Title: Evaluation of Anxiolytic Effects of Justicia Secunda Methanol Leaf Extract and Chemical Constituents in Mice

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Abstract

The use of benzodiazepines and selective serotonin reuptake inhibitors (SSRIs) to treat depression has been linked with serious adverse effects. Due to previous reports against anxiety and depression in traditional medicine, we designed this study to evaluate the effects of *Justicia secunda's* methanol extract (MLEJS) against anxiety and depression in mice. GC-MS (Gas Chromatography Mass Spectroscopy) phytochemical analysis of MLEJS was performed in this work to verify the different bioactive components. An acute oral toxicity study was performed via the Organization for Economic Co-operation and Development 423 (OECD) guideline. We investigate the antidepressant and anxiolytic effect of MLEJS (12.5, 25, and 50 mg/kg) using lipopolysaccharide LPS-induced depression and flumazenil/benzodiazepine GABA (gamma-amino-butyric acid) receptor interaction. The open field test (OFT), forced swimming test (FST), and tail suspension test (TST) were performed to evaluate the depressive-like behavior in mice and hole-board, light and dark box, elevated plus maze, thiopental sodium, and rota-rod motor coordination test were used as a screening paradigm for the anxiolytic effect of MLEJS. The study's findings indicate that MLEJS had an anxiolytic-like effect by increasing exploration of the open arms and decreasing exploration of the closed arms in the elevated plus maze, light/dark, and hole-board tests. Additionally, lipopolysaccharide (LPS)-induced depressive-like behavior in mice was reversed by MLEJS ($p<0.05$). The significant ($p<0.05$) attenuation of pro-inflammatory mediators and suppression of oxido-nitrosative stress could be responsible for the observed effects. The results obtained in this study suggest that the MLEJS can offer an efficient therapeutic option against anxiety and depression concomitantly.

Keywords: Inflammatory mediators, Oxidative stress, $GABA_A$ receptor, Flumazenil

Introduction

With a lifetime prevalence of over 10%, anxiety and depression are major public health issues that impact a large portion of the general population and greatly increase the worldwide burden of disease [1]. Anxiety disorders are thought to be responsible for 26.8 million disability-adjusted life years, according to the Global Burden of Disease (GBD) research, and depression is thought to affect 300 million people worldwide [2]. Anxiety is a physiological reaction that protects an organism against harm. When it gets severe, it becomes pathological and can encourage the emergence of psychiatric and/or cardiovascular illnesses [3]. Treatments for anxiety and depression are frequently linked to side effects like tachycardia, orthostatic hypotension, digestive problems, weight gain, sexual problems, and visual problems. Memory impairment is one of the main issues that arises after longer exposure [5].

It is thought that dysregulation of some CNS neurotransmitters, including dopamine, serotonin, and gamma amino butyric acid (GABA), causes anxiety and depression [3]. Behavioral interventions, medication therapy, and psychotherapy are the current methods used to treat depression and anxiety [4]. In clinical practice, imipramine, desipramine, amoxapine, benzodiazepines, and selective serotonin reuptake inhibitors (SSRIs) have proven to be the most effective medications for managing anxiety and depression [5]. However, the two groups displayed a number of negative outcomes, including reliance, increased suicidal thoughts, lower alertness, sexual dysfunction, and high costs [5]. It is well known that benzodiazepines interact to GABA receptors' allosteric sites. By binding to GABA receptors, benzodiazepines reduce excitability in the central nervous system. Although long-term benzodiazepine use has been associated with negative effects such as memory loss and cognitive decline, this action will reduce anxiety [5]. Researchers are currently revaluing several plant species for their therapeutic chemical principles

due to the many issues with conventional medications. Justicia secunda is one species of such plant. Leaf extracts are used in traditional medicine to treat a variety of illnesses, including anemia, depression, sickle cell disease, and diabetic symptoms [6]. When J. secunda leaves were screened for phytochemical content, alkaloids and polyphenols including flavonoids, quinones, tannins, and anthocyanins were found [7]. The main secondary metabolites in the leaves, according to Kamso et al. [8], are derivatives of luteolin. Strong anxiolytic substances are therefore required, with fewer side effects and an earlier onset of action than those found in present medications. This study uses gas chromatography/mass spectrometry (GC-MS) to examine the bioactive compounds found in Justicia secunda (MLEJS) methanol leaf extract. It also looks at the anxiolytic-like and anti-depressant activities of MLEJS, the role of GABAA/benzodiazepine mechanisms, and MLEJS's impact on pro-inflammatory cytokines, acetylcholinesterase activity, and biomarkers of depressive-like behavior induced by lipopolysaccharide.

2. Methods and Materials

2.1. Chemicals and Reagents

Flumazenil (Neon Laboratories Ltd, India), Diazepam (Roche, Basel Switzerland), Thiopental sodium (Kwality, India), Lipopolysaccharide (LPS) (*Escherichia coli* serotype, 055:B5 Sigma-Aldrich, USA) are the chemicals used in this study. Imipramine hydrochloride (Sigma-Aldrich, USA), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), acetylthiocholine, Ellman Reagent [5′, 5′-Dithiobis- (2-nitrobenzoate) DTNB], Hydrogen peroxide, Ammonium sulfate, Phosphate buffered saline, Distilled water and all other chemicals used were of analytical grade.

2.2. Extract Preparation

The fresh healthy leaves of *Justicia secunda* were obtained from the staff quarters of Obafemi Awolowo University Ile-Ife, Osun state, it was identified and authenticated at the Department of Forest Herbarium with the voucher specimen FHI number 112604.

After being shade-dried and ground into a powder using a high-capacity grinder, the leaves were immersed in 70% aqueous methanol at a ratio of 1:10 (w/v) for three days. A solid extract with a yield of 23.3 g was produced by filtering the extract with Whitman filter paper and then drying it off in a Rotary evaporator. After that, the dry extract was reconstituted in regular saline at quantities suitable for the different tests.

2.3. GC-MS Phytochemical Analysis

The Agilent Technologies GC-MS (GC-7820A, MS 5975C) was used for the analysis. It was applied to an HP-5 capillary column coated with 5% Phenyl Methyl Siloxane (30 m length × 0.32 mm diameter × 0.25 μm film thickness). The instrument was initially set to 110 ºC and kept there for two minutes. After that, the oven temperature was raised to 280 ºC, at a rate of 4 ºC/min, and kept there for nine minutes. The injection port temperature was guaranteed to be 250 ºC, and the helium flow rate was 1.573 ml/min. The ionization voltage was 70eV with an ion source temperature of 230°C, quadrupole temperature of 150°C, and transfer line temperature of 280°C. Samples were injected in splitless mode, one microliter at 260°C injection temperature. A mass spectral scan range of 30-550 (m/z) was used. Compounds included in the plant sample were identified by matching the spectrum acquired from GC-MS analysis with computer searches on a NIST 14 Mass Spectra data repository. Mass spectrum GC-MS interpretation was carried out with the National Institute of Standards and Technology (NIST) database, which has over 62,000 patterns. A comparison was made between the spectrums of the unknown and known components found in the NIST collection. We determined the components of the test materials' names, molecular weights, and structures.

2.4. Animals

In this study, male Swiss mice weighing 20–25 grammes were employed. The rats were housed in clean, well-ventilated polypropylene plastic cages with bedding made of wood shavings. They were also given free access to water and fed commercial rat chow pellets. The ambient conditions were kept standard. The 3Rs (replacement, reduction, and refinement) of animal experiments were followed in the treatment of the animals, and pain and discomfort were minimized by providing enriching environments. The ethical permission number (UI-ACUREC/19/0158) was granted for the use of animals in this investigation.

2.6. Experimental Designs

The study design comprises:

- Acute toxicity study; 6 mice were used
- Anxiolytic-like study; 77 mice were used
- Antidepressant-like study; 30 mice were used

2.6.1 Acute Toxicity Test

Using the Organization for Economic Co-operation and Development 423 (OECD) guideline, an acute oral toxicity study was carried out. The mice (n = 3) that had fasted overnight were given MLEJS orally at a maximum dosage of 2000 mg/kg. They were then monitored closely for two hours, and then for twenty-four, seventy-two, and finally for up to fourteen days, for any signs of lethality, a state of morbidity, or death concerning their behavioral, neurological, and autonomic profiles. To confirm the results and determine the dangerous class of LD50, the limit test was conducted on three additional mice in another group.

An acute oral toxicity investigation revealed that the MLEJS therapy had no toxicity or moribund state. This implied that the approximate LD50 was more than 2500 mg/kg and that the non-observable adverse effect dose level was larger than 2000 mg/kg.

2.6.1. Anxiolytic-like Experiment

The animals were divided into five groups at random, with seven animals apiece. 60 minutes before the experiment, mice in groups 2-4 were given different dosages of MLEJS (12.5, 25, and 50 mg/kg orally, respectively), while group 5 got a dose of diazepam (1 mg/kg p.o.). Group 1 mice were given normal saline 10ml/kg orally and served as the normal control. Benzodiazepines are known to have anxiolytic effects at low dosages and to cause drowsiness and myorelaxant effects at higher dosages [9]. To check for anxiolytic-like effects, we employed diazepam (1 mg/kg) as a positive control. To clarify the potential participation of GABAA/benzodiazepine mechanisms of the extract, six further groups of mice (n = 7) were pre-treated with 6 mg/kg flumazenil, intraperitoneally, 15 minutes before the treatment with the most effective anxiolytic-like dose of MLEJS (50 mg/kg) or diazepam. Group 1 mice were given normal saline (10 milliliters per kg) orally as the normal control. Group 2 mice received 6 milliliters per kg of flumazenil alone. Group 3 mice received 50 milliliters per kg of MLEJS only. Group 4 mice received 6 milliliters per kg of

flumazenil + MLEJS (50 mg/kg). Group 5 mice received 1 milliliters per kg of diazepam, and Group 6 mice received 1 milliliters per kg of diazepam + 6 mg/kg of flumazenil.

2.6.2. Antidepressant-like Experiment

Mice were assigned to six (6) different experimental groups (n = 5) in a semi-randomized fashion, so that mean body weights were comparable in all groups. Thereafter, mice received orally MLEJS (12.5, 25, and 50 mg/kg) or Imipramine (10 mg/kg) as positive control and vehicle (saline, 10 ml/kg as normal control) once daily for 7 days. On the last day of the treatment, that is, the 7th day, LPS (0.83 mg/kg) was injected intraperitoneally after 30 minutes of respective treatment to all groups except the vehicle control group. After 24 hours of LPS administration, a battery of behavioral tests including an open field test (OFT), forced swimming test (FST), and tail suspension test (TST) were performed to evaluate the depressive-like behavior in mice [10].

Behavioral Procedures

2.7. Myorelaxant and Sedative Anxiolytic Tests

2.7.1. Test for Motor Coordination Using Rota-Rod

The animals were pre-selected in a training session 24 hours before the test based on their ability to stay on the bar (at 12 revolutions per minute) for 120 seconds. The test was conducted using a horizontal rotating rod (Ugo Basile). All four paws of the animals were placed onto the bar sixty minutes after the treatment with vehicle (10ml/kg), MLEJS (12.5, 25, or 50mg/kg), or diazepam (1 or 5mg/kg). The time it took for each mouse to fall was recorded, with a maximum of 120 seconds on the bar, and the number of falls was calculated within 60 seconds, with a maximum of three falls permitted [11].

2.7.2. Sedative Test Using Thiopental Sodium Sleeping Time

All groups received thiopental sodium (20 mg/kg, intraperitoneally, i.p.) an hour after oral dose of MLEJS (12.5, 25, and 50 mg/kg), diazepam (1 and 5 mg/kg), or vehicle. Sleep latency is the amount of time after thiopental sodium delivery that passes before the righting reflex is lost; sleeping time is the amount of time that passes between the loss and the reflex's voluntary recovery. To avoid odor bias, the apparatus was cleaned with 70% ethanol after each animal.

2.8. Anxiolytic Activity Tests

2.8.1. Test for Anxiety Using Hole-Board

The animals were placed in the middle of a perforated board divided into nine equal-sized squares sixty minutes after treatment; during the course of five minutes, the number of head dips into the holes and the number of squares crossed (with all four paws) were recorded. To avoid odor bias, the apparatus was cleaned with 70% ethanol after every animal.

2.8.2. Test for Anxiety Using Light and Dark Box

The participant is exposed to a novel environment consisting of protected (dark compartment) and unprotected (light compartment) locations during the light↔ dark exploration test. It is believed that risk aversion and exploratory desire are inherently at odds, which prevents exploration from occurring. The animals were positioned in the middle of the light area facing the dark area entrance sixty minutes after treatment; the number of times they switched between the two compartments and the amount of time they spent in the light area was counted over five minutes.

2.8.3. Test for Anxiolytic/Anxiogenic Properties Using Elevated Plus Maze

The experiment capitalizes on mice's innate curiosity to investigate new surroundings. The arms of the maze, which were raised to a height of about one meter above the floor, were either open and exposed to the mouse or enclosed and protected. The animals were individually placed in the middle of a plus maze sixty minutes after treatment, and they were watched for five minutes. The animal was tested by counting the number of times it entered and exited the open and enclosed arms, as well as the amount of time it spent there. Anxiety was measured by converting the number of entry into the open arms and the amount of time spent there into percentages of all entries and time, respectively.

2.8.4. Involvement of $GABA_A$/Benzodiazepine Mechanisms

The rats were intraperitoneally pre-treated with flumazenil (6 mg/kg; 15 min pre-treatment), an antagonist of GABAA/benzodiazepine receptors, in order to look into potential mechanisms underpinning the anxiolytic actions of MLEJS.

2.9. Antidepressant Activity Tests

2.9.1. Test for Spontaneous Movement Activity Using the Open Field

This test was run to see if the MLEJS may have an impact on the mice's locomotor activity during the open-field test. After the mouse was put within the device, the number of times it crossed lines and reared over the course of five minutes was used to calculate its locomotor activity. After testing each mouse, the equipment was washed with 70% ethanol and dried to remove any traces of the preceding animal's scent.

2.9.2. Test for Antidepressant Activity Using Forced Swimming Test

Mice were used in the experiment, and an open cylindrical container with a diameter of 10 cm and a height of 25 cm holding 19 cm of water was used. During the test session, mice were kept in this unavoidable cylinder for six minutes, with the final four minutes of immobility being recorded. When they stopped fighting, floated still, and made only the motions required to maintain their heads above the water, they were deemed immobile.

2.9.3. Test for Antidepressant Activity Using Tail Suspension Test

The mice were able to be individually suspended 50 cm above the floor on the edge of a table by applying sticky tape about 1 cm from the tip of the tail. The immobility periods for the different groups were recorded at intervals of six minutes. An animal was hanged if it did not exhibit any movement. It was considered immobile.

2.9.4. Assessment of Oxidative Stress and Antioxidant Status

2.9.4.1. Lipid Peroxidation and Nitric Oxide

According to Afolabi et al. [12], the thiobarbituric reacting substance (TBARS) assay was used to determine the concentration of the LPO end product malondialdehyde (MDA). Using the Griess reagent method as reported by Odebiyi et al. [13], the amount of nitrite present in the brain tissues was calculated.

2.9.4.2. Antioxidant status

Reduced GSH concentration was found in the mice's brain supernatant by applying an older Ellam technique, as explained by Alabi et al. [14]. Superoxide dismutase (SOD) activity was measured according to the Misra and Fridovich procedure, which Campos-Shimada et al. [15] reported. The

procedure that Hadwan and Kadhum[16] had previously outlined was used to measure the amount of catalase.

2.9.5. Estimation of Brain Cytokines and Enzyme

The manufacturer's instructions were followed to determine the amounts of TNF-α (BioLegend, USA; CAT NO 430904) and IL-6 (BioLegend, USA; CAT NO 431304) in the brain supernatant. The Ellman method was used to measure the amount of acetylcholinesterase in brain tissue, as explained by Khalil and Abass [17].

2.10. Statistical Analysis

The data were presented as mean ± standard error of the mean, or S.E.M. All behavioral data were subjected to a normality test, one-way analysis of variance (ANOVA) was used for all analyses (nonparametric analysis was used for the Rota-rod test), and Tukey's post-hoc test was used for multiple comparisons when necessary. One-way ANOVA was used to examine the biochemical data, and Tukey's post-hoc test was then performed. Version 8 of GraphPad Prism (GraphPad Software, Inc. La Jolla, CA 92037 USA) was used to analyze the data. For every test, a p-value of less than 0.05 was deemed statistically significant. Since the study is exploratory in nature overall, the p values should be interpreted as descriptive rather than as a means of testing a hypothesis. Data collection was preceded by the specification of all intergroup comparisons.

3.0 Results

3.1. GC-MS Phytochemical Analysis

A full-scan gas chromatogram of J. secunda's methanol leaf extract is displayed in Figure 1. It verified the existence of several bioactive substances with varying retention periods (RT). Table 1 lists the major chemicals that were determined by their RT and % peak area. 9,12,15-octadecatrienoic acid (18.11%) was found to be the most abundant component by peak area. It was followed by n-hexadecanoic acid (14.94%), phytol (7.92%), stigmasterol (5.39%), squalene (4.83%), neophytadiene (3.95%), campesterol (2.96%), vitamin E (2.51%), methyl stearate (1.21%), β-amyrin (1.17%), 9-octadecenamide (1.10%), 2-palmitoyl glycerol (0.91%), and 4a (2H)-naphthalenol (0.86%).

3.2 Effect of methanol leaf extract of *Justicia secunda* on motor coordination in mice using rotarod apparatus

Diazepam (at 1 mg/kg) significantly increased the number of falls in the rota-rod test, while methanol leaf extract of Justicia secunda (MLEJS 12.5, 25, and 50 mg/kg) did not affect the number of falls [KW=14.60, $p<0.01$; Dunn, $p<0.001$ vs. vehicle group, Figure 2A]. Diazepam (5 mg/kg) significantly prolonged the falling latency from the revolving rod [ANOVA: $F(5,24)=3.779$, $p<0.01$, Figure 2B) ($p<0.0001$ vs. vehicle).

3.3 Effect of methanol leaf extract of *Justicia secunda* on sleeping test

Diazepam (1 or 5 mg/kg) reduced the sleep latency [ANOVA: $F(5,24)=13.17$, $p<0.001$; Figure 3A) ($p<0.05$ and $p<0.001$ vs. vehicle, respectively), while the Justicia secunda methanol leaf extract treatments did not significantly reduce the sleep latency ($p>0.05$ vs. vehicle).

Diazepam (1 and 5 mg/kg) also enhanced the amount of time spent sleeping (ANOVA: $F(5,24)=128.53$, $p<0.001$; Figure 3B) ($p<0.01$ and $p<0.001$ vs. vehicle, respectively).

3.4 Anxiolytic-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) in mice using light and dark boxes.

In the light–dark test, the number of transitions between the light and dark compartmentsOral treatment with MLEJS [25 and 50 mg/kg ($p<0.05$ and $p<0.0001$ vs. vehicle, respectively] and diazepam 1 mg/kg ($P<0.001$ vs. vehicle) increased [ANOVA: $F(4,30)=11.74$, $p<0.0001$; Figure 4]. The duration of time in the light compartment was higher for all MLEJS treatments [(12.5, 25

and 500 mg/kg) ($p<0.05$, $p<0.05$ and $p<0.001$ vs. vehicle, respectively)] (ANOVA: $F(4,30)=6.371$, $p<0.0008$; Figure 4). and (1 mg/kg) diazepam ($p<0.001$ vs. vehicle).

3.5 Anxiolytic-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) in mice using the hole board.

Following oral treatment with MLEJS [25 and 50 mg/kg ($p<0.05$ and $p<0.0001$ vs. vehicle, respectively) and diazepam 1 mg/kg ($P<0.001$ vs. vehicle), [ANOVA: $F(4,30)=11.74$, $p<0.0001$; Figure 4] was enhanced. All treatments with MLEJS [(12.5, 25 and 500 mg/kg) ($p<0.05$, $p<0.05$ and $p<0.001$ vs. vehicle, respectively)] increased the amount of time spent in the light compartment [ANOVA: $F(4,30)=6.371$, $p<0.0008$; Figure 4]. and 1 mg/kg of diazepam ($p<0.001$ compared to vehicle)

3.6 Anxiolytic-like effect of acute and sub-acute administration of methanol leaf extract of *Justicia secunda* (MLEJS) in mice using elevated plus Maze.

Figures 6A–6F depict the outcomes of administering the Justicia secunda methanol leaf extract acutely (Figures 6A–6D) and sub-acutely (Figures 6E–6F) on the elevated plus maze. The percentage of entries in the open arms with acute and sub-acute treatments [acute: $F(4, 30) = 12.49$, $p < 0.0001$; chronic: $F(4, 30) = 17.16$, $p < 0.0001$] and the percentage of time spent in the open arms (acute: $F(4, 30) = 91.83$, $p < 0.0001$; sub-acute: $F(4, 30) = 85.29$, $p < 0.0001$) differed between groups, according to a one-way ANOVA. When compared to the vehicle and the lower dose of MLEJS (12.5 mg/kg), diazepam and MLEJS (25 and 50 mg/kg) enhanced the percentage of entrances into and time spent on the open arms in the acute treatment groups (all $p< 0.05$). Additionally, in the sub-acute trials, the proportion of entry into and duration spent on the open arms compared with the vehicle increased with diazepam and all doses of MLEJS (12.5, 25, and 50 mg/kg) (all $p< 0.05$). Following acute administration [$F(4,30) = 1.639$, $p> 0.05$] and sub-acute administration [$F(4,30) = 2.486$, $p> 0.05$], there were no variations in the overall number of arm entries.

3.7 Effects of flumazenil pretreatment on the anxiolytic-like effect of acute administration of methanol leaf extract of *Justicia secunda* in the elevated plus maze

The effects of Justicia secunda leaf extract diluted with methanol in the raised plus maze following flumazenil pretreatment. The percentage of entries into the open arms varied between groups [ANOVA: $F(4,30)=18.17$, $p<0.0001$; Figure 7A] with [MLEJS (50 mg/kg) ($p<0.001$ vs. vehicle group)], [flumazenil + MLEJS (50 mg/kg) ($p<0.05$ vs. vehicle group)], and [diazepam (1 mg/kg)

(and p<0.0001 vs. vehicle group)] and [flumazenil + MLEJS (50mg/kg) (P>0.05 vs. MLEJS (50 mg/kg) group].

The percentage of time spent in the open arms was also examined using ANOVA (F(4,30)=101.2, p<0.0001; Figure 7B) with the following groups: diazepam (1 mg/kg) (p<0.0001 vs. vehicle group), flumazenil + MLEJS (50 mg/kg) (p>0.05 vs. MLEJS (50 mg/kg) group], and MLEJS (50 mg/kg) (p<0.001 vs. vehicle group)]. There was no significant difference in the total number of entries in closed arms between the groups [ANOVA: F(4,30)=0.3166, p>0.05; Figure 7C].

3.8 Antidepressant-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) on LPS-treated mice using open field test.

The effects of Justicia secunda leaf extract in methanol on rearing and total line crossing in an open field experiment. Total line crossing [ANOVA: F(5,24)=12.84, p<0.0001; Figure 8A] showed differences between groups when it came to imipramine (10 mg/kg) + LPS (p<0.0001 vs. LPS group), MLEJS+LPS (12.5, 25 and 50 mg/kg) (p<0.01, p<0.01 and p<0.001 vs. LPS group), and significantly lower LPS (0.83 mg/kg) (p<0.0001 vs. vehicle group). Additionally, MLEJS+LPS (25 and 50 mg/kg) (p<0.05 and p<0.001 vs. LPS group, respectively), imipramine (10 mg/kg) + LPS (p<0.001 vs. LPS group), and a significant decrease in LPS (0.83 mg/kg) (p<0.001 vs. vehicle group) were found in rearing [ANOVA: F(5,24)=5.656, p<0.001; Figure 8B).

3.9 Antidepressant-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) on LPS-treated mice using forced swimming test.

The impact of Justicia secunda leaf extract in methanol on forced swimming immobility time. There were differences between the groups [ANOVA: F(5,24)=27.12, p<0.0001; Figure 22] for imipramine (10 mg/kg) + LPS (p<0.001 vs. LPS group), MLEJS+LPS (12.5, 25 and 50 mg/kg) (p<0.05, p<0.01 and p<0.01 vs. LPS group), and a significant increase in LPS (0.83 mg/kg) (P<0.001 vs. vehicle group).

3.10 Antidepressant-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) on LPS-treated mice using tail suspension test.

Groups MLEJS+LPS (12.5, 25 and 50 mg/kg) (p<0.01, p<0.01 and p<0.0001 vs. LPS group, respectively), imipramine (10 mg/kg) + LPS (p<0.0001 vs. LPS group), and a significant increase

in LPS (0.83 mg/kg) ($p<0.0001$ vs. vehicle group) showed differences [ANOVA: $F(5,24)=19.11$, $p<0.0001$; Figure 10].

3.11 Antidepressant-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) on LPS-treated mice on in vivo oxidants and antioxidant parameters.

When compared to the vehicle-treated group, Figure 11A demonstrates that 24 hours later, LPS significantly ($p<0.05$) increased the level of thiobarbituric acid reactive substances (TBARS) [ANOVA: $F(5,24)=5.222$, $p<0.001$]. The pretreatment with imipramine (10 mg/kg) and MLEJS (50 mg/kg) significantly decreased the amount of TBARS ($p<0.05$). Additionally, pretreatment with MLEJS (12.5 and 25 mg/kg) had a positive but non-significant impact on the amount of TBARS in brain homogenate. When compared to the LPS-treated group, Figure 11B demonstrates that MLEJS (50 mg/kg) pretreatment considerably ($p<0.05$) increased GSH levels; however, MLEJS (12.5, 25 mg/kg) pretreatment was not significant [ANOVA: $F(5,24)=5.280$, $p<0.001$]. On the other hand, compared to mice treated with LPS, imipramine markedly ($p<0.001$) increased GSH levels.

\ Figure 11C shows that pretreatment with imipramine ($p<0.05$) and MLEJS (50 mg/kg) significantly increased SOD activity after LPS induced a considerable drop in it. [$F(5,24)=4.988$, $p<0.01$] in an ANOVA However, neither of the MLEJS concentrations (12.5 or 25 mg/kg) significantly countered the decline in brain homogenate SOD caused by LPS.

Moreover, imipramine administration increased the CAT activity of LPS-challenged mice ($p<0.05$) [ANOVA: $F(5,24)=6.474$, $p<0.01$; Figure 11D]; however, no significant effect was observed with MLEJS pretreatment at any dose. After administering LPS to mice for 24 hours, Figure 11E demonstrated a significant increase in nitrite levels ($p<0.001$) in their brain homogenate as compared to the vehicle-treated group. Pretreatment with imipramine (10 mg/kg) significantly ($p<0.001$) inhibited the increase in nitrite levels in the brain homogenate caused by LPS [ANOVA: $F(5,24)=14.88$, $p<0.0001$]. Additionally, MLEJS (12.5, 25, and 50 mg/kg) showed a protective effect against the LPS-induced decrease in nitrite levels in mice ($p<0.01$, $p<0.01$, and $p<0.001$ vs. LPS group, respectively).

3.12 Antidepressant-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) on LPS-treated mice on cytokines (IL-6 and TNF-α) level

LPS after 24 hours showed a significant ($p<0.0001$) increase in the brain TNF-α level, another proinflammatory cytokine in mice [ANOVA: $F(5,24)=9.344$, $p<0.001$; Figure 12B]. Pre-treatment with imipramine and MLEJS (12.5, 25, and 50 mg/kg) significantly ($p<0.001$, $p<0.01$, and $p<0.001$ vs. LPS group, respectively) decreased TNF-α level.

3.13 Antidepressant-like effect of methanol leaf extract of *Justicia secunda* (MLEJS) on LPS-treated mice on Acetyl-cholinesterase activity.

Compared to the vehicle-treated group, the AChE activity was considerably higher ($p<0.01$) in the mice that were challenged with LPS [ANOVA: $F(5,24)=4.260$, $p<0.001$; Figure 13]. AChE activity was considerably decreased ($p<0.05$, $p<0.05$, and $p<0.01$ vs. LPS group, respectively) by imipramine and MLEJS (12.5 and 50 mg/kg).

Discussion

Globally, anxiety and depression are the primary causes of disability due to their complex and varied psychiatric illnesses. The results of the behavioral tests would most likely be impacted by a motor coordination deficiency. The rota-rod test was used in this investigation to examine the motor effects of MLEJS. The findings indicated that, in contrast to diazepam (5 mg/kg), MLEJS (12.5–50 mg/kg) had no discernible effect on motor coordination.

One of the most popular tests used to assess anxiolytic/anxiogenic qualities is the elevated plus maze test. Based on the observation that rats and mice naturally exhibit a dislike of open spaces, this test interprets avoidance of open arms as anxiogenic behavior [18]. An increase in open arms exploration (time and entry into open arms) is a sign of anxiolytic-like efficacy, but this is not the case for medications with anxiogenic-like effects [18]. In both the acute and sub-acute

administrations of this trial, MLEJS increased the number of entry into open arms and the amount of time spent in them without changing the number of entries into closed arms, suggesting an anxiolytic-like effect without compromising motor function. Anxiety in the light-dark box test is measured by counting the number of transitions into and out of the light chamber; an increase in these parameters is thought to indicate anxiolytic-like qualities. Anxiety is caused by the tension between the urge to explore and to withdraw from an unfamiliar, well-lit environment. This investigation demonstrated that these two parameters rose with MLEJS treatment.

An increase in head-dipping behaviors may be an indication of an anxiolytic-like condition in the hole-board test, which is helpful for simulating anxiety in animals [19]. The data obtained supported the anxiolytic-like effect previously demonstrated in the elevated plus maze and light-dark box tests, demonstrating that MLEJS increased the frequency of head dips without changing the number of squares crossed. Consequently, the outcomes of these three techniques demonstrate that MLEJS exhibits anxiolytic-like activity without altering the sedative effects' typical pattern of locomotion. The crossing number was not changed by MLEJS administration, according to the open-field test results (Table 1), which confirms the prior observation that there was no change in motor coordination from the rota-rod test. The reduction in rearing behavior appears to be due to a central depressive activity rather than a loss of motor coordination or neuromuscular blockage. An increase in these parameters may be a sign of anxiolytic-like effects [19]. MLEJS treatment also raised the preference for the central area, improving the crossing number and the amount of time spent in the apparatus's central section (Table 1). The anxiolytic effect of Pimpinella anisum leaf was reported by Es-Safi et al. [41] using open-field, elevated plus maze, and light-dark box tests. These results are comparable to their work.

The benzodiazepine system is involved in the underlying mechanism of MLEJS's anxiolytic-like actions. Flumazenil, a well-known competitive antagonist that binds to the GABAA receptor's benzodiazepine site and is known to counteract the anxiolytic, sedative, and hypnotic effects of benzodiazepines, was administered to the animals prior to their pretreatment [20]. Flumazenil (6 mg/kg) partially reduced the overall anxiolytic-like action of MLEJS, although not considerably. Our findings demonstrate that flumazenil administration had no discernible impact on the effects of MLEJS, suggesting that the benzodiazepine site of the GABAA receptor may not be involved in MLEJS's anxiolytic-like effects.

In the second part of the investigation, which was the anti-depressant phase, mice that had been exposed to LPS for 24 hours showed depressive-like behavior, which was examined using OFT, FST, and TST. The anti-oxidant biomarkers, pro-inflammatory cytokines, and acetylcholinesterase activity in the mice's brains were also measured.

The primary criterion, which was proposed to show an animal's hopelessness and poor mood (behavioral despair), linked to depressive-like behavior, is the length of immobility or despair behavior produced in both FST and TST. Both of these behavioral despair tests are dependable and have been widely utilized in the screening process for novel antidepressants [21].

According to Barua et al. [21], an LPS dose of 0.83 mg/kg (i.p.) was used in this investigation because it can cause the entire spectrum of the acute illness response and depressive-like behavior, and it has been demonstrated to cause depression 24 hours after LPS injection [22].

The current study's findings showed that MLEJS shortened the time that LPS-treated mice underwent TST and FST immobility. According to Ohgi et al.'s [23] and Ji et al.'s [24] research,

MLEJS's capacity to shorten the period of immobility in LPS-treated mice implies that animals may have antidepressant-like action.

Increased levels of oxidative stress and neuroinflammation have been linked to the genesis of major depression, despite the fact that altered brain levels of monoamines have long been recognized as the primary pathological hallmark of the condition [25]. Cell death, decreased neurogenesis, decreased neural plasticity, and increased autoimmune responses have all been linked to oxidative stress. These factors then start and spread neuroinflammation, which intensifies tissue destruction [26].

It is well known that brain cells are particularly vulnerable to the harmful effects of free radicals, and that the degree of tissue damage is correlated with elevated MDA and nitrite levels and reduced antioxidant defense systems in the cells [25]. Patients suffering from depressive diseases have been found to have elevated levels of MDA, a significant indicator of oxidative stress [27]. Antidepressant therapies significantly decreased oxidative stress, which was demonstrated to correspond with clinical outcome indicators in preclinical research [28]. Our findings demonstrated that oxido-nitrosative stress caused by LPS had an impact on the mice's brains. When comparing the brains of the LPS-treated mice to the vehicle-treated group, the MDA level a hallmark of LPO was found to be considerably higher in the former group.

Numerous past research projects have indicated that NO plays a major role in the pathophysiology of depression. Numerous NO inhibitors have antidepressant effects by either decreasing the amount of cyclic guanosine monophosphate or inhibiting the NO synthase enzyme in the HC [29].

Additionally, it has been revealed that a number of neuroinflammation biomarkers, such as TNF-α and IL-6, are up-regulated in depressive disorders, indicating that inflammatory is the primary underlying cause in the condition [30].

These cytokines cause microglial activation and neuroinflammation, which alter the pathophysiology of depression [31]. We have demonstrated in this study that the treatment of LPS causes a rise in the levels of pro-inflammatory cytokines (TNF-α and IL-6). Here, our findings unequivocally demonstrated that MLEJS pretreatment inhibited pro-inflammatory reactions and lessened the depressive-like behavior that was subsequently brought on by the administration of LPS.

Stress and inflammation can cause the enzymes that break down or inactivate a lot of neuromodulators to become sensitive. It has been demonstrated that oxidative stress and the proinflammatory cytokine IL-1 increase acetylcholinesterase expression [32]. The effects of LPS-mediated neuroinflammation on cortical inhibition and elevated ACh-E activity have an impact on neuromodulation.

MLEJS is a complex mixture of several metabolites, including linolenic acid, triterpenoids, fatty acids, vitamins, aromatic compounds, and steroids, according to the chromatogram. The presence of multiple compounds that may act in an additive manner to enhance the potency of active constituents or multiple active ingredients that may act synergistically, acting through independent but ideally complementary pathways to confer maximal effects, may be the cause of J. secunda's anti-anxiety and anti-depressant effect.

9,12,15-Octadecatrienoic acid, also known as linalenic acid, is the main compound found in MLEJS by GC-MS. It is an essential fatty acid derived from plants and a member of the omega-3

(τ-3) polyunsaturated fatty acid group, from which humans can synthesize other omega-3, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). According to a study by Perez et al. [33], omega-3 PUFAs cause a strong anxiolytic-like effect in the elevated plus maze (EPM) when combined with picrotoxin pretreatment. This suggests that GABAA receptors are involved in these effects. Additionally, the forced swim and tail suspension tests [34] demonstrate an antidepressant-like effect. Additionally, it has been demonstrated that omega-3 PUFAs ameliorate anxiety- and depressive-like phenotypes in a variety of animal models of depression [35, 36]. Additionally, SSRIs in combination with omega-3 PUFAs seem to be more efficacious than antidepressant medications alone in lowering behaviors associated with depression. Additionally, the study demonstrates that MLEJS has similar concentrations of other unsaturated fatty acids, such as oleic acid (omega-9 τ-9) and linoleic acid (omega-6 τ-6). Anxiolytic, antidepressant, anti-inflammatory, hypocholesterolemic, hepatoprotective, cancer-preventive, antihistaminic, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic, and anticoronary properties have all been documented for this [37]. By increasing brain antioxidant state, this compound's capacity to scavenge free radicals may have an indirect relationship with MLEJS's depressive and anxiolytic properties. The previous study by Onochie et al. [42], who also show the abundant content of flavonoids in the leaves of Justicia secunda, is supported by the association between the antioxidant role of MLEJS and its depressive and anxiolytic effects.

Another component found in GCMS is squalene, a naturally occurring triterpene with demonstrated neuroprotective and oxygen scavenging properties. Because squalene lowers neuroinflammation and increases brain antioxidants, it may help explain J. secunda's reported antidepressant potential. It has anticancer qualities as well. A diterpene called phytol, a branched-chain unsaturated alcohol molecule also present in J. secunda, suggests the potential antibacterial,

anti-inflammatory, and anticancer effects of plants. According to Fedotova et al. [38], phytol interacts with and involves the GABAA /benzodiazepine receptor to provide sedative and anxiolytic-like effects in mice.

The extract contains a triterpene called alpha (α) amyrin, which may also have antidepressant and anxiolytic properties. Prior studies confirm that α-amyrin shortens the mice's immobility period during the behavior despair test [39]. Different animal models have revealed the anxiolytic effects of the mixture of α/β-amyrin, which was isolated from the stem bark resin of Protium heptaphyllum. It has been claimed that the positive allosteric regulation of BDZ-sensitive and -insensitive types of GABA-A receptors mediates these effects [40]. The study's findings demonstrated that while α/β-amyrin enhanced the period of permanence and the number of entrances in the open arms, it dramatically lowered the number of crossings, grooming, rearing, and the number of crossings in the closed arms.

The study's findings suggest that, without affecting motor coordination, acute administration of Justicia secunda methanol leaf extract had anxiolytic-like effects in the elevated plus maze, light/dark, and hole-board tests. This effect is probably not mediated by GABAA/BDZ receptors because it did not show tolerance after brief, repeated dosing, nor did it cause peripheral neuromuscular blocking or sedation. Furthermore, in the mouse model of LPS-induced depression, MLEJS shows a great ability to reverse depressive-like behavior. It is possible for the antidepressant effect to be achieved via reducing pro-inflammatory mediators or by blocking oxido-nitrosative stress. As a result, our research raises the possibility that MLEJS could be a helpful therapeutic strategy for the management of mental illnesses linked to oxidative damage and neuroinflammation.

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