Test (FST). The data in Figure 6 revealed that animals subjected to induction of PPD exhibited a depression-like behavior in FST, characterized by significant ($P < 0.05$) increase in duration of immobility by 155.2% and decrease in active swimming and climbing by 19.7% and 44.7%, respectively, versus sham-operated group. In contrast, daily treatment with the flaxseed oils of G9 or G10 at a fixed dose of 270 mg/Kg b.wt for 15 days displayed a significant ($P < 0.05$) decrease in durations of immobility by 37.6% and 23.4%, respectively, versus untreated PPD rats. Treatment with FLX significantly ($P < 0.05$) decreased the immobility by 32.0% and increased the active swimming time by 26.3% but insignificantly ($P > 0.05$) affected climbing duration versus untreated PPD rats. In contrast, flaxseed oil (G9 and G10) supplementation significantly ($P < 0.05$) increased the climbing time by 49.7% and 35.2%, respectively, during 5 min, versus untreated PPD rats.

3.3. Effect of Different Treatments on Body Weight and Immune Organ Index. The results depicted in Table 3 revealed that the weight gain significantly decreased ($P < 0.05$) by 2.2-fold in untreated PPD-induced female rats as compared with sham-operated rats. Treatment of PPD rats with flaxseed oils (G9 and G10) and FLX exhibited a significant increase ($P < 0.05$) in body weight gain by 2.0-, 1.97-, and 2.2-fold, respectively, versus untreated PPD rats. In addition, Table 3 elucidated that, in PPD rats, thymus and spleen indexes were significantly ($P < 0.05$) decreased by 14% and 17%, respectively, as compared with sham-operated rats, while they were significantly increased in PPD rats receiving either G9, G10 flax oils, or FLX by 11, 10, and 13%, respectively, for thymus, and by 15, 9, and 17%, respectively, for spleen versus untreated PPD-induced female rats.

3.4. Effect of Different Treatments on Brain Monoamine Oxidase, Serum Corticosterone, and Proinflammatory Cytokines. Data in Table 4 revealed that, in PPD rats, the levels of brain MAO-A enzyme activity, serum levels of CORT, and proinflammatory cytokines TNF-α, IL-1β, and IL-6 were significantly ($P < 0.05$) increased by 1.47-, 1.92-, 2.0-, 1.95-, and 3.93-fold, respectively, versus sham-operated rats. Conversely, MAO-A activity and CORT were significantly decreased in rats receiving G9, G10 flaxseed oils or FLX by 25.5, 13.9, and 20%, respectively, for MAO-A, and by 31, 29, and 33%, respectively, for CORT versus untreated PPD rats. Also, treatment of PPD rats with G9, G10, or FLX levels for 15 days significantly suppressed serum levels of proinflammatory cytokines by 42, 39, and 43% and TNF-α, by 34, 34, and 38% for IL-1β, respectively, and by 52, 48, and 59%, respectively, for IL-6 versus untreated PPD rats.

3.5. Effect of Different Treatments on the Hippocampal Redox State. The obtained results illustrated in Figures 7(a)–7(e) and Table 5 illustrated that the hippocampal SOD, CAT, reduced GSH, and vitamin C contents were significantly decreased by 27, 24, 18, and 21%, respectively, while hippocampal PC, MDA, and NO contents (Figures 7(e)–7(g)) were significantly increased by 31, 25, and 81%, respectively, in PPD rats versus sham group. On the contrary, hippocampal SOD and CAT enzyme activity were significantly increased in rats administrating G9, G10, or FLX by 26, 23, and 17%, respectively, for SOD, and by 21, 19, and 22%, respectively, for CAT against untreated PPD rats. Also, treatment of PPD rats with G9, G10, and FLX levels significantly increased the hippocampal reduced GSH and vitamin C content by 16, 14, and 19%, respectively, for GSH and by 18, 15, and 18%, respectively, for vitamin C versus untreated PPD rats. Furthermore, hippocampal PC, MDA, and NO content was significantly decreased in rats treated with G9, G10, or FLX treated PPD rats to 17, 16, and 15%, respectively, for PC, by 14, 11, and 17%, respectively, for MDA, and by 38, 35, and 37%, respectively, for NO versus untreated PPD group.

4. Discussion

4.1. Chemical Assessment (Fatty Acids Profile). Flax cultivars have been evaluated regarding yield, yield components, quality of fibers, and seeds in addition to oil chemical composition [39]. Many investigators indicated significant differences among flax genotypes [40]. Flaxseed is the richest source of α-linolenic acid (ALA), with five times more ALA than any other plant food. The relative high percentage of unsaturation in fatty acids especially omega-3 is beneficial as

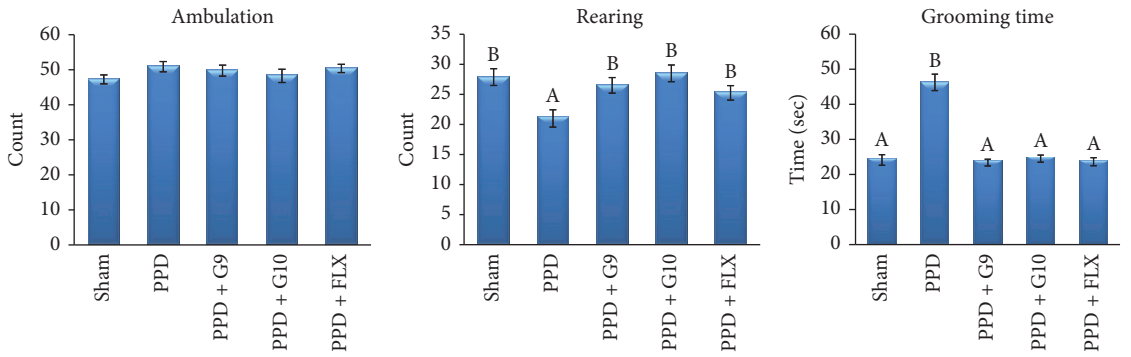

FIGURE 5: Effect of different treatments on ambulation, rearing, and self-grooming behavior in open field test. Each value represents the mean of 8 rats ± SE. The different capital letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by DMCT Duncan's multiple-comparison test at $P < 0.05$. PPD: postpartum depression; G9 and G10: flaxseed oils from cultivars Giza 9 and Giza 10; and FLX: fluoxetine.

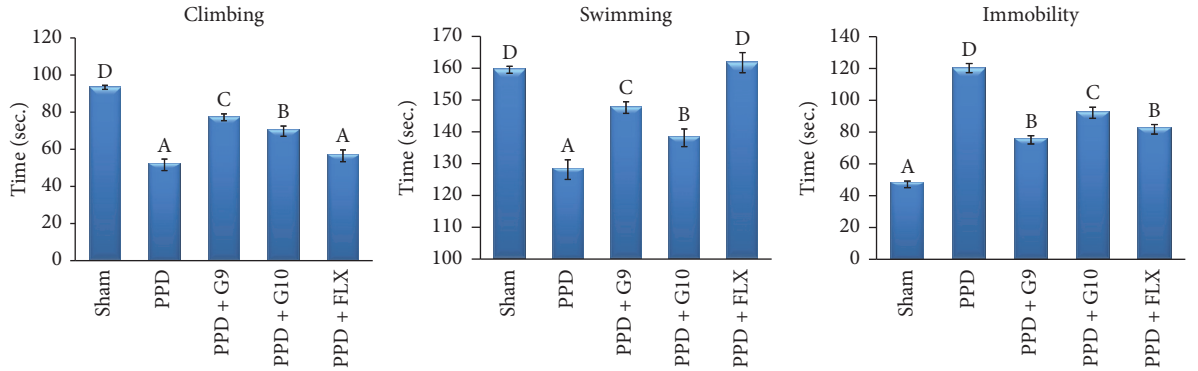

FIGURE 6: Effect of different treatments on climbing, swimming, and immobility in forced swim test. Each value represents the mean of 8 rats ± SE. The different capital letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by DMCT Duncan's multiple-comparison test at $P < 0.05$. PPD: postpartum depression; G9 and G10: flaxseed oils from cultivars Giza 9 and Giza 10; and FLX: fluoxetine.

a food supplement in treating hyperlipidemia and/or hypercholesterolemia [41].

The fixed oil yield of the examined Egyptian cultivars (Table 1) ranged from 33.2 to 42.5%, which are nearly similar to the reported data (38 to 44%) due to genotype and environmental parameters variation [42]. Fatty acid profiles of the oil samples were similar qualitatively and vary quantitatively among the examined flaxseed oil cultivars. GC/MS analysis showed that all the tested samples of flax oil cultivars contain comparable amounts of the ω-3, ω-6, and ω-9 of unsaturated fatty acids. Results of the fixed oil derived from the Egyptian cultivars are in agreement with the previously reported data of Bartram (2013) [15]. The highest percentage of ω-3 was detected in the oil samples derived from Giza 9 and Giza 10 cultivars (59.04 and 53.88%, resp.). Oleic acid (ω-9) was detected in the highest percentage in oil derived from Giza 10. Oil samples derived from Giza 9 (G9) and Giza 10 (G10) cultivars were found to contain a relative percentage of unsaturated fatty acid ω-3 (53.30–58.48%), ω-6 (12.30–11.87%), and ω-9 (20.84–18.54%), respectively. Moreover, the higher ratio of omega-3 fatty acids (ALA) relative to omega-6 (linoleic acid) is very important for prostaglandins metabolism which in turn pivotal in the regulation of inflammation, hormone synthesis, and steroid production [43].

A previous study demonstrated the antidepressant effects of omega-3 fatty acid in postpartum model of depression in rats [3]; however, no studies are available to suggest the benefits of flaxseed oil for improving mood even in the general population. Therefore, we test the hypothesis that daily oral administration of oils of Egyptian cultivars of flaxseed G9 and G10 containing the highest percentage of ω-3 would exhibit antidepressant-like effect by amelioration of the behavior and biochemical changes in PPD model female rat.

4.2. Biological Assessment of the Antidepressant-Like Effect of Flaxseed Oil of Selected Cultivars Using Postpartum Depression (PPD) Model in Rat. Anxiety and depression-like behaviors are associated with postpartum depressive-like symptoms [44]. In the present study, the behavioral responses of PPD rats treated with flaxseed (G9 and G10), compared with FLX as a standard antidepressant drug, were assessed in EPM test, OFT, and FST, commonly considered as standard models of depression in animals.

4.2.1. Effect of Flaxseed Oil Cultivars on PPD Rat Behavior. EPM served as the behavioral model to evaluate anxiety and learning and memory improvement properties [3, 28]. In the

Table 3: Effect of different treatments on body weight and immune organ index.

Groups	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Weight of thymus (mg)	Thymus index (mg/g)	Weight of spleen (mg)	Spleen index (mg/g)
Sham	155.4 ± 1.69^{A}	211.5 ± 1.20^{B}	56.1 ± 1.78^{B}	195.6 ± 4.70^{C}	0.93 ± 0.021^{B}	769.3 ± 25.2^{C}	3.64 ± 0.11^{C}
PPD	157.3 ± 2.23^{A}	182.6 ± 2.31^{A}	25.3 ± 3.14^{A}	146.8 ± 3.50^{A}	0.80 ± 0.017^{A}	552.6 ± 8.09^{A}	3.03 ± 0.07^{A}
PPD + G9	160.5 ± 1.65^{A}	211.1 ± 1.09^{B}	50.6 ± 1.71^{B}	188.7 ± 2.60^{BC}	0.89 ± 0.014^{B}	733.3 ± 10.9^{BC}	3.47 ± 0.05^{BC}
PPD + G10	158.3 ± 2.61^{A}	208.1 ± 3.16^{B}	49.8 ± 3.02^{B}	183.5 ± 2.20^{B}	0.88 ± 0.015^{B}	685.3 ± 22.5^{B}	3.29 ± 0.08^{B}
PPD + FLX	155.1 ± 1.25^{A}	210.9 ± 2.81^{B}	55.8 ± 2.48^{B}	190.4 ± 3.63^{BC}	0.90 ± 0.018^{B}	746.9 ± 15.7^{C}	3.54 ± 0.05^{C}

Each value represents the mean of 8 rats ± SE. In the same column, different letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by Duncan's multiple-comparison test at $P < 0.05$. PPD: postpartum depression; G9 and G10: flaxseed oils from cultivars Giza 9 and Giza 10; and FLX: fluoxetine.

Table 4: Effect of different treatments on brain monoamine oxidase (MAO-A), serum corticosterone (CORT), and proinflammatory cytokines.

Groups	MAO-A (nmol/mg protein)	CORT (ng/ml)	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
Sham	43.2 ± 1.62^{A}	42.5 ± 0.95^{A}	25.6 ± 0.99^{A}	39.4 ± 0.82^{A}	17.2 ± 0.90^{A}
PPD	63.5 ± 1.73^{D}	81.4 ± 1.90^{C}	51.2 ± 1.13^{C}	76.7 ± 1.22^{C}	67.6 ± 2.79^{D}
PPD + G9	47.3 ± 1.70^{AB}	55.9 ± 1.70^{B}	29.6 ± 0.76^{B}	50.4 ± 1.27^{B}	32.7 ± 1.98^{BC}
PPD + G10	54.7 ± 2.23^{C}	58.0 ± 1.88^{B}	31.4 ± 0.98^{B}	50.8 ± 1.35^{B}	35.4 ± 1.51^{C}
PPD + FLX	50.8 ± 1.87^{BC}	54.7 ± 0.98^{B}	29.0 ± 0.97^{B}	47.7 ± 1.40^{B}	27.7 ± 1.44^{B}

Each value represents the mean of 8 rats ± SE. In the same column, the different letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by Duncan's multiple-comparison test at $P < 0.05$.

Table 5: Effect of different treatments on the hippocampal redox state.

Groups	Enzymatic defense system		Nonenzymatic defense system		Markers of oxidative stress		
	SOD	CAT	GSH	Vit. C	PC	MDA	NO
Sham	4.23 ± 0.09^{C}	71.2 ± 1.36^{C}	1.20 ± 0.02^{B}	1.54 ± 0.08^{B}	1.36 ± 0.05^{A}	13.7 ± 0.45^{A}	4.11 ± 0.16^{A}
PPD	3.09 ± 0.11^{A}	54.1 ± 1.74^{A}	0.98 ± 0.05^{A}	1.21 ± 0.02^{A}	1.78 ± 0.10^{B}	17.1 ± 0.47^{C}	7.44 ± 0.15^{C}
PPD + F5	3.90 ± 0.08^{BC}	65.7 ± 1.55^{B}	1.14 ± 0.03^{B}	1.43 ± 0.05^{B}	1.47 ± 0.06^{A}	14.7 ± 0.47^{AB}	4.59 ± 0.25^{AB}
PPD +F6	3.81 ± 0.11^{BC}	64.2 ± 1.88^{B}	1.12 ± 0.03^{B}	1.39 ± 0.04^{B}	1.49 ± 0.06^{A}	15.2 ± 0.25^{B}	4.83 ± 0.16^{B}
PPD + FLX	3.63 ± 0.25^{B}	65.8 ± 1.75^{B}	1.17 ± 0.03^{B}	1.43 ± 0.02^{B}	1.52 ± 0.07^{A}	14.2 ± 0.29^{AB}	4.70 ± 0.19^{B}

Each value represents the mean of 8 rats ± SE. In the same column, the different letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by Duncan's multiple-comparison test at $P < 0.05$. PPD: postpartum depression; G9 and G10: flaxseed oils from cultivars Giza 9 and Giza 10; and FLX: fluoxetine.

present study, HSP induced depressive-like behavior in PPD rats is manifested by increased transfer latency (TL) value of learning and retention (Table 2) and increase in the time spent in closed arm (Figure 4) in EPM test. Administration of flaxseed oil or FLX significantly decreased TL values of learning and retention, indicating better memory retention and significant memory improvement as compared with untreated PPD group.

EPM task in the second experiment is assessing the anxiety-like behaviors in rodents. The obtained data in Figure 4 revealed that PPD rats tend to spent more time in closed arm. The task is based on an approach-avoidance conflict, meaning that the animal is faced with a struggle between a propensity to discover a new environment and an unconditioned fear of high and open places. Treatment with either flaxseed oil (G9 and G10) or FLX significantly decreased the time spent in the closed arms, while it increased the time in open arm reflecting a reduction in anxious behavior or antianxiety behavior. These beneficial effects were decreased in the order of FLX ≥ G9 > G10 in treated groups.

OFT provides simultaneous measures of locomotion, exploration, anxiety, and emotionality by observing the ambulation, rearing, and self-grooming behaviors. A high frequency of these behaviors indicates increased locomotion and exploration and/or a lower level of anxiety [45]. In the present study, untreated PPD significantly decreased the numbers of rearing and increased the time of self-grooming versus control group (Figure 5). However, a significant increase in numbers of rearing and decrease in grooming time without affecting locomotion activity were observed in PPD-treated groups with either FLX or flaxseed oil (G9 and G10) confirming their antidepressant-like effect.

FST is commonly used behavioral despair in rat models to detect antidepressant potential by measuring the decrease in immobility periods. In the present study, female rats undergoing estradiol withdrawal show greater immobility periods in FST as compared to control animals and thus showed depression-like behavior. Administration of flaxseed oil of G9 and G10 cultivars through the postpartum period significantly enhanced escape-directed behaviors (climbing or swimming) with significant longer duration than control group (Figure 6). This active response was considered as behavioral profiles consistent with an antidepressant-like action. This depression-like response could not be explained by the change in general locomotor activity, as these rats were active in the open field test. Continual treatment with either flaxseed (G9 and G10) or FLX was able to reverse the depression-like behaviors in the FST. The improvement effect was more pronounced in G9- and FLX-treated groups.

4.2.2. Effect of Oral Administration of Flaxseed Oil on the Immune Organ Index. The results of Table 3 revealed that HSP induced decrease in body weight gain and immune organ indexes. To a certain degree, the thymus index and spleen index reflect immune function in the body. Slowed body weight increase in PPD untreated females is also being used as a proxy measure of the impact of postpartum depression symptoms [46]. As compared with sham group, the thymus and spleen indexes and body weight gain in PPD rats were significantly decreased within two weeks after hormone withdrawal [28]. Administration of oil samples (G9 and G10) or FLX for two weeks significantly increased body weight gain and improved the hypofunctional immune status

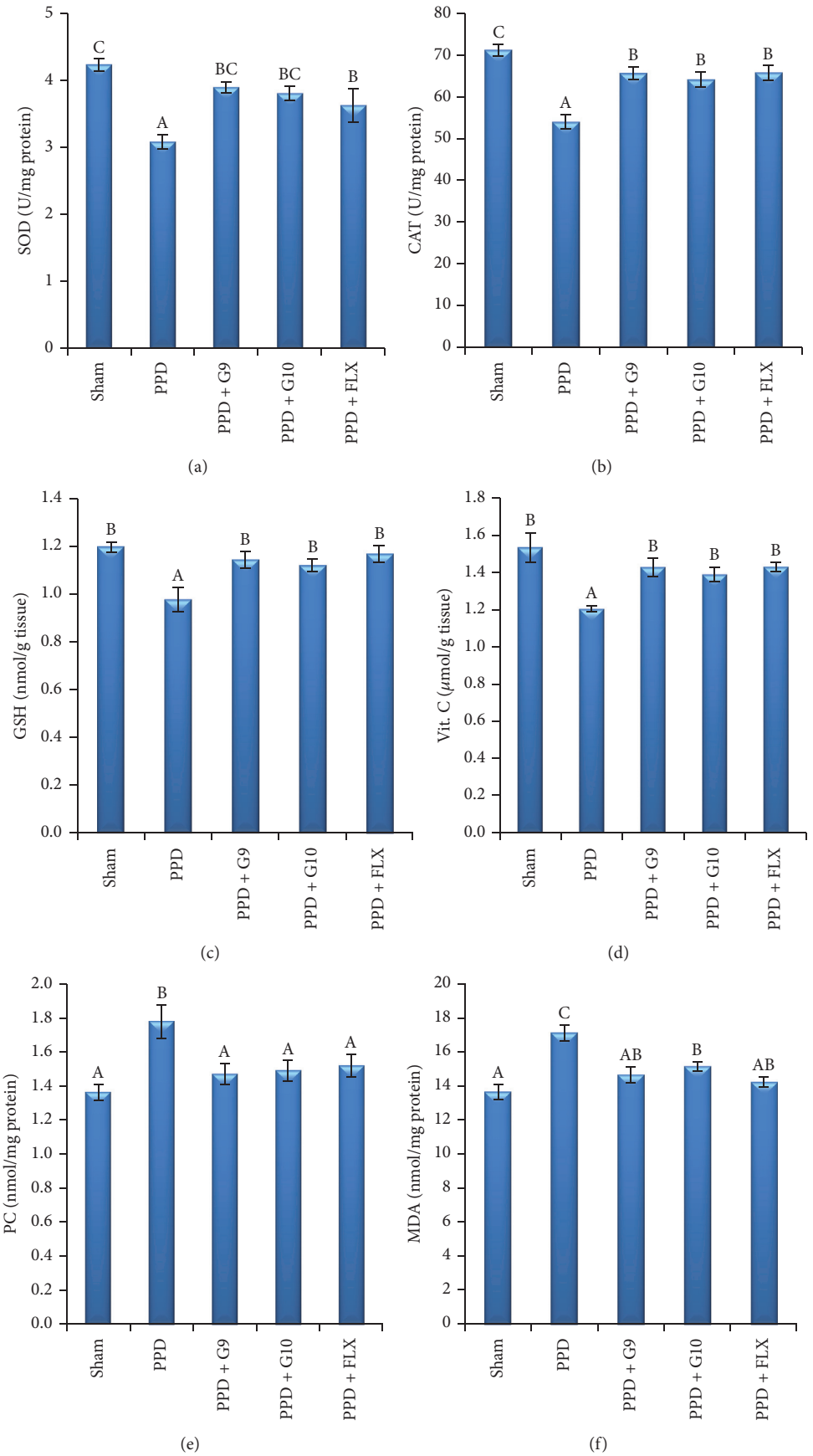

Figure 7: Continued.

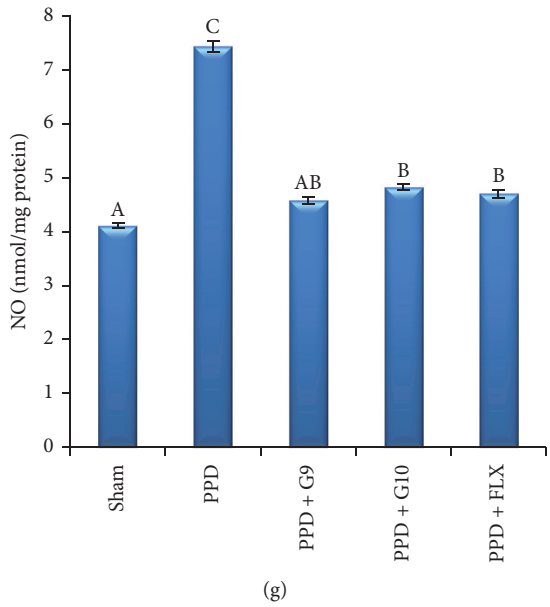

Figure 7: Effect of different treatments on the hippocampal redox state. Each value represents the mean of 8 rats ± SE. In the same column, the different letters indicate a significant difference between groups using one-way ANOVA by using SPSS software (version 22) followed by Duncan at $P < 0.05$. Superoxide dismutase (SOD, U/mg protein); catalase (CAT, U/mg protein); reduced glutathione (GSH, nmol/mg protein); vitamin C (vit C, μmol/g tissue); protein carbonyl (PC, nmol/mg protein); malondialdehyde (MDA, nmol/g tissue); and nitric oxide (NO, μmol/mg protein). One unit of SOD corresponds to the enzyme required to inhibit half of the oxidation of pyrogallol; one unit of CAT is defined as the amount of enzymes required to decompose 1 m mole of hydrogen peroxide in 1 min.

in thymus and spleen in PPD rats by increasing the mass of immune organs; however, flaxseed oil of G9 cultivar exhibited more pronounced effect than G10, which may be due to its higher PUFA content. These results confirmed the potential regulatory role of flaxseed oil in immune function of PPD rat. Our results are also in agreement with the previous study, which reported that omega-3 PUFA supplementation improved the performance of cognitive parameter and prevented the mood and anxiety disorders [19]. Furthermore, many animal and human studies indicated that supplementation with ω-3 PUFA containing products could be of great values for the development of a healthy brain, memory, and learning [28, 47].

4.2.3. Effect of Oral Administration of Flaxseed Oil on the Level of Biochemical Markers in Postpartum Depression (PPD) Rat Model. Depression is multifactor disorder and its etiology includes genetics, environmental, psychological, and biological factors. Several molecular mechanisms play a role in pathogenesis of depression [48]. Although selective serotonin reuptake inhibitors as FLX and tricyclic antidepressants are indicated for the treatment of PPD, their safety is not well established on neurological development of infants during the breastfeeding period. Hence, a safe antidepressant is warranted in the treatment of PPD [49].

Corticosterone (CORT) is an important stress hormone in animals and has been involved in major depressive disorder. CORT is commonly used as an endocrinological diagnostic marker based on its elevation in chronic stress in animal models [50]. Two isoforms of monoamine oxidase (MAO-A and MAO-B) exist, with higher substrate preference of MAO-A isoform for serotonin; in addition, it is the main target for the antidepressant MAO inhibitors. MAO-A was found to regulate the metabolic degradation of serotonin and catecholamines in noradrenaline and serotonin neurons. This is thought to be the site of therapeutic action in depression [51]. In the present study, PPD rats showed significant increase in serum level of CORT and brain activity of MAO-A relative to sham rats (Table 4). This effect was nearly restored by oral administration of both oil samples (G9 and G10) as well as by FLX relative to PPD rats, which reflected the antidepressant-like potential.

The imbalance in proinflammatory cytokines plays a role in the etiopathology of mood shifts in PPD [28, 52]. Among the proinflammatory cytokines believed to be at the top of the stress-induced inflammatory cascade is the proinflammatory cytokine interleukin-1 beta (IL-1β), which has been shown to be involved in inflammatory responses leading to stress-related cellular damage. It was demonstrated that IL-1β likely plays a role in initiating the inflammatory cascade in response to stress [53]. Based on this concept, the obtained results showed that PPD induced significant increase in MAO-A activity, levels of CORT, and proinflammatory cytokines (TNF-α, IL-1β, and IL-6); however, daily administration of both flaxseed oil at a dose of 270 mg/kg b.wt and FLX to PPD groups for 2 weeks effectively decreased MAO-A, CORT and effectively suppressed the biochemical markers of inflammation, as compared to sham-operated group. These observations indicate that the PPD model of rats, which produce depression-like behavior, is related to high brain MAO-A activity, serum CORT, and proinflammatory cytokines. Therefore, flaxseed oil could be considered as a natural way

to reduce the levels of MAO-A, CORT, and proinflammatory cytokines in PPD rats. This is supported by earlier study of Dhingra and Bhankher (2014) [30], who reported that antidepressant-like activity is mediated by inhibition of MAO-A activity and suppresses the production of proinflammatory cytokines. In addition, our results are in agreement with previous studies which reported elevated level of CORT in both humans [54] and rats [3] during the postpartum period. In addition, it has been confirmed that higher levels of CORT during the postpartum period showed increase in depressive-like behavior in female rats and decrease in maternal care, hippocampal cell proliferation, and body weight [55].

Clinical and experimental tests proved that the immune system is dysregulated in PPD [7, 28] and omega-3 fatty acid deficiency increases constitutive proinflammatory cytokines production in rats [56]. It was reported that proinflammatory cytokines (e.g., IL-6 and IL-8) are activated by nuclear factor κB (NFκB), a transcription factor. Activity of this transcription factor is affected by levels of ROS and glutamate. NFκB itself increases oxidative stress in the organism and causes inflammatory reaction which can lead to neuroprogression [57]. Overproduction of proinflammatory cytokines can be related to insufficient activity of antioxidant enzymes and low level of antioxidants. Overproduction can lead to pathological changes in brain that can escalate to cognitive dysfunction or neuropsychiatric disorders [48]. However, supplementation of omega-3 fatty acids can decrease the level of CORT and proinflammatory; therefore, omega-3 fatty acids attenuate the depressive and anxiolytic effects of proinflammatory cytokines in postpartum-induced rats [3].

The present study may provide *in vivo* evidences of antidepressant-like activity mediated through inhibition of MAO-A, antioxidant, anti-inflammatory, and neuroprotective potential of flaxseed oil of Egyptian cultivars of G9 and G10 at a dose of 270 mg/kg b.wt (equivalent to a human dose of 3 g/day) for 15 days comparable to FLX as a reference antidepressant drug in PPD rat model.

4.2.4. Effect of Flaxseed Oil on the Hippocampal Antioxidant Defense State. Lipid peroxidation occurs more frequently in brain tissues due its high capacity in oxygen consumption and its high content of lipids and transition metals [58]. The hippocampus is one of the few brain areas that facilitate adult neurogenesis, and disruption of that process has been implicated in many mental disorders. It is involved in learning, memory, motivational control, and emotional control. The hippocampus also mediates the hypothalamic-pituitary-adrenal axis and expresses a high number of serotonin and GABAergic receptors, making it a popular therapeutic target for antidepressant action [59]. It is particularly sensitive to insults, such as stress, ischemia, and aging [60]. Oxidative stress, an imbalance between oxidative and antioxidative systems, is implicated in depression through its free radicals by-products including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Lipid peroxidation occurs when ROS production exceeds the antioxidant capacity [44]. Products of oxidative stress represent pivotal markers for detecting and measuring of depression status as well as for determining effectiveness of antidepressants [48]. In the brain tissue, antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and the nonenzymatic endogenous reduced glutathione GSH are responsible for detoxification of ROS [61]. Lowered concentration of ascorbic acid (vit C) was observed also in patients with depression and its intravenous administration not only amplifies antidepressants efficacy, but also act as antidepressant itself [62]. Deficiency of several nonenzymatic antioxidants is connected with worsening depression severity and anxiety [62]. Changes in activity of oxidative stress-related enzymes are associated with depression and inflammation [48].

From the aforementioned discussion, it seems necessary to measure the hippocampal antioxidant defense state. The results in Table 5 demonstrated that PPD induced by HSP in rats was associated with significant decrease in hippocampal SOD and CAT activity and depletion in levels of GSH and ascorbic acid as compared with normal control rats. In addition, the markers of lipid peroxidation (MDA) and protein oxidation (PC) as well as NO were significantly elevated in PPD group, which confirmed the susceptibility of brain tissues to oxidative stress. These results suggested that decreased antioxidant enzyme activities result in accumulation of ROS and negatively correlated with severity of depression. On the contrary, oral administration of flaxseed oil cultivars (G9 or G10) at a dose of 270 mg/kg b.wt for 2 weeks to rats with PPD induced by HSP significantly restored the hippocampal redox balance state and attenuated PPD-induced oxidative damage (Table 5). The best oxidative stress prevention effect was obtained with G9, followed with FLX and then G10 in case of SOD, PC, and NO, while for enzyme activity of CAT and level of GSH and MDA, the following order was recorded: FLX > G9 > G10. Also, it should be pointed out that administration of FLX and G9 showed the same improvement degree in hippocampal content of vit C. These results are consistent with data reported on multifaceted action of omega-3 from fish oil through anti-inflammatory, antioxidative, and antiapoptotic effects which improved the learning and memory in diabetic rats [58, 63].

The potential of diet for the preservation of hippocampal function was investigated by Labrousse et al., 2012 [64], who demonstrated that the treatment with EPA/DHA for eight weeks increased the long-chain ω-3 PUFAs in the brain tissues, prevented cytokines expression and alteration in astrocytes morphology of the hippocampus, and enhanced the spatial memory and Fos-associated activation in the hippocampus of aged mice. Thomas et al. [60] demonstrated that dietary supplementation regime of resveratrol or docosahexaenoic acid (DHA) or their combination effectively altered the hippocampal gene expression through anti-inflammatory mechanisms. Furthermore, supplementation with EPA, DHA, and α-ALA prevents the cognitive impairment by maintaining membrane integrity, enhancing neurogenesis, decreasing neuroinflammation, and increasing the cerebral blood flow [65]. On the other hand, overproduction of nitric oxide (NO) is strong damaging-free radical. However, the low concentration NO is considered as an important neurotransmitter connected to pathophysiology of depression, anxiety, epilepsy, and schizophrenia.

NO affects sexual and aggressive behavior and via synthesis pathway also takes part in anxious behavior, inflammation, and depression elicited by interferon alpha [66]. Ren and Chung (2007) revealed that anti-inflammatory effect of ALA is mediated through the inhibition of NO production and inducible nitric oxide synthase gene expression [67]. Elevated concentration of NO was detected in patients with depression [68]. Antidepressant-like effect can be induced by inhibition of NO synthesis in brain; inhibition of inducible nitric oxide synthase (iNOS) leads to increased effectiveness of serotonergic antidepressants and can be applied to patients suffering from drug resistant depression [69].

Furthermore, anxiety-like behaviors assessed by elevated plus maze and open field tests and depression-like behaviors evaluated by the forced swim test confirmed the antidepressant potential of tested cultivars of flaxseed oil. It should be mentioned that this study was the first study which demonstrated the efficacy of flaxseed oil of the Egyptian cultivars G9 and G10 in the treatment of postpartum depression. On the other hand, the fewer side effects of flaxseed oil compared with the classical antidepressant FLX confirm application of flaxseed oil as an alternative treatment of depression in traditional medicine.

5. Conclusion

In line with our hypothesis, this study supported the correlation between oxidative stress and inflammatory responses in PPD and provides *in vivo* evidences of efficacy of Egyptian cultivars of flaxseed oil G9 and G10 in alleviating depression- and anxiety-like behavior and biochemical changes in PPD-induced female rats. Accordingly, flaxseed oil may be a useful, cheap, and an alternative therapeutic agent for treating stress-related disorders such as postpartum depression. However, further studies are required to clarify this hypothesis in humans, which might support a novel preventive strategy to slow down the symptoms of PPD.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Anxiolytic Effect of *Citrus aurantium* L. in Crack Users

Gabriel Chaves Neto,[1] João Euclides Fernandes Braga,[2] Mateus Feitosa Alves,[3] Liana Clébia de Morais Pordeus,[1] Sócrates Golzio dos Santos,[4] Marcus Tullius Scotti,[3] Reinaldo N. Almeida,[1] and Margareth de Fátima Formiga Melo Diniz[1]

[1]*Postgraduate Program in Neuroscience Cognitive and Behavior, Federal University of Paraíba, João Pessoa, PB, Brazil*
[2]*Department of Nursing and Collective Health, Federal University of Paraíba, João Pessoa, PB, Brazil*
[3]*Postgraduate Program in Natural and Synthetic Bioactive Products, Federal University of Paraíba, João Pessoa, PB, Brazil*
[4]*Institute of Research in Drugs and Medicines, Federal University of Paraíba, João Pessoa, PB, Brazil*

Correspondence should be addressed to Mateus Feitosa Alves; mateusfalves@gmail.com

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The objective of this study was to investigate the anxiolytic effects of the essential oil (EO) of *Citrus aurantium* L. in patients experiencing crack withdrawal. This was developed with internal users in therapeutic communities in Paraíba, Brazil. The test population consisted of 51 volunteers, subdivided into three groups. To elicit anxiety, the Simulated Public Speaking (SPS) method was used. Physiological measures were assessed at specific phases during the experiment using appropriate equipment. Psychological measures of anxiety were assessed using the Trait-State Anxiety Inventory (IDATE) and the Analog Smoke Scale (HAS). EO was administered by nebulization. The experiment was developed in individual sessions and consolidated to four phases. The results demonstrated that the test subjects in the groups that were given the EO maintained controlled anxiety levels during SPS, when compared to the Control Group (no treatment). Subjects who used the EO also maintained levels of "discomfort" and "cognitive impairment" during SPS. It was concluded that individuals who are experiencing internal crack cocaine withdrawal present high anxiety traits and that nebulization of the EO of *Citrus aurantium* L. provided an acute anxiolytic effect in crack cocaine users exposed to SPS.

1. Introduction

Chemical dependency is a widely discussed phenomenon, since abusive use of psychoactive substances has become a serious social and public health concern. Throughout the twentieth century, this problem has gained increased relevance in the world and today is characterized as a chronic disease classified among psychiatric disorders [1].

Crack is one of the distinct forms of cocaine, a substance extracted from the leaf of a plant called coca (*Erythroxylum coca*), which is found in the Andes. When the drug is smoked in crack form, a large amount of cocaine molecules reach the brain almost immediately following use, producing an explosive effect. This speed of action is due to the fact that the smoke begins in the lungs, which are highly vascularized organs, and this quickly transports the drug to the brain. The drug is, subsequently, rapidly eliminated from the body, producing a sudden interruption of the sense of wellbeing, followed immediately by immense displeasure and an overwhelming desire to reuse the drug [2].

The use of *crack* has increased due to the high potential for addiction, pleasant effects, easy administration, low cost, and not being injected (becoming a safer route to HIV infection), among other reasons. This high dependence potentially triggers the craving (or cracking) effect. In the specific case of crack dependence, craving is an uncontrollable phenomenon by users, leading them to compulsive use with a daily pattern of consumption, which continues for several days in a row. This is only concluded when the physical, psychological, or financial exhaustion is achieved [3].

Crack addicts seeking treatment for drug withdrawal encounter a battle during abstinence, a period that is surrounded by anxiety and an intense craving for drug use. If there is not adequate monitoring, as well as therapeutic management of these manifestations, users eventually return to the use of crack [4].

Anxiety often presents itself as a state of tension, apprehension, and discomfort, involving emotional and physiological factors [5]. Anxiety disorders have been related both to hyperactivity in the amygdala and to a decreased hippocampal response. The pharmacological treatment of anxiety consists of conventional drugs, such as benzodiazepines and antidepressants. Additionally, barbiturates, carbamates, noradrenergics, antihistamines, glutamic acid, and buspirone are other commonly used therapies. Although a number of medications are presented, treatment is still plagued with limitations, side effects, and dependence and often does not have a standardized success rate across the population [6].

Aromatherapy, which consists of the therapeutic application of essential oils (EO) by inhalation, has been quite effective in relieving anxiety symptoms in research conducted in Brazil [7, 8]. In the United States, the use of herbal and medicinal plants by the population varies from 16.5 to 42.0%, with 5.5 to 20.5% being used for anxiety-related conditions [9]. Traditionally, populations of several countries use preparations based on Citrus species in the treatment of nervous system disorders, especially anxiety or insomnia [10].

Among the species, *Citrus aurantium* L. is popularly known as "bitter orange" [11], and its EO is rich in limonene [12]. Data has indicated that using *Citrus aurantium* L. EO has achieved results indicating anxiolytic effects, both in animals and human studies [11, 13, 14]. These findings highlight a new path in the scientific field for novel research evaluating results that corroborate and amplify the knowledge about the EO of *Citrus aurantium* L. and its anxiolytic effects in diverse population groups.

There is a high number of crack dependents in the world, and they face numerous difficulties to remain in treatment. Therefore, it is necessary to search for alternative measures that aid and contribute to the effectiveness of treatment. The objective of the present study was to investigate the anxiolytic potential of *Citrus aurantium* L. EO as a complementary therapy to reduce anxiety in patients enduring the withdrawal of crack cocaine.

2. Materials and Methods

This is an experimental study of an acute pharmacological clinical trial, controlled and randomized, and was developed at the Federal University of Paraíba (UFPB), Postgraduate Program in Neuroscience Cognitive and Behavior [16]. The population groups consisted of nonusers of crack cocaine. This study occurred in two therapeutic communities of recovery and treatment of chemical dependents in the state of Paraíba, Brazil, with voluntary users of *crack* in abstinence.

The study was approved under protocol number 094/1115. CAAE: 42619715.2.0000.5188 from the Ethics and Research Committee of the Health Sciences Center of the Federal University of Paraíba, in compliance with Resolution number 466/12 of the National Health Council, which regulates the conduct of research involving human beings.

2.1. Experimental Substance. The substance used for inhalation was the essential oil (EO) of *Citrus aurantium* L., produced and marketed by the company "By Samia Aromatherapy." The species *Citrus aurantium* L., popularly known as bitter orange, laranjeira-amarga, or laranjeira cavalo, is a native plant of Southeast Asia, introduced in Brazil in the period of colonization [17].

The EO was administered by nebulization, 2 drops (0.1 mL) of EO in 1.9 mL of distilled water solution with an emulsifier (Tween 80 at 12%), for each subject. The subjects of the "Control Group" experienced the same procedure; however, they received only the distilled water with an emulsifier. An electric nebulizer inhaler (Inalar®) was used; each group had an inhalation kit, exempting remnants of the EO during administration in the "Control Group." Following each experiment, the inhalation kits underwent a disinfecting process [18].

2.2. Gas Chromatography. In order to confirm the composition of the *Citrus aurantium* L. EO and guarantee product quality, an analysis was performed at the Pharmaceutical Products Quality Control Laboratory at the UFPB. Chromatograms were obtained by gas chromatography (Shimadzu GC-MS-QP5050A) using a 5% phenyl and 95% dimethylpolysiloxane capillary column with a length of 30 m, 0.25 mm internal diameter, and 0.25 μm film thickness, manufactured by J & W Scientific (Santa Clara, CA, USA).

2.3. Participants. The population consisted of 51 volunteers, who were subdivided into three groups according to the treatment. The "Control Group," non-crack users who were not internal to the therapeutic communities ($n = 17$), and two experimental groups, the "Nonuser EO Group," non-crack users who were not internal to the therapeutic communities ($n = 17$), and the "User EO Group", who were users of crack that were internal to the therapeutic communities ($n = 17$).

The eligibility criteria of the "User EO Group" included: presenting with a chemical dependence, being internally abstinent and having crack as a drug reason for hospitalization, male gender, being older than 18 years old, not making use of substances that affect the central nervous system, lack of cardiovascular problems, lack of upper airway obstruction problems, and lack of neurological and/or psychiatric comorbidities that affect cognition. The subjects of the "Control Group" and "Nonuser EO Group" were randomly selected in the general population; the subjects have a sociodemographic profile similar to the "EO User Group" group, following the same eligibility criteria; however, they are subjects without chemical dependence and are not internal to the therapeutic community.

2.4. Experimental Anxiety Induction Model. To induce anxiety, the Simulated Public Speaking (SPS) method [19] was used. The SPS has been shown to cause physiological and psychological changes. Briefly, the subject is requested to

deliver a speech in front of a video camera with its image being displayed on a TV screen. The speech, with a fixed time of 4 minutes, should describe anxious moments in your life.

2.5. Psychological Measures of Anxiety Assessment. To assess anxiety levels, the State Trait Anxiety Inventory (IDATE), an inventory developed by Spielbergert et al. (1970) [20], was translated and validated for the Portuguese language by Biaggio and Natalício (1979) [21]. It is a tool composed of two self-assessment subscales: the IDATE-Trait (IDATE-T), which defines the trait of anxiety of the individual and differentiates the tendency to react to situations identified as threatening. This is intended to be a more stable characteristic. The second subscale is the state IDATE (IDATE-E), which identifies the state of anxiety in relation to a situation considered anxious or distressing and is intended to be a transitory characteristic. Each of the subscales presents 20 questions, with four possible degrees of intensity of response, ranging from 1 to 4, in which the scores added by each volunteer oscillate between 20 and 80 points.

To accurately measure levels of anxiety, cognitive impairment, sedation, and discomfort, the Humor Analog Scale (HAS), a self-assessment scale originally proposed by Norris (1971) [22], was translated and validated for the Portuguese language By Zuardi and Karniol (1981) [23]. Consisting of 16 items, each composed of a straight line of 100 mm connecting two adjectives of opposite directions; the center of the line corresponds to the habitual state of the individual.

2.6. Physiological Measures for Anxiety Assessment. Physiological measures were measured during specific phases of the experiment using appropriate equipment. The model I-330-C2 + Plus Clinical System (J & J Engineering®) was used for the measurements of End Temperature (TEMP) and levels of electric conductance of the skin (ECS). The measurements of systolic blood pressure (SBP), Diastolic Blood Pressure (DBP), and heart rate (HR) were measured using the pulse sphygmomanometer (TeshLine®).

2.7. Procedures. The experiment was developed for individual sessions. The locations of the therapeutic communities provided adequate rooms to perform the experiment on volunteer users of crack. The "Nonuser EO Group" and the "Control Group" had the sessions developed in a room provided by the Health Sciences Center, UFPB. Prior to initiating the experiment, brief explanations were provided discussing the objectives of the study and free and informed consent of the participants was obtained; the subjects were not informed about the type of OE to be inhaled and the purpose of it. A semistructured interview was conducted to characterize the sample population and to identify variables such as, age, sex, drug consumption, and time of consumption. The clinical trial was consolidated to four phases: (I) Basal, (II) Stressor, (III) During, and (IV) Final, adapted from the model of Guimarães et al. (1987) [19]. The four phases are described below.

Baseline Phase (BP). The IDATE-T, IDATE-E, and HAS were measured along with the physiological measures of SBP, DBP, HR, TEMP, and ECS. The differentiation between the groups occurred at the end of this phase. The "Nonuser Group" and the "User EO Group" participants inhaled *Citrus aurantium L.* EO by nebulization for 5 minutes while the "Control Group" subjects inhaled only the distilled water with emulsifier by nebulization during the same time period.

Stressor Phase (SP). The subjects were informed they would have two minutes to prepare a speech focusing on situations that contributed to anxiety during their lives and four minutes to deliver the speech in front of a video camera having their image displayed on a TV. Following the two minutes of preparation and prior to the beginning of the speech, the IDATE-E, the HAS, and the physiological parameters (SBP, DBP, HR, TEMP, and ECS) were measured.

During Phase (DP). Following the two minutes of speech, the subject was interrupted and the IDATE-E and HAS measured the physiological parameters (SBP, DBP, HR, TEMP, and ECS), and, quickly after collecting the data, the speech was resumed.

Final Phase (FP). The IDATE-, HAS, and the physiological parameters (SBP, DBP, HR, TEMP, and ECS) were measured fifteen minutes following the end of the discourse.

2.8. Statistical Analysis. Statistical analysis was performed with the help of the Graph Pad Prism statistical software (version 6.00, Graph Pad Software Inc., San Diego, CA, USA). Hypothesis tests were defined according to the normality of the data and the classification of variables, using parametric methods (ANOVA, followed by the Bonferroni test) and nonparametric methods (Kruskal-Wallis, followed by Dunn's).

Data were presented with mean and standard error of the mean (e.o.m.) for the parametric methods and in the median and percentiles (25–75th percentile) for the nonparametric, when $P < 0.05$ was considered significant.

3. Results

3.1. Analytical Control of Essential Oil of Citrus aurantium L. Figure 1(a) illustrates the peaks of the analyzed compounds. Limonene displayed the highest peak, indicating its role as the major compound. Data demonstrated a retention time of 8.9 min and area 48124161, corresponding to 97.99% of the essential oil analyzed. Figure 1(b) shows the mass spectrum of the limonene compound having a molecular weight of 136 *m-z*, and a base peak of 68 *m-z*. The Kovats index (1029) was calculated and compared with the literature according to Table 1.

3.2. Characterization of Individuals Participating in the Study. The "Control Group" had a mean age of 28 years (±2.01), the "Nonuser EO Group" had a mean age of 24 years (±0.7282), and the "User EO Group" had a mean age of 30 years (±2,125). Anxiety-Trace scores resulted in the "User EO Group" presenting with the highest score, a median of 45

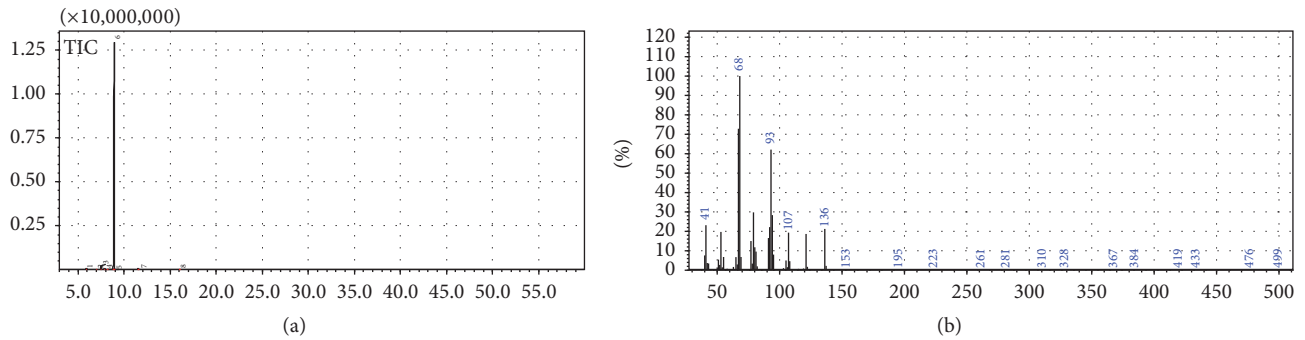

Figure 1: (a) Chromatogram of the essential oil of *Citrus aurantium* L. (By Samia); (b) limonene mass spectrum, showing the base peak at 68 *m/z*.

Table 1: Identification of the major compound [15].

Name	Peak	Retention time	Area	Area%	Kovats index, calculated	Kovats index, literature
Limonene	68	8,9 min	48124161	97,99	1029	1031

Table 2: Presentation of the Medians (Percentile 75%, Percentile 25%) of the IDATE-E scores between the phases of the Simulated Public Speaking (SPS).

Groups	Phases of application of SPS, median (Percentile)			
	Basal	Stressor	During	Final
Anxiety				
Control	38 (29–43)	46 (36–58)	38 (35–52)	33 (30–35)
OE Nonuser	33 (30–37)	32[1] (29–37)	34[2] (30–36)	32 (29–35)
OE User	36 (32–40)	34[1] (31–39)	38 (31–40)	31 (30–35)
P value	0,1732	0,0023	0,0279	0,9555

Source: Direct Research 2015. Statistical Test: Kruskal-Wallis and Dunn's posttest. [1]Significantly different from the "Control Group" at the time Stressor. [2]Significantly different from the "Control Group" at the time.

(37–57), followed by the "Control Group" with a median of 41 (38–53), and "Nonuser EO Group" with 37 (30–40).

3.3. Evaluation of the Effects of the Essential Oil of Citrus aurantium L. and Placebo on the Psychological Parameters Measured. When analyzing the anxiety levels measured by the IDATE-E and the HAS, similar variations of the scores are observed, which reinforces the confidence of the measurements. The IDATE-E scores between the phases of Simulated Public Speaking are presented in Table 2; the groups presented uniform levels of anxiety in the baseline phase (BP), with no significant differences observed. In the Stressor Phase (SP), a significant difference was observed between the groups, where the groups treated with the EO had controlled anxiety levels, while the "Control Group" demonstrated an increase in these levels. In the During Phase (DP), only the "Nonuser EO" group maintained a significant difference against the "Control Group." At the Final Phase (FP), the groups had similar medians, with no significant differences.

The Cognitive Impairment Factor scores did not indicate a significant difference between the groups in the BP. In the EP, the groups treated with the EO presented a significant difference in comparison to the "Control Group" ($P = 0.0018$). In the DP, only the "Nonuser EO Group" maintained a statistical difference ($P = 0.0075$) when compared to the "Control Group"; the "User EO Group" did not display a statistical difference in relation to the "Control Group." In the FP, no statistical difference between groups was observed (Table 3).

The discomfort factor presented a score with no statistical difference between the groups in the BP (Table 3). In the EP and DP, a statistical difference was identified between the "Nonuser EO Groups" and "Control Group." However, the "User EO Group" did not present statistical differences in comparison to the "Control Group" at any time. Regarding the sedation factor, no variations were observed that could be attributed to the inhalation of the EO.

3.4. Evaluation of the Effects of the Essential Oil of Citrus aurantium L. and Placebo on the Measured Physiological Parameters. Regarding the physiological parameters evaluated, Table 4 presents the variation of the SBP. The groups did not display significant differences in the BP. In the EP, the "Control Group" experienced an increase in mean SBP and a significant difference was observed between the "Control Group" and the "Nonuser EO Group." Although the "User EO Group" did not display a statistical difference in relation to the "Control Group" in the EP, the mean of the SBP was reduced.

Table 3: Presentation of the mean and standard error (Ep) of the HAS factors (anxiety, cognitive impairment, and discomfort) in the different phases of the Simulated Public Speaking (SPS).

Groups	Phases of application of the SPS, mean (Ep)			
	Basal	Stressor	During	Final
Anxiety				
Control	33 (4,921)	50 (5,615)	44 (6,058)	26 (4,28)
OE Nonuser	26 (4,213)	23* (3,369)	27# (4,834)	23 (4,389)
OE User	29 (3,829)	35* (3,52)	34 (3,014)	25 (3,409)
P value	0,5603	0,0002	0,0530	0,8730
Cognitive impairment				
Control	37 (2,123)	46 (5,314)	41 (5,152)	35 (4,147)
OE Nonuser	30 (3,928)	25* (2,873)	22[2] (3,293)	23 (3,71)
OE User	30 (3,212)	30* (3,66)	29 (3,709)	30 (3,579)
P value	0,1893	0,0018	0,0075	0,0937
Discomfort				
Control	25 (2,925)	35 (3,665)	35 (3,755)	25 (3,254)
OE Nonuser	21 (2,296)	21[1] (2,425)	22[2] (2,558)	20 (2,244)
OE User	31 (4,767)	28 (4,571)	29 (4,574)	27 (4,937)
P value	0,1746	0,0344	0,0361	0,3051
Sedation				
Control	35 (3,306)	26 (3,056)	29 (3,393)	26 (3,851)
OE Nonuser	32 (3,661)	30 (4,811)	25 (4,076)	25 (3,946)
OE User	34 (4,837)	37 (5,715)	37 (5,094)	34 (5,521)
P value	0,4426	0,3428	0,1201	0,3066

Source: Direct Research 2015. *Statistical Test:* ANOVA and Bonferroni posttest. [1]Significantly different from the "Control Group" at the time Stressor. [2]Significantly different from the "Control Group" at the time. ∗ is equivalent to number 1. # is equivalent to number 2.

Table 4: Presentation of the mean and standard error (Ep) of the systolic blood pressure in the different phases of the Simulated Public Speaking (SPS).

Groups	Phases of application of SPS, mean (Ep)			
	Basal	Stressor	During	Final
SBP				
Control	130 (3,146)	141 (3,997)	140 (3,127)	130 (3,285)
OE Nonuser	125 (3,122)	125[1] (2,516)	125[2] (3,113)	121 (2,361)
OE User	135 (2,942)	133 (3,338)	135 (2,806)	130 (3,149)
P value	0,0744	0,0071	0,0041	0,0624

Source: Direct Research 2015. *Statistical Test:* ANOVA and Bonferroni posttest. [1]Significantly different from the "Control Group" at the time Stressor. [2]Significantly different from the "Control Group" at the time.

In the DF group, a statistical difference between the "Control Group" and the "Nonuser EO Group" was observed. In the FP, all groups presented with a lower mean SBP, although no statistical differences were found.

The physiological measures of Diastolic Blood Pressure, Heart Rate, Electrical Conductance of the Skin, and End Temperature were also measured and analyzed. However, there were no significant differences between the variables in any of the test groups at any of the phases evaluated that could be attributed to the inhalation of the EO.

4. Discussion

Anxiety, specifically generalized anxiety disorder (GAD), has increased in drug users [24]. Crack users are also presenting with increased anxiety disorders at a high frequency. These disorders are identified in studies as a frequent comorbidity among users and regular use of the substance presents a significant relationship with the presence of anxiety disorders [25–27].

It has been previously reported that crack users demonstrate increased anxiety levels. Studies indicate that younger individuals have higher anxiety scores compared to older ones [28, 29]. In the present study, results indicated that a mean age of 30 years presented with greater anxiety levels than what had previously been reported. The data indicated that active crack users had the highest average of trait anxiety scores when compared to the "Nonuser EO Group" and the "Control Group."

Crack results in an increase in dopamine concentration in the reward system at a much greater level than natural stimuli. The repeated activation of the reward system generates a

learning mechanism that modulates behavior in a progressive way to seek the drug. In a short period of time, which varies between individuals and amount of consumption, the individual enters into a neurophysiological exhaustion of sensations of reward and sensitization of the mesolimbic pathways. This subsequently compromises additional neurobiological systems; among the systems, we can mention that the hypothalamic-pituitary-adrenal system is normally activated during drug withdrawal. Alteration of this system is directly related to changes in the state of anxiety and stress and, depending on the intensity and frequency, alters the anxiety traits of the individual [30].

Crack users also try to control the drug-obsessed quest and anxiety with the help of individual harm reduction strategies. In the studies conducted, users have reported strategies for craving relief and pharmacological and behavioral tactics to avoid their development such as, eating, having sex, playing soccer, working, avoiding the social context of using crack, and using drugs that cause drowsiness [31].

During the experiment, the groups that received *Citrus aurantium* L. EO by inhalation, "User EO Group" and "Nonuser EO Group," demonstrated lower scores of the IDATE-E during the EP of the SPS. Conversely, the "Control Group" showed a higher score and a significant difference with the other groups during that phase. Although the crack user group is more likely to react anxiously to a situation identified as threatening, anxiety is maintained at controlled levels at the time of SPS intervention.

Levels of anxiety measured by HAS in the "Control Group" were increased during the EP and MP, with a significant difference in comparison to the groups that inhaled the EO. The "Nonuser EO Group" had reduced anxiety levels in the EP and these levels remained controlled in the DP. The "User EO Group" presented a slight increase in anxiety levels in the EP and DE. The difference in anxiety levels of the groups reinforces the anxiolytic hypothesis of inhaled *Citrus aurantium* L. EO prior to the SPS experiment. Although the participants in this study did not know which EO they were inhaling, they stated that the EO odor was not unknown to them. As pleasant aromas may induce a state of wellbeing in people, do not rule out a possible placebo effect.

It is reasonable to believe that the anxiolytic effects observed in this study were due to EO; nonclinical studies have shown anxiolytic effects of inhaled *Citrus aurantium* L. EO in rats. The effect was observed on the behavior of the animals when developing specific tests [13, 14]. In one study, in addition to observing significant anxiolytic activity of the EO, they presented results that strongly suggest the involvement of 5-HT1A receptors, a subtype of serotonin receptors, presenting a possible pathway of action [10].

A clinical study performed on patients awaiting dental care consisted of exposing subjects to the inhalation of orange EO in the waiting room. Subjects who were exposed to the EO were found to be calmer with a lower level of anxiety state [32]. Results indicated anxiolytic properties of sweet orange EO in healthy subjects while developing an anxiogenic task, evidenced by the significant difference in state anxiety levels between the group exposed to the aroma and the Control Group [8].

Clinical trials that used *Citrus aurantium* L. on anxiety reduction obtained satisfactory results [33, 34]. Preoperative patients received a *Citrus aurantium* L. bloom two hours prior to the procedure, using the IDATE as an instrument for measuring anxiety and, comparing with the Control Group, the authors identified a reduction in preoperative anxiety in outpatient surgery of the experimental group [33]. Additionally, a study performed on patients with chronic myeloid leukaemia exposed patients to the EO prior to the procedure for collecting medullary material. Data indicated that patients who inhaled the EO *Citrus aurantium* L. presented a decrease in the IDATE-E score and remained relaxed during the procedure. EO, even used in only a single dose, presented similar performance levels to the anxiolytic diazepam and demonstrated efficacy in the anxiety control of patients undergoing an unpleasant diagnostic procedure [11].

This study focused on crack users; the EO also presented an anxiolytic effect in a group that lives daily with anxiety at different levels, one of the largest problems experienced in the period of abstinence and maintenance of drug abandonment. Research has been performed to evaluate alternative anxiety control methods that could be utilized by crack users. Cooperative games and respiratory relaxation have been effective in reducing cravings and anxiety levels in addicted crack users, and the results allow a novel therapeutic approach, suggesting viable and effective strategies for the management of cravings and anxiety symptoms in crack dependents [3, 34, 35].

Cognitive impairment was another factor measured by HAS. The Control Group presented increased cognitive impairment at test times, which was different from the EO treated groups. The "User EO Group" maintained a level of cognitive impairment at the Stressor Phase and decreased at the During Phase. The "Nonuser EO Group" reduced the level at the two intermediate stages of the test.

The increase in anxiety levels reflects the cognitive changes that impair the individual's performance against certain tasks. The results demonstrate that individuals who inhaled the EO of *Citrus aurantium* L. showed no change in cognitive impairment. The effect of EO on crack users is highlighted, since drug abuse causes cognitive alterations. Results identified neurocognitive impairments in crack dependents, such as changes in attention tests, verbal fluency, visual memory, verbal memory, learning ability, and executive functions [36].

Anxiety is accompanied by a sense of discomfort due to the anticipation of danger or something that is unknown. During SPS, the "Control Group" indicated an increase in discomfort levels with a significant difference in comparison to the "Nonuser EO Group." Throughout the phases of the test, although the "User EO Group" did not present with statistical differences compared to the "Control Group," the crack users began at a higher level of discomfort than the other groups and reduced this level in the following phases. The EO of *Citrus aurantium* L. controlled the levels of anxiety, in addition to allowing the individuals to remain comfortable during the accomplishment of the anxiogenic task.

The physiological measures evaluated indicated that SPS produced experimental anxiety, as demonstrated in previous studies [6, 37], evidenced by changes from the baseline values

of each measurement, mainly between the BP and EP. A transient emotional state, such as anxiety, is strongly marked by tension, apprehension, and activation of the autonomic nervous system and tends to increase blood pressure, heart rate, skin conductance, and lower extremity temperature [38, 39].

Among the measured physiological measures, only SBP presented variations that could be attributed to the inhalation of *Citrus aurantium* EO. In one study, a decrease in SBP was observed by the group exposed to *Citrus aurantium* L. EO, with a similar effect to the group that received diazepam [11]. Based on the results presented, researchers observed satisfactory results of *Citrus aurantium* L. EO in decreasing the parameters of DBP and HR, postulating that the effects of EO on the physiological parameters suggest a decrease in autonomic excitability. Aromatherapy appears to modulate the activities of the autonomic nervous system toward equilibrium [40].

5. Conclusion

By analyzing the results obtained in this clinical trial, it can be concluded that individuals who experience crack withdrawal present a high anxiety trait. Administration by nebulization of *Citrus aurantium* L. EO is indicated to be efficient in controlling the psychological parameters of anxiety in individuals exposed to an anxiogenic task. As for the physiological measures, only the SBP of the "Nonuser EO Group" remained constant during the test. The EO of *Citrus aurantium* L. showed no effect on the additional measured physiological measures, suggesting that the pathways of the EO action do not have an effect on the autonomic system.

The EO of *Citrus aurantium* L., administered by nebulization, showed an acute anxiolytic effect in crack users in abstinence. This previously unreported finding has great clinical relevance when presenting a viable alternative of complementary therapy in the control of anxiety in users who are abandoning the use of drugs. Additional studies are required to increase the knowledge of the use of aromatherapy, the duration, and the time of action of the antianxiety effect of the EO in the control of anxiety in users of crack at times other than withdrawal.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Modified Suanzaorentang Had the Treatment Effect for Generalized Anxiety Disorder for the First 4 Weeks of Paroxetine Medication: A Pragmatic Randomized Controlled Study

Ming-Fen Song,[1,2] Lin-Lin Hu,[3] Wen-Juan Liu,[3] Yi Liu,[3] Xiao-Yun Tao,[1] Ting-Ting Wang,[1] Sheng-Dong Wang,[2] Long Zhang,[2] and Yong-Hua Zhang[1,3]

[1]*Internal Medicine of Traditional Chinese Medicine, The 2nd Clinical Medical College, Zhejiang Chinese Medical University, 548 Binwen Road, Hangzhou, Zhejiang 310053, China*
[2]*Molecular Biology Laboratory, Hangzhou Seventh People's Hospital, 305 Tianmushan Road, Hangzhou, Zhejiang 310013, China*
[3]*Psychosomatic Disorders Department, Hangzhou Seventh People's Hospital, 305 Tianmushan Road, Hangzhou, Zhejiang 310013, China*

Correspondence should be addressed to Yong-Hua Zhang; zyh16916@163.com

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Background. Paroxetine does not show satisfactory therapeutic effect for generalized anxiety disorder (GAD) patients for the first 2–4 weeks of medication. Diazepam is always concurrently used although it has some shortcomings such as physical dependence and withdrawal reactions. In this study, we aimed to identify whether modified Suanzaorentang (MSZRT), a combined Chinese formula including Suanzaorentang (SZRT) and Zhizichitang (ZZCT), could control the anxiety of GAD for the first 4 weeks of paroxetine medication. *Methods*. 156 GAD patients were randomized to the treatment of paroxetine, paroxetine-diazepam, or paroxetine-MSZRT for 4 weeks. Hamilton Anxiety Scale (HAMA) Test and Self-Rating Anxiety Scale (SAS) Test were determined each week as the evaluation of clinical efficacy. Adverse events (AEs) were also closely observed by performing the Treatment Emergent Symptom Scale (TESS) Test. *Results*. Both paroxetine-MSZRT and paroxetine-diazepam decreased more HAMA and SAS total scores than paroxetine from weeks 1 to 3. Paroxetine-MSZRT as well as paroxetine-diazepam had an obviously higher onset rate than paroxetine in each week. After 4 weeks' treatment, the overall effectiveness rate in the paroxetine-MSZRT group (90.00%) was obviously higher than those of the paroxetine group (74.42%) but did not significantly differ from the paroxetine-diazepam group (93.88%). *Conclusion*. MSZRT had the treatment effect for GAD when paroxetine was used for the first 4 weeks.

1. Introduction

Generalized anxiety disorder (GAD) is one of the common neuroses with lifetime prevalence rate of 4–7% in general population [1, 2] and characterized by excessive, uncontrollable, and often irrational worry [2–4]. Chemical drugs have been widely used for the treatment of GAD worldwide. For example, benzodiazepines, which enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) via targeting $GABA_A$ receptor, are classic anxiolytics for GAD [5, 6]. But benzodiazepines are not recommended for a long-term treatment, because they are associated with tolerance, psychomotor impairment, cognitive and memory changes, physical dependence, and withdrawal reaction on discontinuation [7]. However, 40% of GAD patients had reported illness duration lasting >5 years [8] and a long-term efficacious and safe protocol is required [9, 10].

In recent years, paroxetine is widely used for the long-term continuing treatment of GAD [8, 11]. However, its anxiolytic response in GAD patients represents an obviously

delayed process [12]. This delayed response causes patients to doubt paroxetine treatment and then decrease their compliance. In order to control anxiety as soon as possible, benzodiazepines such as diazepam are still concurrently given with paroxetine for a short time although they have some shortcomings mentioned above.

Chinese herbal medicine, as one of the most popular complementary therapies of Western medicine, usually in herbal formula, has been commonly used and widely accepted. Recently, our research group has been trying to establish an alternative to benzodiazepines by using Chinese herbal medicine when paroxetine is used for GAD treatment.

Suanzaorentang (SZRT), a formula of five medicinal Chinese herbs including Semen Zizyphi Spinosae (Suanzaoren), Sclerotium Poriae Cocos (Fuling), Radix Ligustici Chuanxiong (Chuanxiong), Rhizoma Anemarrhena (Zhimu), and Radix Glycyrrhizae (Gancao), is beneficial for the replenishment of yin and proposed to have the tranquillizing effect according to *Jin Gui Yao Lve* written by Zhong-Jing Zhang in approximately 210 AD. Thus, SZRT was selected as the basic Chinese medicine for the treatment of GAD in this study. Besides SZRT, we added Zhizichitang (ZZCT), which was also documented in *Jin Gui Yao Lve*, comprising Zhizi (*Gardenia jasminoides* fruit), Dandouchi (Fermented Soybean), Chanyi (periostracum cicada), and aids in eliminating internal fire. We named this new combined formula of SZRT and ZZCT as modified SZRT (MSZRT) in this study. Yin-deficiency and fire-excess syndrome are common in GAD patients and often exhibit somatic symptoms such as insomnia, palpitation, restlessness, headache, and rapid pulse, flash, and excessive sweating. Therefore, the combination of these two classic compounds may perfectly complement each other in sedative effect for GAD patients. Based on our experience, we predicted that MSZRT may display its sedative effect right after medication. However, the evidence about the treatment efficacy and safety of MSZRT on GAD patients is still lacking.

In the present study, we aimed to identify whether MSZRT could control the anxiety and solve the delayed therapeutic effect when paroxetine is used for GAD patients. 156 subjects were recruited and randomly assigned to receive the treatment of paroxetine, paroxetine-diazepam, or paroxetine-MSZRT. The treatment efficacy was compared among groups. In addition, adverse events (AEs) were also closely observed to ensure the tolerance of MSZRT.

2. Materials and Methods

2.1. Recruitment of Subjects. This study was approved by the Ethics Committee of Hangzhou Seventh People's Hospital. The written informed consent from each subject was obtained before study. They were allowed and free to withdraw from this study for any reasons at any time.

First of all, inpatients of Psychosomatic Disorders Department in our hospital, diagnosed as GAD by two experienced psychiatrists based on DSM-V and treatment-free within 2 months, were recruited. Next, patients were estimated with Hamilton Anxiety Scale (HAMA) Test by a trained clinician and Self-Rating Anxiety Scale (SAS) by themselves. In this study, participants were required to have a score ≥ 14 on HAMA and ≥ 50 on SAS at baseline.

It was reported that GAD patients with a depressive episode, either of MDD or of BPD, can also have a high HAMA total score [12]. To purify the subjects, we also determined Hamilton depression scale (HAMD) for each participant and those with score ≥ 7 were excluded in the present study. Furthermore, those patients with evidence of drug abuse, drinking, cognitive impairment, and physical illness such as diabetes, severe hypertension, cardiovascular and cerebrovascular diseases, malignant diseases, respiratory diseases, or autoimmune infections were also excluded.

156 subjects (69 men and 87 women) from January, 2015, to March, 2016, meeting our inclusion and exclusion criteria, were recruited. All subjects were randomly assigned to receive the treatments of paroxetine, paroxetine-diazepam, or paroxetine-MSZRT. Finally, 14 subjects quit this study due to serious AEs or anxiety deterioration during the whole study period (9: anxiety deterioration from the paroxetine group; 1: dizziness, 2: constipation from the paroxetine-diazepam group; 2: diarrhea from the paroxetine-MSZRT group). Finally, 43 cases (17 men and 26 women) in the paroxetine group, 49 cases (21 men and 28 women) in the paroxetine-diazepam group, and 50 cases (22 men and 28 women) in the paroxetine-MSZRT group were analyzed.

2.2. Drug Preparation. Daily dose of MSZRT formula for each patient comprised Suanzaoren (Semen Zizyphi Spinosae) 15 g, Zhimu (Rhizoma Anemarrhena) 12 g, Fuling (Sclerotium Poriae Cocos) 15 g, Chuanxiong (Radix Ligustici Chuanxiong) 10 g, Zhizi (*Gardenia jasminoides* fruit) 10 g, Dandouchi (Fermented Soybean) 6 g, Chanyi (periostracum cicada) 6 g, and Zhigancao (Radix Glycyrrhizae) 6 g. All herbs were purchased from Medicinal Materials Co. Ltd. (Lin'an City, Zhejiang Province, China). They were mixed and prepared as 400 ml of decoction solution according to traditional methods and packed into two bags. Paroxetine (20 mg/tablet) was obtained from Tianjin Smith Kline & French laboratories Ltd., China. Diazepam (2.5 mg/tablet) was purchased from Beijing Yimin Pharmaceutical Co., Ltd., China.

2.3. Patient Treatments. Subjects in three groups took paroxetine 20 mg/day half an hour after breakfast in the first week. From second week, they were allowed to increase paroxetine dose. The maximum dose during the study period was 60 mg/day if judged clinically necessary by the investigator. Meanwhile, the paroxetine-diazepam group received 2.5 mg of diazepam three times daily as recommended by the manufacturer. The paroxetine-MSZRT group received two bags of the MSZRT decoction per day and drank them half an hour after breakfast and supper based on the traditional administration method for Chinese herbal formula. No other medications or psychotherapy were permitted during study period.

2.4. Efficacy Evaluation. HAMA total scores at baseline and weeks 1, 2, 3, and 4 after treatment were evaluated as the

Table 1: The demographic characteristics of subjects.

Variables	Paroxetine	Paroxetine-diazepam	Paroxetine-MSZRT	Statistical analysis	
				F/χ^2 value	P value
Number of subjects (n)	43	49	50		
Sex (male/female)	17/26	21/28	22/28	0.20	0.91
Marriage (married/single)	32/11	29/20	35/15	2.63	0.27
Age in year	50.60 ± 12.84	47.94 ± 12.10	48.96 ± 12.87	0.56	0.57
BMI (kg/m^2)	21.82 ± 2.49	21.12 ± 2.30	20.94 ± 2.15	2.00	0.14
Education (year)	14.34 ± 4.34	13.69 ± 4.75	14.74 ± 4.92	0.63	0.53

Data are expressed as mean ± standard deviation.

primary outcome measurement by a trained clinician, who was blind to the treatment for each patient. Subjects also performed SAS test at all observation points as the secondary outcome measurement to confirm the results obtained from HAMA test.

When the reduction rate of HAMA total score first reached ≥25% as compared with baseline, the treatment onset was considered.

At the end of the observation, we judged the treatment efficacy for each participant based on the reduction rate of HAMA total score compared to baseline. The reduction rate ≥ 75% was considered as clinical control, 50–75% was regarded as marked effectiveness, 25–50% was viewed as effectiveness, and <25% was defined as ineffectiveness. Thus, when study is finished, we set a reduction rate ≥ 25% in the HAMA total score as overall effectiveness and <25% as ineffectiveness. We also considered HAMA total score ≤ 7 at the final observation as clinical remission. The overall effectiveness rate and clinical remission rate were calculated for each group.

2.5. Estimation of AEs. Throughout the study, the subjects were monitored closely for AEs. We used the Treatment Emergent Symptom Scale (TESS) to evaluate AEs including behavioral toxicity, laboratory examination, nerve system, autonomic nervous system, cardiovascular system, and others such as skin symptom, body weight, headache, and appetite. The value for each item on the scale ranged from zero to four: zero meant no AE, one indicated mild AE, two represented moderate AE, three showed severe AE, and four expressed very severe AE.

2.6. Statistical Analysis. All statistical analyses were conducted using SPSS 19.0 software package (SPSS Inc., America). Baseline demographic characteristics were compared by using independent sample ANOVA for continuous variables and χ^2 test for categorical variables. Repeated measurement ANOVA test was carried out for the comparisons of HAMA or SAS total scores at all observation points among three groups. χ^2 test was performed for the comparisons of onset rates, overall effectiveness rates, and clinical remission rates among three groups. Kruskal-Wallis H test followed by Nemenyi test was run to analyze the distribution difference of treatment efficacy among three groups.

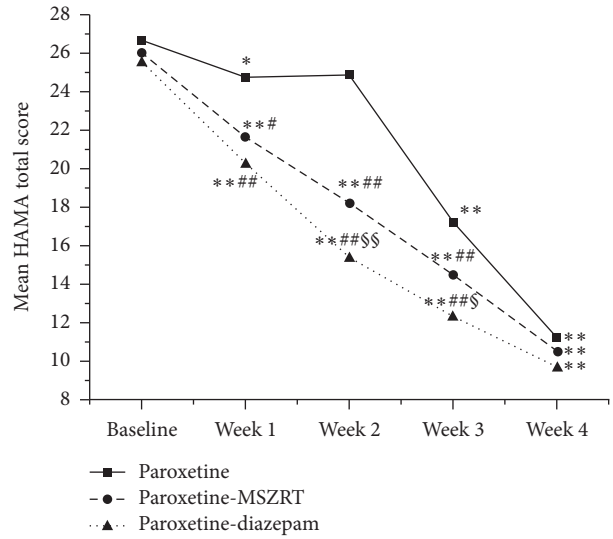

Figure 1: The mean HAMA total scores among the three groups. ∗ means $P < 0.05$ and ∗∗ depicts $P < 0.01$ as compared to the former weeks in each group. # means $P < 0.05$ and ## depicts $P < 0.01$ as compared to the paroxetine group. § means $P < 0.05$ and §§ depicts $P < 0.01$ as compared to the paroxetine-MSZRT group.

3. Results

3.1. Baseline Characteristics. The information about sex, marriage, age, BMI, and education of all analyzed subjects was collected and shown in Table 1. No significant difference in all demographic characters was observed among the three groups ($P > 0.05$). All subjects included in the present study were nonsmokers and nondrinkers to avoid their possible influences.

The doses of paroxetine were (40.40 ± 8.80) mg in the paroxetine group, (38.98 ± 7.70) mg in the paroxetine-diazepam group, and (38.00 ± 9.69) mg in the paroxetine-MSZRT group. They were not statistically different among the three groups ($F = 0.62$, $P = 0.54$).

3.2. Comparison of HAMA Total Scores. The change of HAMA total scores in three groups was shown in Figure 1. At week 1, as compared to those of baseline, the paroxetine group had slightly decreasing effect on HAMA total score (from 26.66 to 24.72, $P < 0.05$), while two combined treatments

Table 2: The onset case numbers in each week.

Onset numbers	Week 1 n (%)	Week 2 n (%)	Week 3 n (%)	Week 4 n (%)
Paroxetine	3 (6.98%)	6 (13.95%)	27 (62.79%)	32 (74.42%)
Paroxetine-diazepam	41 (83.67%)[a]	36 (73.47%)[d]	44 (89.80%)[g]	46 (93.88%)[j]
Paroxetine-MSZRT	34 (68.00%)[b,c]	39 (78.00%)[e,f]	43 (86.00%)[h,i]	45 (90.00%)[k,l]

At week 1, [a]$\chi^2 = 53.99$ and $P = 0.000$; [b]$\chi^2 = 35.94$ and $P = 0.000$ as compared with the paroxetine group. [c]$\chi^2 = 3.31$ and $P = 0.07$ as compared with the paroxetine-diazepam group.
At week 2, [d]$\chi^2 = 32.70$ and $P = 0.000$; [e]$\chi^2 = 37.92$ and $P = 0.000$ as compared with the paroxetine group. [f]$\chi^2 = 0.28$ and $P = 0.60$ as compared with the paroxetine-diazepam group.
At week 3, [g]$\chi^2 = 9.48$ and $P = 0.002$; [h]$\chi^2 = 6.69$ and $P = 0.01$ as compared with the paroxetine group. [i]$\chi^2 = 0.34$ and $P = 0.56$ as compared with the paroxetine-diazepam group.
At week 4, [j]$\chi^2 = 6.72$ and $P = 0.01$; [k]$\chi^2 = 3.94$ and $P = 0.047$ as compared with the paroxetine group. [l]$\chi^2 = 0.50$ and $P = 0.48$ as compared with the paroxetine-diazepam group.

exhibited obviously decreasing effect (from 26.02 to 21.63 in the paroxetine-MSZRT group, from 25.48 to 20.20 in the paroxetine-diazepam group, both $P < 0.01$). At week 2, the paroxetine group did not show a statistical change in HAMA total score while two combined groups were found to have a significant decrease (both $P < 0.01$) as compared to those of week 1. At weeks 3 and 4, each group had a continuous decrease in HAMA total score as compared with their former weeks (all $P < 0.01$). Paroxetine-diazepam seemed to decrease HAMA scores more than paroxetine-MSZRT at weeks 2 and 3 ($P < 0.01$ or $P < 0.05$). Following treatment for 4 weeks, no obvious difference in HAMA total scores was observed among the three groups ($P > 0.05$).

3.3. Comparison of Onset. The treatment onset in each group was shown in Table 2. Both paroxetine-MSZRT and paroxetine-diazepam had obviously higher onset rates than paroxetine alone from weeks 1 to 4 (all $P < 0.05$), especially at weeks 1 and 2. However, the difference between paroxetine-MSZRT and paroxetine-diazepam did not reach statistical significance ($P > 0.05$).

3.4. Comparison of SAS Scores. The results of self-reported SAS scores were similar to clinician-rated HAMA scores (Figure 2). In brief, paroxetine did not show obvious therapeutic effect at week 1 ($P = 0.13$) and week 2 ($P = 0.22$) as compared with baseline. From weeks 3 and 4, a significant decrease of SAS score was observed in this group ($P < 0.01$ comparing week 3 versus week 2 and week 4 versus week 3). However, paroxetine-diazepam and paroxetine-MSZRT kept decreasing SAS scores from week 1 to week 4 ($P < 0.01$). Paroxetine-diazepam was also observed to decrease SAS scores more than paroxetine-MSZRT at weeks 2 and 3 ($P < 0.01$ or $P < 0.05$). At the final observation, there was no statistical difference among the three groups ($F = 2.61$, $P = 0.08$).

3.5. Comparison of Treatment Efficacy. The treatment efficacy in the three groups was showed in Table 3.

The overall effectiveness rate was 74.42%, 93.88%, and 90.00% for the paroxetine, paroxetine-diazepam, and paroxetine-MSZRT group, respectively. Although the two

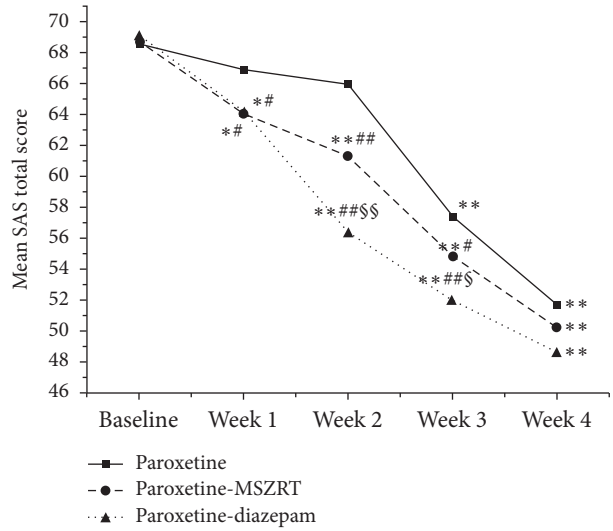

Figure 2: The mean SAS total scores among the three groups. * means $P < 0.05$ and ** depicts $P < 0.01$ as compared to the former weeks in each group. # means $P < 0.05$ and ## depicts $P < 0.01$ as compared to the paroxetine group. § means $P < 0.05$ and §§ depicts $P < 0.01$ as compared to the paroxetine-diazepam group.

combined treatment groups did not have obvious difference in overall effectiveness rate ($\chi^2 = 0.50$, $P = 0.48$), they were significantly higher than that of the paroxetine group ($\chi^2 = 5.54$, $P = 0.02$ for the paroxetine-diazepam group; $\chi^2 = 3.94$, $P = 0.047$ for the paroxetine-MSZRT group). After 4 weeks' treatment, the distribution of overall effectiveness among the three groups was not statistically different ($\chi^2 = 0.48$, df = 2, $P = 0.79$).

The clinical remission rates in the three groups were 9.30%, 12.24%, and 10.00%. There was no significant difference among the three groups ($\chi^2 = 0.24$, $P = 0.89$).

3.6. Evaluation of AEs. The subjects in this study reported AEs mainly in gastrointestinal system and some feelings such as drowsiness, dizziness, and headache as shown in Table 4. We did not calculate the mean score of each item because most of them were evaluated as 0. The incidence rates of

Table 3: The treatment efficacy among three groups.

Efficacy	Paroxetine *n* (%)	Paroxetine-diazepam *n* (%)	Paroxetine-MSZRT *n* (%)
n	43	49	50
Overall effectiveness	32 (74.42%)	46 (93.88%)[a]	45 (90.00%)[b,c]
Clinical control	7 (16.28%)	7 (14.29%)	9 (18.00%)
Marked effectiveness	20 (46.51%)	27 (55.10%)	26 (52.00%)
Effectiveness	5 (11.63%)	12 (24.49%)	10 (20.00%)
Ineffectiveness	11 (25.58%)	3 (6.12%)	5 (10.00%)
Clinical remission	4 (9.30%)	6 (12.24%)[d]	5 (10.00%)[e,f]

[a] $\chi^2 = 5.54$, $P = 0.02$ as compared with the paroxetine group.
[b] $\chi^2 = 3.94$, $P = 0.047$ as compared with the paroxetine group.
[c] $\chi^2 = 0.50$, $P = 0.48$ as compared with the paroxetine-diazepam group.
[d] $\chi^2 = 0.21$, $P = 0.65$ as compared with the paroxetine group.
[e] $\chi^2 = 0.01$, $P = 0.91$ as compared with the paroxetine group.
[f] $\chi^2 = 0.13$, $P = 0.72$ as compared with the paroxetine-diazepam group.

Table 4: Adverse events in the three groups.

Groups	Nausea *n* (%)	Loss of appetite *n* (%)	Diarrhea *n* (%)	Constipation *n* (%)	Drowsiness *n* (%)	Dizziness *n* (%)	Headache *n* (%)	Sexual dysfunction *n* (%)
Paroxetine	6 (13.95%)	10 (23.26%)	4 (9.30%)	13 (30.23%)	11 (25.58%)	10 (23.26%)	5 (11.63%)	7 (16.28%)
Paroxetine-diazepam	6 (12.24%)	6 (12.24%)	3 (6.12%)	17 (34.69%)	13 (26.53%)	9 (18.37%)	6 (12.24%)	6 (12.24%)
Paroxetine-MSZRT	5 (10.00%)	7 (14.00%)	6 (12.00%)	15 (30.00%)	9 (18.00%)	6 (12.00%)	2 (4.00%)	8 (16.00%)
χ^2	0.35	2.32	1.03	0.31	1.20	2.05	2.48	0.39
P value	0.84	0.31	0.60	0.86	0.55	0.36	0.29	0.83

nausea, loss of appetite, diarrhea, constipation, drowsiness, dizziness, headache, and sexual dysfunction were not significantly different among the three groups ($P > 0.05$).

No abnormal laboratory changes were observed in any of the patients from the initial screen to the last evaluation.

4. Discussion

In the present clinical trial, we determined whether MSZRT could control the anxiety and solve the delayed therapeutic response when paroxetine is used for GAD patients for the first 4 weeks of medication.

Clinical trial can be designed to be either pragmatic or explanatory [13]. Explanatory trials are designed to find out whether a treatment has any efficacy (usually compared with placebo) under ideal, experimental conditions [13]. However, it is a big challenge to perform an explanatory clinical trial in the study of Traditional Chinese Medicine due to the fact that a suitable solution as the placebo of Chinese herbal medicine is still lacking. Fortunately, a widely accepted study design, pragmatic clinical trial, has been developed for this situation. Pragmatic trials are designed to find out about how effective a treatment actually is in routine, everyday practice. They are used to test an overall "package" of care, including the contribution of the therapeutic relationship, patients' expectations, and any specific therapy that is used. They compare the effect of this package of care with another treatment, not with a placebo. Thus, the pragmatic design is especially useful where the use of a placebo control to separate specific from nonspecific effects is problematic. In addition, blindness in this type of study is not as strict as explanatory trial. Participants are allowed to know what are the treatments they receive [13].

Our present study is a pragmatic trial, which exactly reflects our everyday clinical practice. The reasons why we designed this study as a pragmatic trial are as follows. First, we could not find a satisfactory solution as the placebo for MSZRT, which is similar to MSZRT in color, taste, and smell. Second, we wanted this study to reflect the routine treatment of Chinese herbal medicine in the medication method. Recently, some researchers manufactured the extract of Chinese herbal medicine into powder or granules, while their placebo was prepared with starch or artificial pigments in the same form and similar color, smell, and taste [14, 15]. It was a great way to solve the problem of placebo. However, we have never used powder or granules of MSZRT in our clinical practice before. Because powder or granules may present different treatment effects from decoction solution based on the Traditional Chinese Medicine theory. The decoction solution drinking is believed to be the best way to exert treatment effects of Chinese herbal medicine. That is why the decoction solution has been used for the medication method for a long history about thousands of years and it still is the most classical and widely accepted method in Traditional Chinese Medicine. Thus, in this study, we chose the decoction solution of MSZRT for the paroxetine-MSZRT group in order to keep the same medication method between research and clinical real use.

Although paroxetine is an efficacious approach for GAD, it has obvious delayed therapeutic onset. A study showed that the effect of paroxetine was seen in patients with somatic anxiety after 3-4 weeks and in patients with cognitive anxiety after 3–6 weeks [16]. The mechanism of this delayed effect is still unknown. It was reported that the steady state levels of paroxetine in body were achieved after 4–14 days of medication [17]. The time required for steady levels of paroxetine to exert their full effects through a reaction cascade after drug intake was considered to contribute the delayed effect of paroxetine [17]. On the other hand, 5-HT_{1A} autoreceptors were also thought to be involved. It was reported that 5-$HT_{1A/1B}$−/− mice induced a strong anxious-like behavioral state [18]. The 5-HT_{1A} receptor antagonist pindolol had been combined with SSRIs in patients with anxiety disorders to shorten the onset of the clinical action and increase the proportion of responders [19]. It was believed that serotonergic negative feedback mediated by 5-HT_{1A} autoreceptors to decrease the synthesis and release of 5-HT after SSRI medication was associated with the delayed therapeutic effect [20–22]. Thus, 5-HT_{1A} autoreceptors desensitization was required before SSRI exerting effect [23]. In the present study, we also observed a deterioration of anxiety during paroxetine treatment in some cases. Its cause or mechanism remains unknown. A paper reported that 5-HT_{2A} receptors were considered to be involved and their activation may attenuate paroxetine-induced anxiety [24].

In our previous study, we found that SZRT together with Zhi Zi Chi Tang, which was the same formula as MSZRT in the present study, could decrease SAS scores and improve daytime function in insomniacs with anxiety [25]. Insomnia is one of the common symptoms of GAD [26] or it is frequently cooccurring [27]. In our current study, we hypothesized that MSZRT may also have treatment effect for GAD due to its role of tranquillization according to TCM theory. Our results showed that paroxetine-MSZRT decreased HAMA and SAS total scores obviously during the study period (Figures 1 and 2). In addition, paroxetine-MSZRT had obviously higher onset rates (Table 2) and overall effectiveness rates (Table 3) than paroxetine. These suggested that MSZRT had the ability to control the anxiety of GAD for the first 4 weeks of paroxetine medication. However, paroxetine-MSZRT showed less effective actions in decreasing HAMA and SAS total scores as compared to those of paroxetine-diazepam at weeks 2 and 3. This may be due to the fact that Chinese herbal formula focuses on adjusting system balance in body and always shows moderate role for disease treatment.

The mechanism of MSZRT on GAD treatment is still unknown. It was reported that the effect of SZRT may be associated with serotonergic system based on the fact that SZRT exhibited binding affinity for serotonin receptors [28] and the sleep regulation effect of SZRT was blocked via using 5-HT_{1A}, 5-HT_2, and 5-HT_3 antagonists [29]. GABAergic system was also considered to be involved in the mechanism of SZRT effect through $GABA_A$ receptor associated chloride channel [30]. In addition, Yang et al. reported that the components of amino acid and fatty acid in SZRT would also be in response to the treatment effect through immune and nervous system [28]. Although ZZCT is also a common Chinese formula used for sedation and has the synergistic effect together with SZRT, its underlying mechanism with/without SZRT has seldom been studied. Thus, further researches to elucidate the possible mechanism of MSZRT for GAD treatment are needed.

The most AEs in the paroxetine-MSZRT group were some mild gastrointestinal reactions such as nausea, diarrhea, anorexia, dry mouth, and some whole body feelings such as drowsiness, dizziness, and headache, which were also observed in the other two groups of this study and reported by other authors previously. For example, Lai et al. found that dizziness, headache, stomach ache, and diarrhea were probably related to SZRT treatment [31]. In this study, we did not find any significant difference in the incidence of each AE item among three groups (Table 4). These results suggested that MSZRT was generally well tolerated for GAD patients. Sexual side effect is commonly associated with SSRI treatment. Some evidence indicated that the activation of 5-HT_{1A} receptors mitigated SSRI-induced sexual dysfunction [32, 33]. However, MSZRT seemed to have no alleviation effect on sexual side effect in the present study (Table 4).

However, our study has limitations. First, MSZRT treatment effect included some placebo effect and we could not exclude this part in the analysis of data. This is the main limitation of a pragmatic design as compared with an explanatory design. Second, in order to protect patients' right to receive effective treatments and avoid conflict with the ethics, we did not use MSZRT alone to treat GAD patients due to its uncertain effect so far. Third, this study was carried out for only 4 weeks because the delayed effect of paroxetine usually occurs within 4 weeks and if paroxetine still did not work after 4 weeks' medication, the treatment protocol should be changed according to the Ethics Committee of our hospital. Fourth, we have not performed the mechanism study of MSZRT on GAD treatment in the present study. Further investigations with placebo, MSZRT-alone treatment, longer observation, and the underlying mechanism of MSZRT are required.

5. Conclusions

Our results suggested that MSZRT exhibited the anxiety-controlling effect for GAD by decreasing HAMA and SAS total scores, enhancing onset rate and overall effectiveness rate for the first 4 weeks of paroxetine treatment, which was also observed when diazepam was used. Thus, we recommend MSZRT as an alternative to diazepam and concurrent use of MSZRT and paroxetine as a new protocol for the treatment of GAD during this period.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Anticonvulsant and Neuroprotective Activities of *Phragmanthera austroarabica* Extract in Pentylenetetrazole-Kindled Mice

Hibah M. Aldawsari,[1] Basma G. Eid,[2] Thikrayat Neamatallah,[2] Sawsan A. Zaitone,[3] and Jihan M. Badr[4,5]

[1]*Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia*
[2]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia*
[3]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt*
[4]*Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia*
[5]*Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt*

Correspondence should be addressed to Jihan M. Badr; jihanbadr2010@hotmail.com

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Anticonvulsant and neuroprotective activity of *Phragmanthera austroarabica* extract were tested in pentylenetetrazole-kindled mice. All the chemical constituents of the plant extract were identified. Additionally, the extract was standardized and proved to contain total phenolic contents equal to 379.92 ± 1.32 mg gallic acid equivalents/g dry plant extract. Induction of kindling was achieved by repeated intraperitoneal administration of pentylenetetrazole (35 mg/kg) twice weekly. Male albino mice were given *P. austroarabica* extract (200, 400, or 800 mg/kg). The two higher doses (400 or 800 mg/kg) of the extract significantly caused notable reduction in seizure activity and hippocampal malondialdehyde level compared to pentylenetetrazole control group. The highest dose enhanced cortical GSH level and showed intact DNA in the laddering assay. Upon studying the neuroprotective effect, mice treated with the higher dose of the extract demonstrated an improvement in the percent of surviving neurons in the cortex and hippocampus. We concluded that *P. austroarabica* extract ameliorated seizure activity and protected cortical and hippocampal neurons against pentylenetetrazole-induced kindling in mice.

1. Introduction

A crucial challenge is to discover the causes of the variety of neurodegenerative diseases. This will allow the prevention or slowing of the resultant disorders. In many neurodegenerative diseases, oxidative stress is considered as a common factor. Additionally, it is recognized as the most probably proposed mechanism for degenerative processes related to age [1, 2]. A considerable number of previous studies illustrated a strong evidence linking oxidative stress to a number of neuronal diseases as Alzheimer's disease [3, 4], Parkinson's disease [5, 6], and multiple sclerosis [7, 8]. Damage of the neuronal cells is mediated by reactive oxygen species. Accumulation reactive oxygen species causes peroxidation of lipid and damage of DNA and protein and finally may lead to cell death [9]. Antioxidants have the ability of inhibiting reactive oxygen species. A considerable number of natural extracts proved to possess neuroprotective activity [10, 11]. There is evidence that seizures lead to neuronal loss and brain damage. Moreover, death of cell in discrete brain regions is considered as one of the characterizing anatomical features of epilepsy [12]. Brain regions that are more vulnerable to seizure disorders include the amygdala and the hippocampus in addition to the neighboring piriform and entorhinal cortex [13, 14]. The role of oxidative stress and free radicals in seizure disorders has been documented

extensively [15, 16]. Pentylenetetrazole (PTZ) is chemical toxin reported to interact with GABA-A receptor complex in the adult brain [17]. It can reach the brain through successful transportation via blood-brain barrier [18]. Furthermore, animal data suppose that even a single seizure can have damaging effect and may lead to fragmentation of DNA; a feature characterizing apoptosis. *Phragmanthera austroarabica* (Loranthaceae) is a semiparasitic plant collected from Saudi Arabia. To date, there is no information about the effect of *P. austroarabica* extract on animal models of neurologic disorders and epilepsy. So, this study was performed to examine the possible neuroprotective effect of the extract in PTZ-kindling model in mice. Previous chemical study of the plant led to isolation and chemical identification of twelve compounds. Ten out of the twelve compounds exhibited potent free radical scavenging property when tested against DPPH reagent. These compounds, namely, are chrysophanic acid and its 8-*O*-glucoside, emodin and its 8-*O*-glucoside, methyl gallate, pectolinarigenin, quercetin, catechin and its 4′-*O*-gallate, and dillenetin-3-*O*-glucoside. Additionally, the total plant extract revealed significant antioxidant effect [19]. In the present work, two compounds (gallic acid and β-sitosterol-3-*O*-glucoside) were identified. In addition, the extract was standardized where total phenolic contents were assessed by the use of Folin-Ciocalteu reagent.

2. Materials and Methods

2.1. In Vivo Determination of Anticonvulsant and Neuroprotective Activity

2.1.1. Animals and Housing Conditions. Thirty albino male mice were used in this study with access to water and food. Mice were housed in polyethylene cages under a normal light/dark cycle and allowed to acclimatize for the experiment conditions for one week before starting of drug injection. Seizure scoring was done at 14:00–17:00 h to reduce influence of circadian on seizure susceptibility. All possible efforts were done to reduce animals suffering. Study protocol was approved by the committee of research ethics at Faculty of Pharmacy in Suez Canal University *(License number 20146A7)*.

2.1.2. Pentylenetetrazole-Induced Kindling in Mice. Pentylenetetrazole was obtained from Sigma-Aldrich (MO, USA) and then prepared by dissolving in sterile saline. For induction of kindling, mice were injected intraperitoneally with PTZ (35 mg/kg) trice a week to reach a total number of thirteen injections [20] to produce the model of chemically induced chronic epilepsy. Mice in the vehicle group were treated by injection with saline parallel to PTZ injection.

2.1.3. Study Groups. Thirty mice were divided into five groups in a random way, six mice each as follows: *Group I (control saline group)*: injection by saline was done every second day to reach a total of 22 injections. *Group II (PTZ control group)*: mice were injected with subconvulsive doses of PTZ (30 mg/kg, i.p.) every second day for a total of 22 injections [21]. *Groups III–V (PTZ + P. austroarabica groups)*: mice were injected with PTZ as mentioned above and on the same days, they will receive protective doses of the extract (200, 400, or 800 mg/kg, p.o.), respectively, 45 min before each PTZ injection. The total number of protective doses was 22. Importantly, the selected doses of the extract were determined according to those reported in previous studies either for the same plant or for closely related plants belonging to the same family [22–25].

2.1.4. Evaluation of Seizure Activity in PTZ-Kindled Mice. The observation of convulsive behavior was done along 30 min in each mouse after PTZ injection. The severity of the seizures was ranked based on Racine scale [26] which is commonly used for assessment of seizure activity in rodents [27]. Mice were scored as follows: (0): if there is no seizure response, (1): if there exist immobility, closure of the eye, twitching of the ear, and appearance of facial clonus, (2): if there is nodding of head together with highly severe facial clonus, (3): if there is clonus of one forelimb, (3, 5): if there is bilateral forelimb clonus and no rearing, (4): if there is bilateral forelimb clonus accompanied with rearing, (4, 5): if there is no rearing and animals fall on a side, and righting reflex was lost in addition to generalized tonic-clonic seizures, and (5): if rearing occurred and animals fell on back together with generalized tonic-clonic seizures. After the last PTZ injection, an average of thirteen seizure scores recorded throughout the course of the experiment in each experimental group were calculated and compared. In addition, the percent of mice survival in each experimental group was determined.

2.1.5. Dissection of Brain and Processing of the Two Hemispheres. After completing the experiment, the mice were anaesthetized using thiopental sodium (50 mg/kg) and then were sacrificed by cervical dislocation. This was followed by removal of brains and one hemisphere was frozen immediately. The frozen hemisphere was used for isolation of the cortex and hippocampus. Those were used for DNA laddering assay and calculation of oxidative stress parameters. Other hemisphere was fixed for 24 h in 10% paraformaldehyde solution. This was followed by cutting of the brain into sections on a vibratome. Two coronal sections at the same hippocampal level were stained with hematoxylin and eosin (H&E) or Cresyl violet stain and protected with a cover-slip; the stain helps to declare the cytoarchitecture of cortex and hippocampus and to evaluate cell degeneration.

2.1.6. Determination of Oxidative Stress Parameters. The cortex and hippocampus from each brain were isolated while frozen. In order to measure oxidative stress parameters, one part of tissues was homogenized in one ml of phosphate-buffered saline (pH = 7.4) by the use of a Teflon homogenizer (Glass Col homogenizer system, Vernon hills, USA). Centrifugation of the tissue homogenate was performed at 4°C at 3000 ×g for 15 min; this step was followed by collection of the supernatant to be used in the different assays. Homogenates were assayed for malondialdehyde (MDA) and reduced glutathione (GSH) using commercial colorimetric

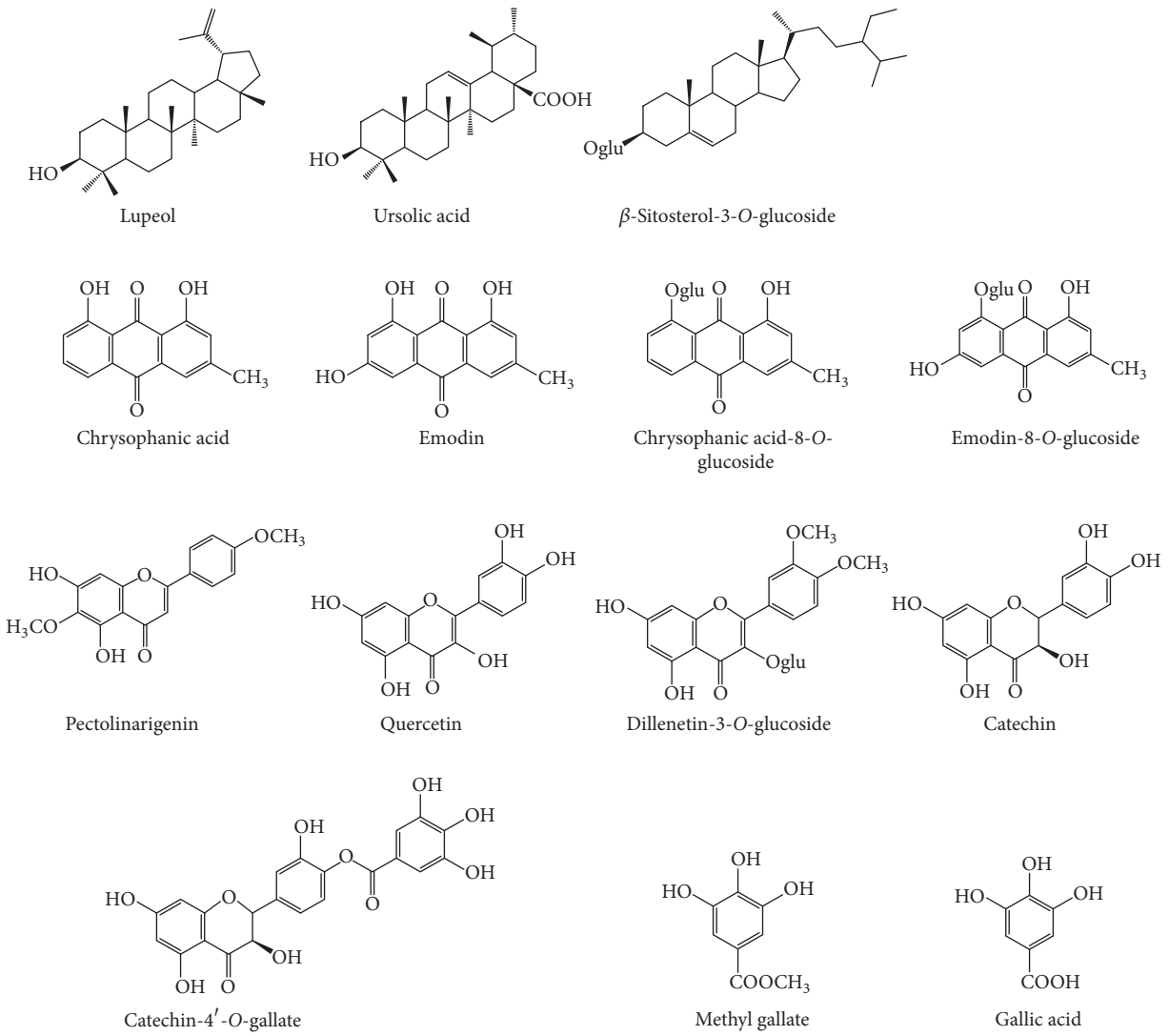

Figure 1: Structure of the compounds of *Phragmanthera austroarabica*.

kits obtained from Biodiagnostic Company (Giza, Egypt). Instructions reported by the manufacturer were followed. MDA was measured according to the methods based on its reaction with thiobarbituric acid (TBA) in an acidic medium and temperature equals 95°C for 30 minutes. The yielded product is TBA-reactive. Measurement of absorbance of the reaction product was done at 534 nm as reported previously. Tissue GSH was estimated using the method depending on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with reduced GSH to form a yellow colored product. This color is directly proportional to GSH concentration. Absorbance was determined colorimetrically by measurement at 405 nm.

2.1.7. Extraction of DNA and DNA Ladder by Gel Electrophoresis. Assessment of endonuclease-dependent ladder-like DNA fragmentation was done. Gel electrophoresis technique was used. Extraction of genomic DNA from the brain tissues was achieved by the use of Bio Basic EZ-10 spin column genomic DNA kit (Markham, Canada), following the manufacturers protocol. The samples of extracted genomic DNA were subjected to electrophoresis by the use of 0.8% (w/v) agarose gel at 90 V and 110 mA (2 h). After that, samples were stained using ethidium bromide; this was followed by visualization under UV light. The 100 bp DNA ladder kit (Solis Biodyne, Tartu, Estonia) is a molecular weight marker for ready use. Photos for the gel were captured by the aid of gel documentation system and interpreted using Gel Docu advanced ver. 2 software.

2.1.8. Quantification of Surviving Neurons in the Cortex and Hippocampus of Rats. The solution used for staining consisted of Cresyl violet (0.5 g) in distilled water (100 ml). Removal of the matrix was done by washing of microtome sections by tap water, followed by their dipping three times in distilled water. This step was followed by staining at room temperature (for ten minutes) using Cresyl violet stain dye and then drying in air for one hour. The dry sections were

dipped shortly in alcohol. Sections were cleared using xylene and protected using Entellan cover-slip. Finally, they were microscopically checked.

Cresyl violet staining was performed for staining of Nissl's substances, which are the clumps of ribosomes associated with membrane of endoplasmic reticulum in the nerve cells [28]. The method depends on detection of Nissl substance in sections of the tissue which are fixed with formalin and embedded in paraffin. Staining is usually used for identification of basic neuronal structure within the brain [29]. The cortex and hippocampus were chosen since they are the most sensitive area to toxic insults and important for maintaining epileptic seizures. Quantification of the surviving cells was performed within the cortex and hippocampus from all animals cut at the same level.

Neuronal morphology was performed in ten regularly spaced sections among the entire surface of cortex and hippocampus. Counting of the neurons was based on identification of a clear and distinct nuclear membrane. The only neurons considered viable are those with visible nuclei and with the entire outline of the cell appearing complete. The mean of viable neurons in the ten regularly spaced sections was calculated for each mouse in each group [30, 31] and calculated as % from the total number of the cells. The numbers of cells in each of the cortical or hippocampal areas are as those counted from photographs captured at 40x magnification [32–34].

Pycnotic cells in different hippocampal area were counted. Identification of the dead cells was done by morphological criteria by the blebbing in plasma membrane and shrunken cytoplasm, surrounded by perineuronal vacuoles, neuron size alteration, and triangular shape of the cells. For microscopic evaluation, ten sections covering the entire surface of the cortex and hippocampus were analyzed. Then, the sum of cell counts from the ten sections was calculated as % from the total number of cells and then averaged for each animal.

2.1.9. Statistical Analyses. The data taken from this study were tabulated and expressed as mean ± standard error. One-way analysis of variance was used for analysis of the results, followed by Bonferroni's post hoc test. The percent of surviving mice was compared applying the Chi square test. The statistical package for social science, version 16 (SPSS Software, SPSS Inc., Chicago, USA) was employed for this purpose. All P values were two-tailed and $P < 0.05$ was considered statistically significant.

2.2. Phytochemical Study

2.2.1. General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were obtained in CD_3OD on Bruker Avance DRX 600 spectrometers at 600 MHz for hydrogen NMR (^{1}HNMR) and 150 MHz for carbon NMR (^{13}C NMR). For separation by column chromatography, silica gel (Merck, 70–230-mesh ASTM) and Sephadex LH-20 (Pharmacia) were utilized. Further, precoated silica gel 60 F-254 plates (Merck) were used for thin-layer chromatography (TLC). Spots were visualized by exposure to NH_3 vapour, UV radiation, and *P*-anisaldehyde/sulfuric acid.

2.2.2. Plant Material. Collection of the plant was done in March (2011) from Abha, Khamis Mushait at South Saudi Arabia. Identification of the plant was confirmed by Dr. Nahed Morad (Faculty of Science at King Abdulaziz University). A specimen was deposited at Natural Products Department, Faculty of Pharmacy, King Abdulaziz University, with a code number 2011-Phaa.

2.2.3. Chemical Study. The air dried plant was powdered (0.5 kg) and macerated with methyl alcohol (2 × 1000 mL), concentrated, and fractionated using hexane, chloroform, and ethyl acetate, respectively. Two grams of chloroform extract was fractionated on a silica gel column. Then, the extract was eluted with chloroform : methanol gradient. After that, fractions eluted by methanol : chloroform (3 : 97) were further fractionated on a silica gel column with gradient elution using chloroform-methanol, followed by repeated crystallization from methanol to give 15 mg of a white powder (A). Four grams of the ethyl acetate extract was chromatographed on a column packed with silica gel and then eluted with chloroform : methanol gradient. Fractions eluted by 20% methanol in chloroform were then purified on Sephadex, eluted with methanol to give 65 mg of creamy powder (B).

2.2.4. Assessment of Total Phenolic Content. Total phenolic contents were determined by spectrophotometry, using gallic acid as a standard (prepared to cover the range of 0–200 mg%). Briefly, 20 mg of the dry extract was dissolved in 15 ml methanol; the volume was completed to 20 ml using methanol. Extract (0.1 mL) was mixed with distilled water (2.8 ml), 2% sodium carbonate solution (2 ml), and Folin-Ciocalteu's reagent (0.1 ml). The mixture was left for 30 minutes. Absorbance was measured at 750 nm against distilled water blank [35].

3. Results

3.1. Effect of P. austroarabica Extract on Seizure Activity in Pentylenetetrazole-Kindled Mice. In the current study, repetitive injection of PTZ resulted in kindling in mice. Mice showed progressively increased seizure scores and were compared at the final score. The final seizure score exhibited by mice in the PTZ control group was greater than that exhibited by the saline group (4.33±0.31 versus 0±0, $P < 0.05$, Figure 2). Treatment with diazepam significantly reduced the final seizure score compared to the PTZ control group. Treatment with *P. austroarabica* extract (200 mg/kg) did not significantly ameliorate the final seizure score in comparison to PTZ control group. However, the middle and high doses of *P. austroarabica* extract (400 or 800 mg/kg) significantly reduced the final seizure score compared to PTZ group (2.6 ± 0.22 or 2 ± 0.29 versus 4.2 ± 0.34, resp., $P < 0.05$, Figure 2). Importantly, the score recorded in mice treated with *P. austroarabica* extract (800 mg/kg) was not significantly different from that recorded in mice treated with diazepam.

3.2. Effect of P. austroarabica Extract on Cortical Malondialdehyde and Reduced Glutathione and Integrity of Cortical DNA Pentylenetetrazole-Kindled Mice. The results of the current

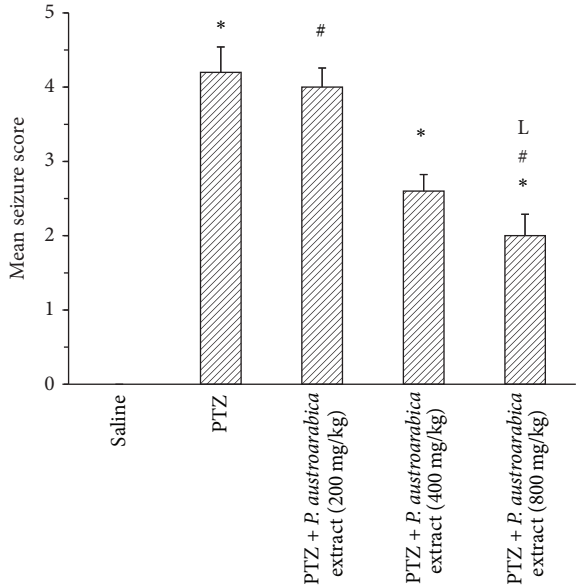

Figure 2: Mean seizure score in the experimental groups. Mice were injected with PTZ (35 mg/kg) trice a week for a total of 13 injections. Seizures were scored (0) if there is no seizure response, (1) if immobility, eye closure, ear twitching, or facial clonus appears, (2) if head nodding associated with more severe facial clonus, (3) if there is clonus of one forelimb, (3, 5) if there is bilateral forelimb clonus without rearing, (4) if there is bilateral forelimb clonus with rearing, (4, 5) if animals fall on a side (without rearing), loss of righting reflex with generalized tonic-clonic seizures, and (5) if rearing and falling on back occur accompanied by generalized tonic-clonic seizures. Results are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test at $P < 0.05$. *Significant difference from saline group. #Significant difference from PTZ group. LSignificant difference from PTZ + *P. austroarabica* extract (200 mg/kg) group, $n = 4$–6.

study indicated that PTZ control group showed lower cortical GSH and greater MDA level compared to saline group. Treatment with *P. austroarabica* extract (200 or 400 mg/kg) did improve cortical GSH level; however, the higher dose of the extract (800 mg/kg) enhanced cortical GSH level compared to PTZ control group ($P < 0.05$, Figure 3(a)). Regarding cortical MDA level, treatment with *P. austroarabica* extract (400 or 800 mg/kg) reduced the detected level in comparison to the PTZ control group (Figure 3(b)). Furthermore, gel electrophoresis for DNA samples from the study groups demonstrated high degree of fragmentation in PTZ control group. Meanwhile, mice treated with *P. austroarabica* extract (200 mg/kg) did not improve the quality of the DNA ladder (Figure 4).

3.3. Effect of P. austroarabica Extract on Survival of Cortical Neurons in Pentylenetetrazole-Kindled Mice. Figures 5(a)–5(e) are photographs for sections from the cortex of different experimental groups stained with Cresyl violet stain. Figure 5(f) indicates that PTZ control rats showed lower percent of surviving neurons compared to saline group. Treatment with the highest dose of *P. austroarabica* extract (800 mg/kg) increased the percent of surviving neurons compared to PTZ control group (Figure 5(f)).

3.4. Effect of P. austroarabica Extract on Survival of Hippocampal Neurons in Pentylenetetrazole-Kindled Mice. Figures 6(a)–6(e) demonstrate photographs for the hippocampus of different study groups showing different degrees of staining with Cresyl violet stain. In Figure 6(f), the mean percent of surviving neurons showed that PTZ control group exhibited lower percent of surviving neurons in comparison to the saline group. Furthermore, administration of *P. austroarabica* extract (800 mg/kg) enhanced the percent of surviving neurons in comparison to PTZ control group (Figure 6(f)).

3.5. Effect of P. austroarabica Extract on Pyknosis in Cortical Neurons in Pentylenetetrazole-Kindled Mice. Figures 7(a)–7(e) show some photographs for cortical sections stained with H&E from the different experimental groups. Pyknosis appears in the form of shrunken neurons with dark cytoplasm surrounded by vacuoles in most of cases. Figure 7(f) represents the mean of percent of pyknotic neurons in each group and highlights that PTZ-kindled mice show greater percent of pyknotic neurons in comparison to the percent registered in saline group. Treatment with the high dose of *P. austroarabica* extract (800 mg/kg) resulted in a reduction in the percent of pyknotic cortical neurons compared to PTZ group, but lower doses did not result in a similar effect.

3.6. Effect of P. austroarabica Extract on Pyknosis in Hippocampal Neurons in Pentylenetetrazole-Kindled Mice. Figures 8(a)–8(e) demonstrate photographs for the hippocampal neurons in the study groups. The PTZ-kindled group showed greater percent of pyknotic neurons in comparison to the saline group. Treatment with the moderate or high doses of *P. austroarabica* extract (400 or 800 mg/kg) reduced the percent of pyknotic neurons compared to PTZ-kindled mice (Figure 8(f)).

3.7. NMR Data of the Isolated Compounds

Compound A (β-Sitosterol-3-O-glucoside). ^{13}C-NMR (150 MHz, $CDCl_3$ + CD_3OD): 37.8 (C-1), 29.7 (C-2), 78.1 (C-3), 39.9 (C-4), 139.8 (C-5), 121.7 (C-6), 32.2 (C-7), 31.7 (C-8), 49.7 (C-9), 37.3 (C-10), 21.6 (C-11), 38.2 (C-12), 50.8 (C-13), 56.2 (C-14), 26.1 (C-15), 25.3 (C-16), 56.1 (C-17), 12.2 (C-18), 19.3 (C-19), 36.1 (C- 20), 19.4 (C-21), 33.9 (C-22), 26.2 (C-23), 46.1 (C-24), 30.4 (C-25), 21.1 (C-26), 21.2 (C-27), 23.2 (C-28), 12.1 (C-29), 102.3 (C-1′), 74.1 (C-2′), 76.3 (C-3′), 70.9 (C-4′), 77.7 (C-5′), 61.9 (C-6′).

Compound B (Gallic Acid). Creamy powder, ^{1}H NMR (600 MHz, CD_3OD): δ_H 7.02 (2H, s, H-2,6); ^{13}C NMR (150 MHz, CD_3OD): δ_C 110.1 (C-2,6), 120.0 (C-1), 139.1 (C-4), 145.6 (C-3,5), 168.6 (C=O).

3.8. Total Phenolic Contents. Gallic acid was used as a standard for determination of total phenolic content. The total phenolic content in the plant extract was determined

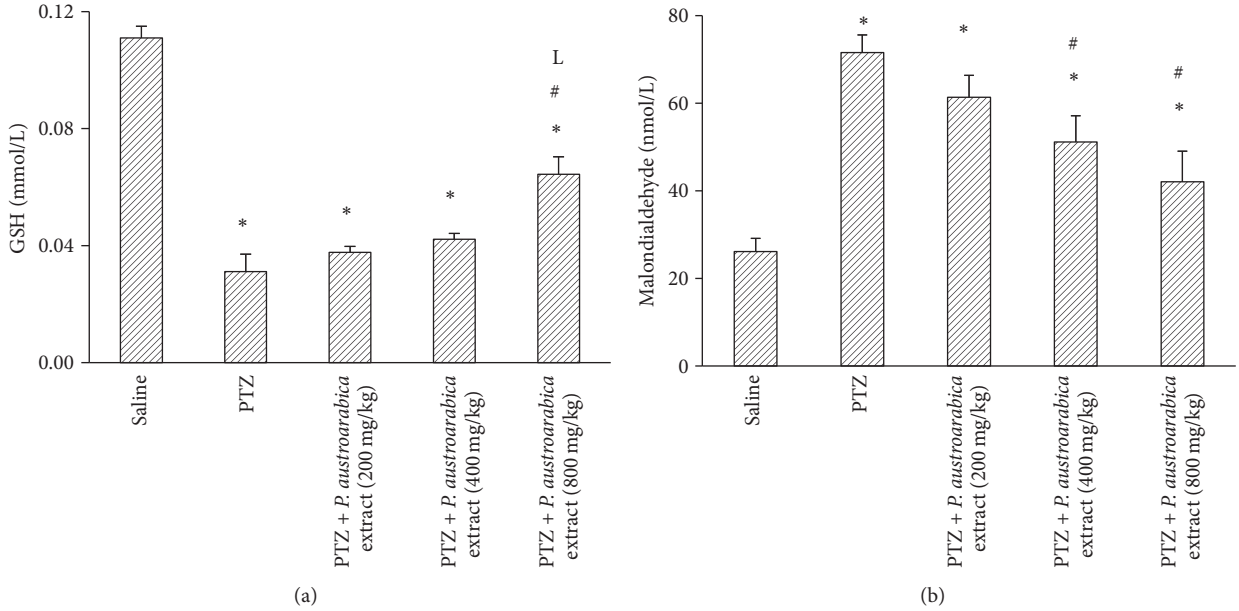

Figure 3: Cortical level of reduced glutathione and malondialdehyde in the experimental groups. Mice were injected with PTZ (35 mg/kg) trice a week for a total of 13 injections. Cortices from frozen brains were homogenized and assayed for reduced glutathione (GSH) and malondialdehyde. Results are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test at $P < 0.05$. *Significant difference from saline group. #Significant difference from PTZ group. LSignificant difference from PTZ + *P. austroarabica* extract (200 mg/kg) group, $n = 4$–6.

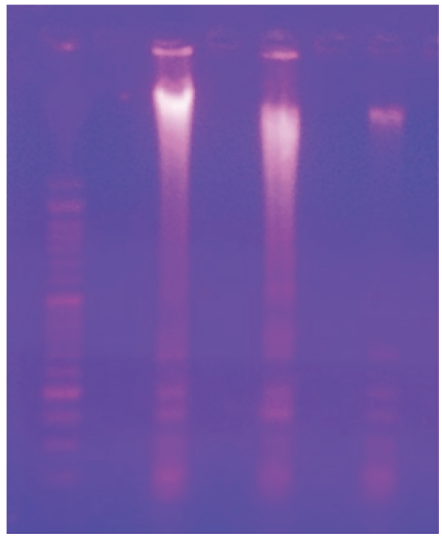

Figure 4: Agarose gel electrophoresis for DNA samples from the experimental groups. DNA was extracted from the cortical homogenate from one frozen brain hemisphere and loaded 100 bp ladder. The first lane is showing smeared DNA from one mouse in PTZ control group. The second lane shows DNA from PTZ + *P. austroarabica* extract (200 mg/kg) group showing internucleosomal DNA fragmentation with mixed smearing. The third lane shows DNA sample from one mouse in PTZ + *P. austroarabica* extract (800 mg/kg) with no laddering. DNA samples were run over agarose gel.

and expressed as mg gallic acid equivalents per 1 g of the dry powdered extract. The method was repeated three times and the relative standard deviation was determined where the total phenolic contents were assessed as 379.92 ± 1.32 mg gallic acid equivalents/g dry plant extract. The linearity range was determined as 4–80 mg/ml. The regression equation was as follows: $y = 0.0083x + 0.071$ (y = the absorbance, x = the concentration); correlation coefficient equals 0.9987.

4. Discussion

The total alcoholic extract of the plant was subjected to different chromatographic techniques as described. Fourteen compounds were detected in this plant, of which twelve were previously identified [19]. Additionally, two compounds were isolated and chemically identified as β-sitosterol-3-*O*-glucoside and gallic acid. The two compounds are known and previously reported from different plants. Structure elucidation of the compounds was based on different NMR data in addition to comparison with the data previously reported in literature [36, 37]. Other compounds were identified by direct comparison using TLC with those previously isolated by the author from the same plant. The chemical constituents of *Phragmanthera austroarabica* are illustrated in Figure 1.

The total phenolic content was found to be 379.92 ± 1.32 mg gallic acid equivalents/g dry plant extract. The validity of the method was proved by the small value of the correlation coefficient (0.9987).

In the current study, repeated injection of PTZ produced a gradual increase in seizure activity during the course of the experiment. This activity was accompanied by oxidative stress, as indicated by high cortical malondialdehyde content

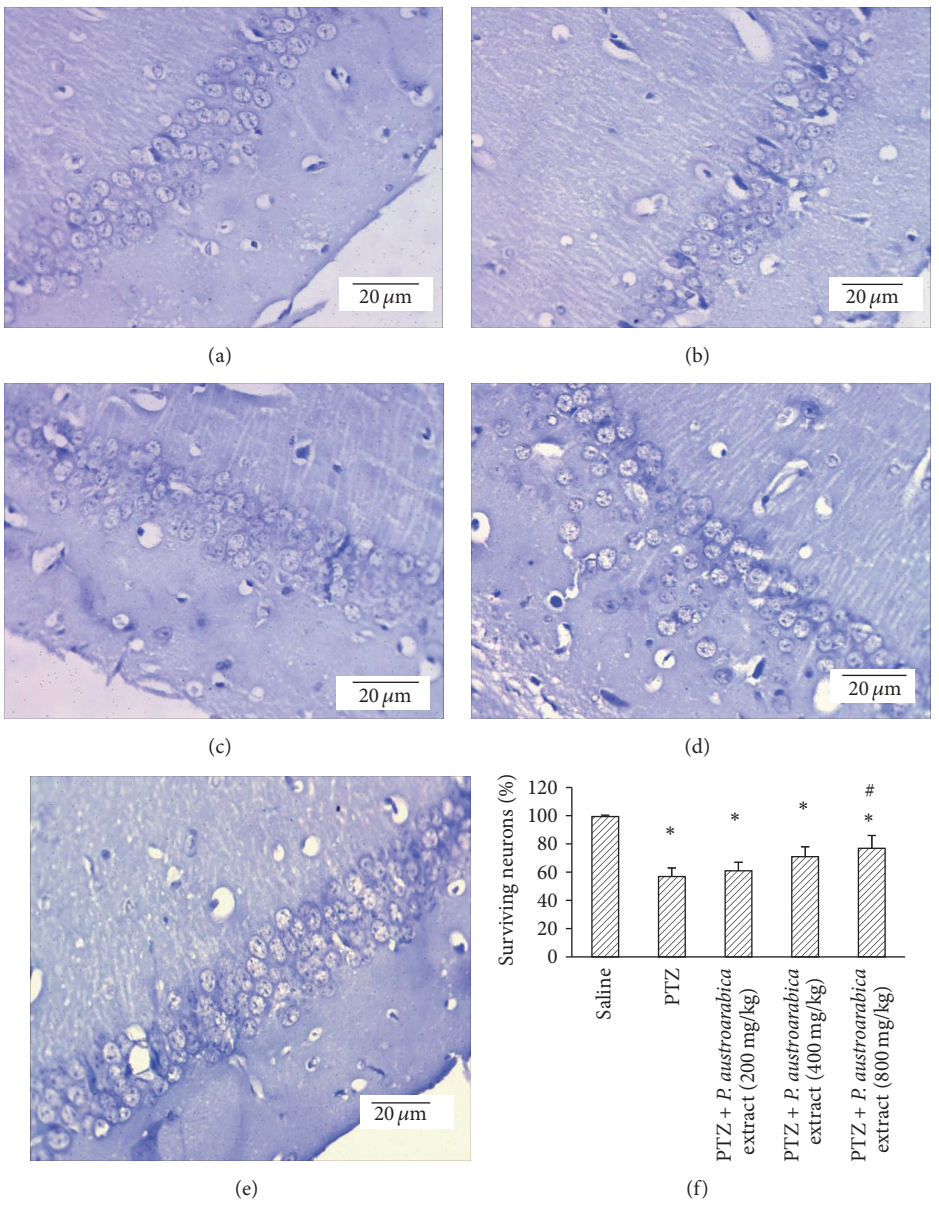

FIGURE 5: Surviving neurons in cortex of the experimental groups. ((a)–(e)) Photographs for cortical sections from the experimental groups stained with Cresyl violet stain. (f) Mean percent of surviving neurons in the study groups. Mice were injected with PTZ (35 mg/kg) trice a week for a total of 13 injections. Cortices from formalin-fixed brains were cut and stained. Results are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test at $P < 0.05$. *Significant difference from saline group. #Significant difference from PTZ group.

and lower percent of surviving neurons in the cortex and hippocampus. The current results declared that the hippocampus was greatly affected by PTZ-kindling compared to the cortex. Many studies reported that repeated injection of subconvulsive doses of PTZ increases the susceptibility of the animals, resulting in a fully kindled state. In this case, brief seizures last for few minutes resembling human epilepsy along with histopathological abnormalities in some brain regions [20]. It is well known that seizures result in and enhance brain damage [14, 32]. For this reason, the kindling model offers a good tool for studying the pathophysiological mechanisms and cumulative changes during epileptogenesis [38]. Biological testing highlighted that the medium and high doses of *P. austroarabica* extract ameliorated seizure activity as indicated by low seizure scores in PTZ-kindled mice. This is the first report to demonstrate the central activity of this extract. However, amelioration of the seizure activity does not necessarily imply a direct anticonvulsant activity for the extract, but this ameliorative effect may arise from other biological activities such as suppression of oxidative

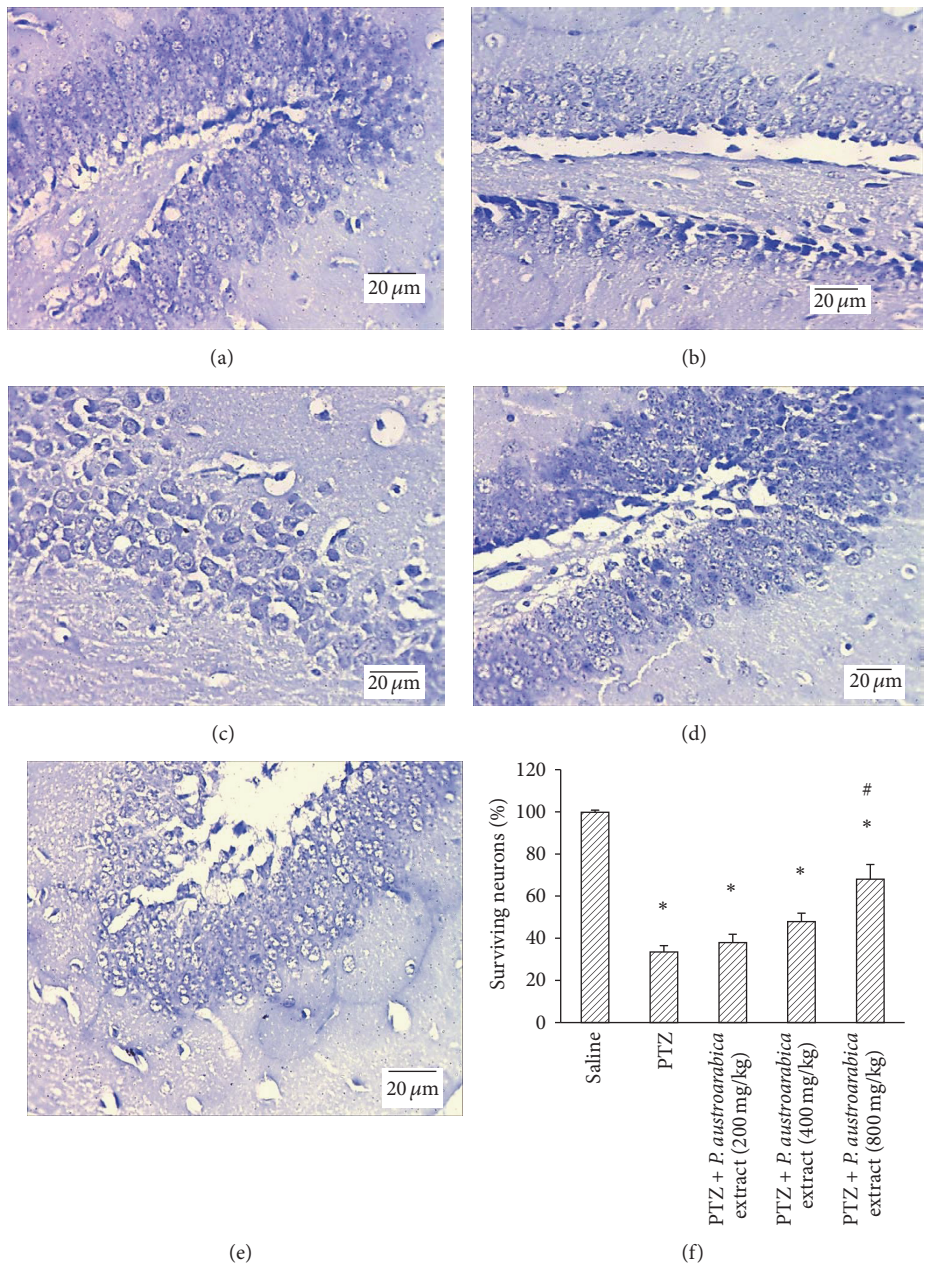

Figure 6: Surviving neurons in hippocampus of the experimental groups. ((a)–(e)) Photographs for hippocampal sections from the experimental groups stained with Cresyl violet stain. (f) Mean percent of surviving neurons in the study groups. Results are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test at $P < 0.05$. *Significant difference from saline group. #Significant difference from PTZ group.

burden. Further, some antioxidant properties were outlined by increasing cortical GSH and reducing MDA level upon treatment with the high dose of the extract. The antioxidant activity may contribute to the observed effect of the extract as similar properties were documented previously in animal models of diabetes [23]. Extensive studies for experimental models reported that continuous seizures or even repetitive brief seizures in kindling provoke pathological neurodegeneration in the brain [39, 40]. At the cellular level, the high dose of the plant extract provided beneficial neuroprotective effect according to the increase of the percent of surviving neurons in the cortex and hippocampus. It is also important to note that the high dose is mostly the dose that produced biological activity. This makes it necessary to determine the safety margin of this extract.

Actually, only two articles were detected in the literature concerning the biological effect of this extract. These reported studies described the beneficial effects of *P. austroarabica* extract in treating experimental diabetes [23]. It is worth mentioning that previous studies were conducted on pure

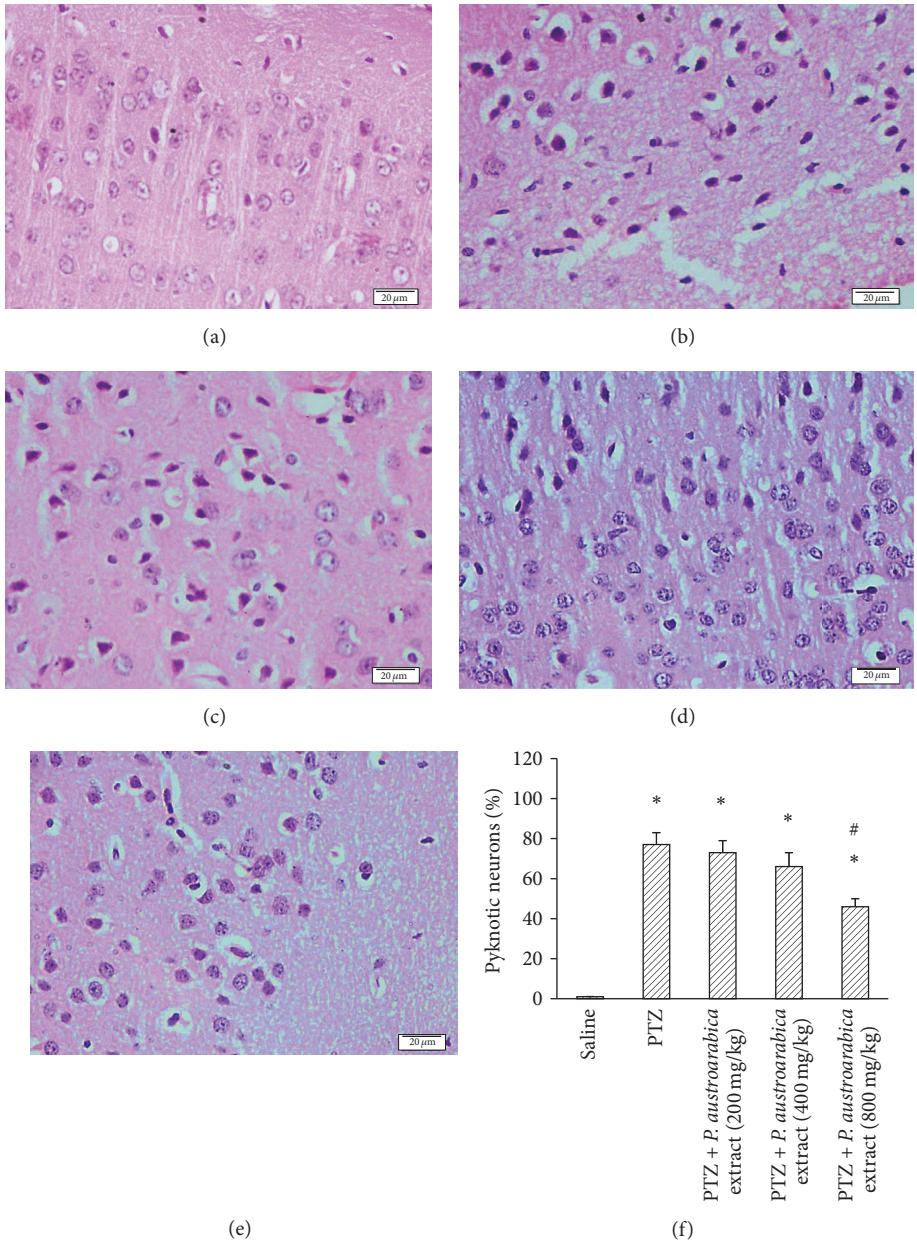

FIGURE 7: Pyknotic neurons in cortex of the experimental groups. ((a)–(e)) Photographs for cortical sections from the experimental groups stained with hematoxylin and eosin stain. (f) Mean percent of pyknotic neurons in the study groups. Mice were injected with PTZ (35 mg/kg) trice a week for a total of 13 injections. Cortices from formalin-fixed brains were cut and stained. Results are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test at $P < 0.05$. *Significant difference from saline group. #Significant difference from PTZ group.

compounds that are present as major constituents in this plant. These studies exhibited their beneficial effect as neuroprotective agents. Among these compounds are quercetin [41], gallic acid [42, 43], catechin [44], and emodin [45]. Additionally, many phenolic compounds were previously reported as neuroprotective [46], whereas *P. austroarabica* extract is considered to accumulate a considerable amount of phenolic constituents. These facts can additionally justify the efficacy of *P. austroarabica* extract as neuroprotective in the studied models in our study.

5. Conclusion

In conclusion, the current study represents a starting point for more research about the neuroprotective effect of *P.*

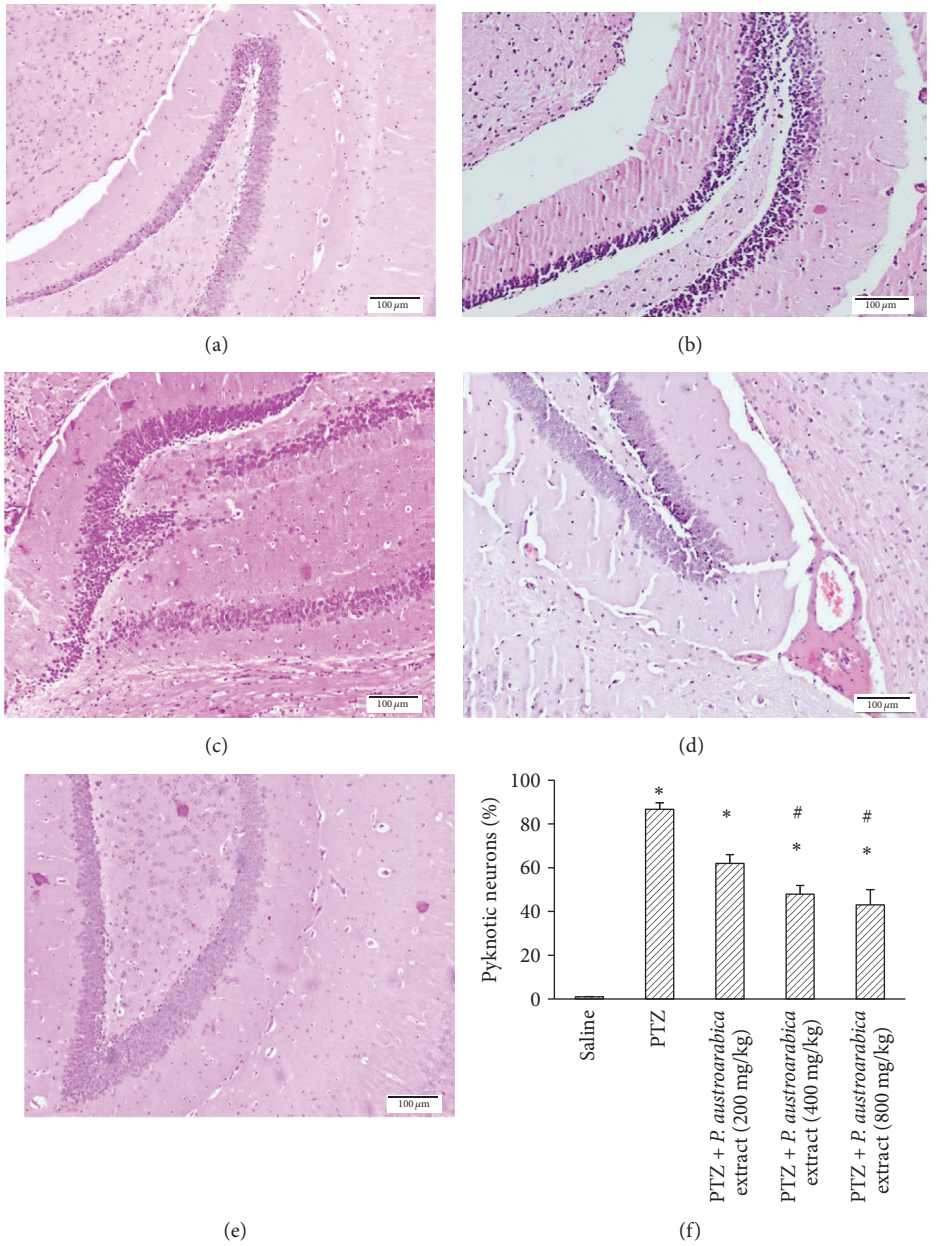

FIGURE 8: Pyknotic neurons in hippocampus of the experimental groups. ((a)–(e)) Photographs for hippocampal sections from the experimental groups stained with hematoxylin and eosin stain. (f) Mean percent of pyknotic neurons in the study groups. Results are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test at $P < 0.05$. *Significant difference from saline group. #Significant difference from PTZ group.

austroarabica extract in various models of epilepsy, neurotoxicity, and neurodegenerative diseases and further elucidates the detailed mechanism of action.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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