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Research Article

Antidepressant-like effects of the leaf extract of *Mallotus oppositifolius* (Geiseler) Müll. Arg. (Euphorbiaceae) in the chronic unpredictable mild stress model: A role of the gut-brain axis

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ABSTRACT

The gut microbiota has been posited as a target for the treatment of major depressive disorder. Herein, we investigated the effect of the hydroethanolic leaf extract of *Mallotus oppositifolius* (MOE) on the gut microbiota of mice and how this contributes to its known antidepressant-like effect. A 6-week chronic unpredictable mild stress (CUMS) procedure was employed in 7 groups of mice to induce depression. From the third week, oral MOE treatments (10, 30, 100 mg/kg) and two reference drugs, fluoxetine (12 mg/kg) and minocycline (40 mg/kg), known to affect the gut microbiota, were administered. The sixth and seventh groups were the vehicle stressed (VEH-S) and non-stressed groups (VEH-NS). Changes in depressive-like behaviors were assessed using sucrose preference test while the forced swimming test (FST) was used to assess sustained antidepressant-effect after treatment discontinuation. Moreover, changes in prefrontal cortex (PFC) and hippocampal serotonin (5-HT) levels were evaluated using enzyme-linked immunosorbent assay (ELISA). The effect of treatment on the profile of the gut microbiota of the groups was elucidated using 16S rRNA Oxford Nanopore sequencing. MOE and reference drugs reversed the depression-associated reduction in sucrose preference when compared to VEH-S. MOE (with peak effect at 30 mg/kg) reduced immobility while increasing swimming and climbing behaviors. MOE reversed CUMS-induced reduction of 5-HT concentration in PFC and hippocampus. The behavioral effects of MOE were associated with shifts in the gut microbiota of CUMS-exposed mice. The study has provided seminal evidence that MOE ameliorates CUMS-induced depressive symptoms by modulating gut microbiota and increasing brain 5-HT levels.

Introduction

Major depressive disorder (MDD) is a leading cause of global disability and morbidity with an estimated 246 million cases across all age groups worldwide (James et al., 2018). Understanding of the pathophysiology of depression has significantly improved, as research indicates that factors such as neurotrophic, inflammatory, and neuroendocrine processes contribute to the development of this condition alongside previously recognized monoamine-related causes (Hasler, 2010). Despite the advancements in our understanding of the neurobiology of MDD and the introduction of alternative antidepressant classes, only 60–70 % of individuals with depression recover with antidepressant therapy (Murphy et al., 2017). Treatment-resistant MDD patients face higher risks of relapse, chronicity, psychosocial impairments and suicide (Miyaoka et al., 2018). The existence of loopholes in the hypotheses of depression suggests that there may be undiscovered target

Abbreviations: 5-HT, 5-Hydroxytryptamine; BDNF, Brain-derived Neurotrophic Factor; CNS, Central nervous system; CUMS, Chronic Unpredictable Mild Stress; DNA, Deoxyribonucleic acid; GABA, γ-aminobutyric acid; GI, Gastrointestinal; GM, Gut microbiota; ICR, Institute of Cancer Research; LEfSe, Linear Discriminant of Effect Size; MDD, Major Depressive Disorder; MOE, *Mallotus oppositifolius* extract; NMDA, N-methyl-D-aspartate; PCoA, Principal Coordinate Analysis; PCR, Polymerase chain reaction; rRNA, Ribosomal Ribonucleic Acid; TRD, Treatment-resistant Depression.

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proteins, beyond those identified by monoamine and monoamine-alternate theories, that novel antidepressants may need to exploit. Discovering these new targets could lead to innovative treatments for MDD and a deeper understanding of the development of the disease.

In this regard, the gut-brain axis is considered a potential target for antidepressant therapy. The gut-brain axis, considered a bidirectional communication pathway between the brain and gut, plays an important role in neuropsychiatry (Cryan et al., 2019). Studies suggest that the microbiota present in the gut are not just a collection of commensal microorganisms, but also a metabolic organ with crucial functions in disease management. Therefore, it may be targeted for treatment of various diseases, including central nervous system (CNS)-related conditions such as depression (Hirschberg et al., 2019). The microbiota-gut-brain axis can modify the autonomic nervous system, gastrointestinal, immune and central nervous systems through the production of microbial metabolites such as short-chain fatty acids (SCFAs), tryptophan as well as neurotransmitters such as serotonin (Cryan et al., 2019). A considerable body of preclinical research has established a correlation between certain species of microorganisms, including *Lactobacillus delbrueckii, Lactobacillus reuterii,* and *Faecalibacterium prausnitzii,* and their potential to mitigate depressive behaviors in rodent models when administered (Hao et al., 2019; Qiu et al., 2021; Xie et al., 2020). In contrast, certain species of microorganisms, exemplified by *Alistepes* spp., have also been associated with depression when their population within the gut microbiota is high (Jiang et al., 2015). This further supports the need to focus on the gut-brain axis in search for enhanced antidepressant therapy.

Mallotus oppositifolius (Euphorbiaceae), a dioecious shrub found in dry forests and secondary growth across West Africa (Nwaehujor et al., 2013), is a subject of interest in pharmacological research due to its potential in treating neuropsychiatric disorders and antimicrobial properties. The leaf extract of *M. oppositifolius* (MOE) has been found to elicit rapid-onset and sustained antidepressant-like effects through enhancement in serotonergic neurotransmission and inhibition of glycine/NMDA receptor activation. These observed effects were without impaired cognitive function or significant weight change (Kukuia et al., 2014, 2016b). In addition to its antidepressant activity, the leaves of *M. oppositifolius* have also shown promising antimicrobial activity. For instance, MOE exhibited potent antibacterial activity against *Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis* and beta-lactam resistant Gram-positive cocci (Chukwujekwu et al., 2005; Gangoué-Piéboji et al., 2009). Furthermore, other studies on *M. oppositifolius* showed potent antifungal activity (Adekunle and Ikumapayi, 2006; Ngouana et al., 2021). Considering these antimicrobial activities, it is plausible that the leaf extract of *M. oppositifolius* may affect the gut microbiota. However, it is unclear if the influence of MOE on the gut microbiota will contribute to the antidepressant-like effects previously reported.

The study investigated the effect of *M. oppositifolius* leaf extract on the gut microbiota and its role in antidepressant activity. These findings will provide a useful resource to develop novel treatment strategies for depression and related mental health disorders.

Experimental procedures

Plant collection and extraction

Leaves of *M. oppositifolius* were obtained from the Centre for Plant Medicine Research (CPMR), Akuapem-Mampong, Ghana without harming the plant. Authentication was done by a botanist at the Department of Botany, University of Ghana, and a voucher specimen (CPMR 314/17) kept. Air-dried leaves were crushed with a hammer mill before 3 kg of powder was extracted using ethanol (70 % v/v) for 72 h. The resulting extract was condensed to a syrupy substance at 60 °C using a rotary evaporator then dried in a water bath. The dried extract was then stored in a desiccator until needed. A weight of 31.5 g of extract was obtained representing 1.05 % yield. The final yield is referred to as *M. oppositifolius* extract (MOE) or extract.

Chemicals and reagents

Sucrose powder ($C_{12}H_{22}O_{11}$), fluoxetine and minocycline were purchased from Sigma-Aldrich Inc. St. Louis, Missouri, United States. ZymoBIOMICS DNA Miniprep Kit was purchased from Zymo Research, USA. Microwell Mouse Serotonin ELISA kit was purchased from Syndro Bioresearch, USA.

Compounds and drug presentation

M. oppositifolius extract (MOE) was dissolved in normal saline (0.9 %) and doses of 10, 30 and 100 mg/kg of not more than 0.5 ml per animal were administered orally with the aid of an oral gavage needle. The doses of MOE used in the study were selected based on results obtained from previous investigations by Kukuia et al. (2014) which stated that neuroactive effects were observed at those doses.

Fluoxetine, a selective serotonin reuptake inhibitor with established influence on the gut microbiota (Sun et al., 2019), and minocycline, a tetracycline antibiotic which has been found to elicit antidepressant activity due to its effect on the gut microbiota (Schmidtner et al., 2019), were used as the reference drugs; normal saline (0.9 %) was used as the vehicle. Minocycline 40 mg/kg and 12 mg/kg fluoxetine were prepared by dissolving in normal saline. The doses of the control drugs were selected based on research by Sun et al., (2019) and Schmidtner et al., (2019) respectively.

Experimental animals and housing

Fifty-six (56) male ICR mice (20–30 g) were acquired from the Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana. They were housed individually in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding and given a commercial pellet diet (GAFCO, Tema, Ghana), along with free access to water. The environment was kept at 25 ± 2 °C temperature, relative humidity between 60–70 %, and a light–dark cycle of 12 h. Mice were acclimatized to their new environment for a week and evaluated for weight and sucrose preference. Animal studies and behavioral tests on mice were conducted at the Animal House Unit and Neuropsychopharmacology Laboratory at the University of Ghana Medical School.

Chronic unpredictable mild stress (CUMS) procedure

The chronic unpredictable mild stress (CUMS) procedure was conducted following the method described by (He et al., 2020). Mice were isolated and subjected to daily stressors (as shown in Table 1), with no predictability between consecutive days. This regimen lasted for six weeks. The reference drugs (minocycline and fluoxetine) and MOE were

Table 1
Schedule of stressors used during CUMS procedure.

	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
WEEK 1	A	B	C	D	E	I	G
WEEK 2	D	H	A	C	E	M	J
WEEK 3	K	A	G	C	F	I	L
WEEK 4	C	E	G	F	L	K	D
WEEK 5	E	C	G	M	F	J	B
WEEK 6	D	H	A	C	E	I	J

Key: A – Overcrowding for 10 h, B – Water Deprivation for 12 h, C- Tilting of cage at 45̊ for 12 h, D – Wet bedding for 15 h, E – Empty cage for 12 h, F – Reverse Day/Night cycle, G – Traffic noise for 4 h, H – Water in cage for 4 h, I- 24 h food/water Deprivation, J – Tail Pinch for 2 mins, K- Unfamiliar Cage, L – Food Deprivation, M- Dampened Sawdust.

administered from Week 3 of the CUMS procedure with the no-CUMS (naïve control) and CUMS-only (negative control) groups receiving an equal volume of normal saline. Oral dosages were given to all groups once daily at 10:00 am (Fig. 1).

The ICR mice were divided into 7 groups, namely:

No-CUMS control group **(VEH-NS)**
CUMS-only group **(VEH-S)**
Three groups administered 10 mg/kg, 30 mg/kg and 100 mg/kg hydro-ethanolic extract of *M. oppositifolius* (MOE) respectively **(MOE −10, MOE −30, MOE −100)**
Fluoxetine group (12 mg/kg/day**) (FLX)**
Minocycline group (40 mg/kg/day) **(MNC)**

Each group consisted of six males assigned randomly and housed individually. To eliminate bias, each mouse was assigned a unique number between 1 and 42. The website http://random.org was then used to generate a randomized list of the numbers. The randomized list was separated into 7 equal segments with each representing a group. Each mouse was then assigned to a group based on its assigned number.

Sucrose preference test (SPT)

The sucrose preference test (SPT) was performed at four time points: Week 0 for acclimatization, before CUMS (Week 1), pre-treatment (Week 3), and after the six-week procedure (Fig. 1). Mice underwent adaptive training on Days 1–4 with two bottles of 200 ml water provided on Day 1 and 2, two bottles of 200 ml 1 % sucrose on Day 3, and one bottle of water and one bottle of 1 % sucrose on Day 4. Following a period of food and water deprivation, mice were given predetermined amounts of both substances to drink freely for an hour each. The location of the bottles changed halfway through each hour-long session to avoid position preference bias. A blinded experimenter collected the data by measuring the weight of liquid consumed. Sucrose preference was calculated as follows:

$$\textit{Sucrose preference} = \frac{\textit{weight of sucrose consumed}}{(\textit{weight of sucrose consumed} + \textit{weight water consumed})} \times 100$$

Forced Swimming Test (FST)

The forced swimming test (FST) is used to evaluate antidepressant efficacy of drugs, with shorter immobility time indicating greater impact (Kukuia et al., 2014). In this study, the FST was conducted prior to sacrificing the animals in week 7 to assess sustained antidepressant-like effect after discontinuation of treatment (Fig. 1). Each mouse underwent testing by being placed in a clear plastic cylinder filled with water at 25 ± 1 °C, and depth of 20 cm. Following 2 min of adaptive swimming, immobility during a 4-minute interval which was digitally recorded with a video camera (Sony 4 K Handy Cam, FDRA×100E). Mice that remained floating without actively keeping their heads above water were categorized as immobile. For assessment of active behavior of the mice, vertical movements against walls were categorized as climbing whilst horizontal movements across the water surface were categorized as swimming. Immobility, climbing, and swimming times were measured and analyzed using a video tracking software (Boris v.8.19.4) (Friard and Gamba, 2016; Kukuia et al., 2022).

Fecal sample collection

Stool samples from the mice that went through the CUMS procedure were processed according to the procedure of Shahi et al. (2019). Stool samples were collected after Week 7 using divider boxes consisting of rectangular plastic bowls partitioned with cards. The fecal pellets were removed from each compartment using sterile forceps and placed in pre-labelled 2 mL microcentrifuge tubes which were stored at −80 °C within 24 h. The collection compartments, forceps and divider boxes underwent sterilization with 70 % ethanol before use. Mice were individually placed in the sterilized compartments for up to an hour to allow natural defecation process while ensuring concurrent sample collection. Once collected, the tubes containing the samples were securely closed and further processed after storage at −80 °C temperature until needed.

Gut microbiota analysis

Stool sample processing

For DNA extraction from fecal samples, frozen samples were thawed at room temperature. A DNA isolation kit blank was included in all extraction steps. The ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA) was used to extract DNA from individual samples following the manufacturer's protocols.

16S rRNA Gene Amplification

The 16S hypervariable regions were amplified using the Ion 16S Metagenomics kit (Thermo Fisher Scientific) and the V2-4–8 primer set. The supplied *E. coli* DNA was used as a positive control, following the kit instructions. After PCR amplification, the products were purified using the AMPure magnetic bead-based purification system (Beckman Coulter) and their quantity was measured with the QuBit dsDNA HS Assay kit and Qubit® 4.0 Fluorometer (Thermo Fisher Scientific).

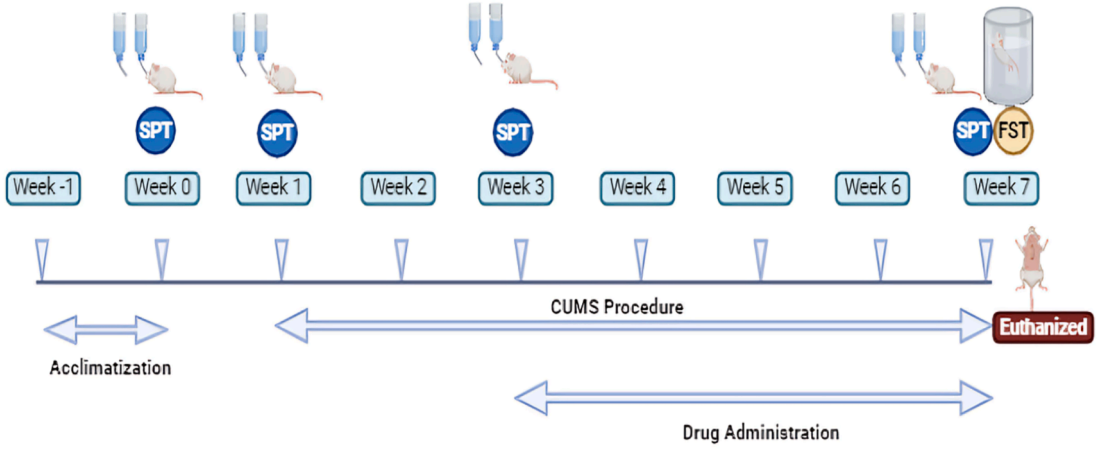


Fig. 1. Timeline of CUMS procedure.

Oxford nanopore next-generation sequencing

DNA libraries were generated using the Oxford Nanopore kit SQK-LSK109 and EXP-NBD196 kit from the purified amplicons that were diluted down to 50 ng, and each library was barcoded according to the manufacturer's protocol. Quality control of the barcoded libraries was performed with the Agilent 4200 TapeStation and Qubit® 4.0 Fluorometer. The barcoded libraries were then pooled together, including one positive control (*E. coli*) and one negative control (nuclease free water), and sequenced on the MinION MK1B (ONT) sequencing machine with a FLO-MIN106 flow-cell.

Hippocampus and prefrontal cortex isolation

After the FST, four mice were randomly chosen from each group. The mice were euthanized following the method described by Kukuia et al. (2022).

The mice were then sacrificed and their whole brains separated from the skull. The prefrontal cortex and hippocampus were rapidly separated on ice plates, quickly frozen using liquid nitrogen, and subsequently preserved in a −80 °C freezer.

Tissue homogenate preparation

The dissected brain tissue (prefrontal cortex or hippocampus) was weighed individually. The tissue was then minced into small pieces and homogenized with a glass homogenizer on ice in PBS in a ratio of 1 mg of tissue: 10 µl of PBS. The resulting mixture was then centrifuged at 1000 × *g* (3000 rpm) for 20 min. The supernatant was subsequently collected and preserved at a temperature of −80 °C.

Enzyme-Linked Immunosorbent Assay (ELISA) of 5-HT

The levels of serotonin (5-HT) in the hippocampus and prefrontal cortex were assayed using Microwell Mouse Serotonin ELISA kits (Syndro Bioresearch, USA) following the manufacturer's instructions and recorded. The optical density (OD) of each well was measured using a ThermoFisher MultiSkan FC microplate reader within 15 min of the addition of the stop solution. A standard curve was obtained by plotting the average OD for each standard concentration against the known concentration of the standard. The plotted standard curve was subsequently used to determine the concentrations of 5-HT in the prefrontal cortex and hippocampus of the various groups by extrapolation.

Bioinformatic analysis

The data files from the sequencing procedure were converted to FASTQ format using Epi2Me. The metagenome reads were then run against a Kraken microbial database to yield report files. This was followed by a Bracken database run against the results to obtain the relative abundances of the respective groups and results of diversity analysis.

Statistical analysis

GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA) was used for data and statistical analysis of the behavioral test findings, 5-HT ELISA test results and the alpha diversity analysis results. All values were recorded as the Mean ± standard error of the mean. To analyze the behavioral test findings and demonstrate statistically significant differences, one-way and two-way analyses of variance (ANOVA) and Tukey's multiple comparisons test and Dunnett's multiple comparison test respectively were used. To analyze the 5-HT ELISA test results and demonstrate statistically significant differences, one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were used. Statistical analysis of the alpha diversity indices was done using Kruskal Wallis test analysis. To analyze the beta diversity indices, principal coordinate analysis (PCoA) plot was used to demonstrate the clustering of the various groups on Python (version 3.8.10). In all test above, a $P<0.05$ was considered statistically significant. Linear Discriminant of Effect Size (LEfSe) analysis was performed on the various groups to determine their respective significant taxa at the Galaxy module website http://huttenhower.sph.harvard.edu/galaxy. The test involved a non-parametric factorial Kruskal-Wallis sum-rank test ($P<0.05$), followed by a Wilcoxon non-parametric multiple comparison ($P<0.05$) and then finally a linear discriminant analysis (LDA) > 2.0 (Segata et al., 2011). Spearman correlation analysis was used to calculate the statistical correlation between the relative abundance of depression-related taxa, results of behavioral tests and levels of 5-HT and the data visualized using Python (version 3.8.10).

Results

Effect of MOE on behavioral tests of depression

The sucrose preference test (SPT) and forced swimming test (FST) were conducted to evaluate the effect of MOE on depression-related behaviors of ICR mice. As shown in Fig. 2A below, the SPT was performed at four time points; namely (Weeks 0, 1, 3 and 7). In Week 0 (before the start of the CUMS procedure), no significant difference in percentage of sucrose preference was observed across the groups. All groups of mice, except the no-CUMS control group (VEH-NS), were subjected to various stressors from the start of Week 1 and these stressed groups showed a significant ($F_{18,\ 178} = 5.062$, $P<0.0001$) reduction in percentage sucrose preference compared to the VEH-NS, indicative of the success of the depression model employed. Compared with the VEH-S group, 4-week administration of MOE (10, 30, 100 mg/kg), fluoxetine (12 mg/kg) and minocycline (40 mg/kg) significantly increased ($P<0.001$) percentage sucrose preference to near baseline levels (i.e., comparable to VEH-NS).

The influence of MOE 10, 30, 100 mg/kg and the reference drugs, fluoxetine and minocycline, on total sucrose preference is depicted as the area under the curve (AUC) graph (Fig. 2B). Here, we show that vehicle stressed mice (VEH-S) showed a significantly reduced total sucrose consumption when compared to the vehicle non-stressed group (VEH-NS). Total sucrose consumption in stressed mice was significantly ($F_{6,\ 49} = 7.907$; $P<0.0001$) increased by MOE, FLX and MNC.

To assess whether the antidepressant-like effect of MOE will be sustained when drug treatment ceases, the FST was conducted in the 7th week. As shown in Fig. 3, MOE (10, 30 and 100 mg/kg), minocycline (40 mg/kg) and fluoxetine (12 mg/kg) significantly ($P<0.05$) decreased immobility behavior (Fig. 3A) while increasing duration of swimming (Fig. 3B) and climbing (Fig. 3C), when compared to the VEH-S group.

Effect of MOE on 5-HT levels of prefrontal cortex and hippocampus of mice

Here, we present the graphs of the effect of MOE on the 5-HT levels in the prefrontal cortex and the hippocampus as violin plots. There was a depression-associated reduction in concentration of 5-HT in the prefrontal cortex (Fig. 4A) and hippocampus (Fig. 4B) of the VEH-S group when compared with the VEH-NS ($P<0.0001$). In contrast, MOE (10, 30 and 100 mg/kg), minocycline (MNC 40 mg/kg) and fluoxetine (FLX 12 mg/kg) significantly ($P<0.0001$) reversed the CUMS-induced reduction in 5-HT concentration in the prefrontal cortex in comparison to the VEH-S (Fig. 4A). Interestingly, the effect of MOE, FLX and MNC on the concentration of 5-HT in the prefrontal cortex was higher than the vehicle non-stressed group (VEH-NS). In addition, all treatment groups (MOE, FLX and MNC) reversed the fall in hippocampal 5-HT concentration, although not to pre-stressed levels (VEH-NS) (Fig. 4B).

Effect of MOE on the gut microbiota of CUMS mice

Here, we present the results of the relative abundances of taxa of microorganisms present in the gut microbiota at the various taxonomic levels after treatment with MOE. Even though the most prevalent taxa were consistent across various treatment groups at the various

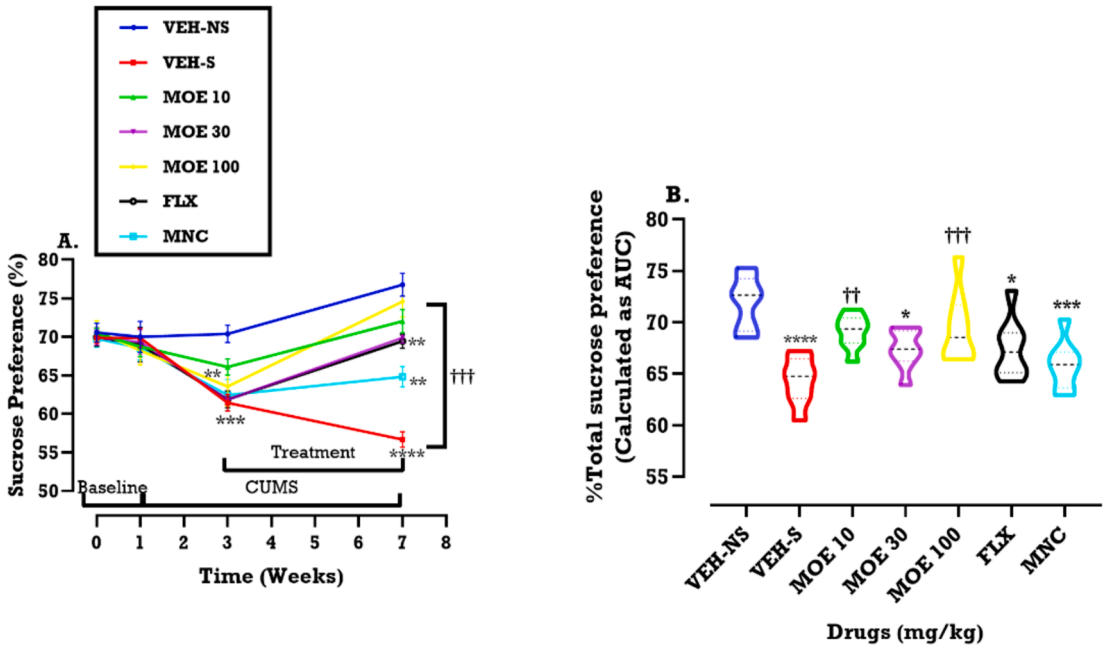


Fig. 2. Effect of *Mallotus oppositifolius* extract (MOE) on sucrose preference of CUMS-treated mice (A) Time-course events and (B) violin plots of area under the curve showing effects of MOE (10, 30 and 100 mg/kg), fluoxetine (FLX 12 mg/kg) and minocycline (MNC 40 mg/kg) treatments on the percentage sucrose preference of ICR mice subjected to the CUMS procedure. Data represented as mean ± SEM (n = 6–8). (A) *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 when compared to no-CUMS control, †††P<0.001, ††††P<0.0001 when compared to the CUMS-only control (Two-way ANOVA followed by Dunnett's multiple comparison test) (B) *P<0.05, ***P<0.001, ****P<0.0001when compared to no-CUMS control, ††P<0.01 when compared to CUMS-only control (One-way ANOVA followed by Tukey's multiple comparison test).

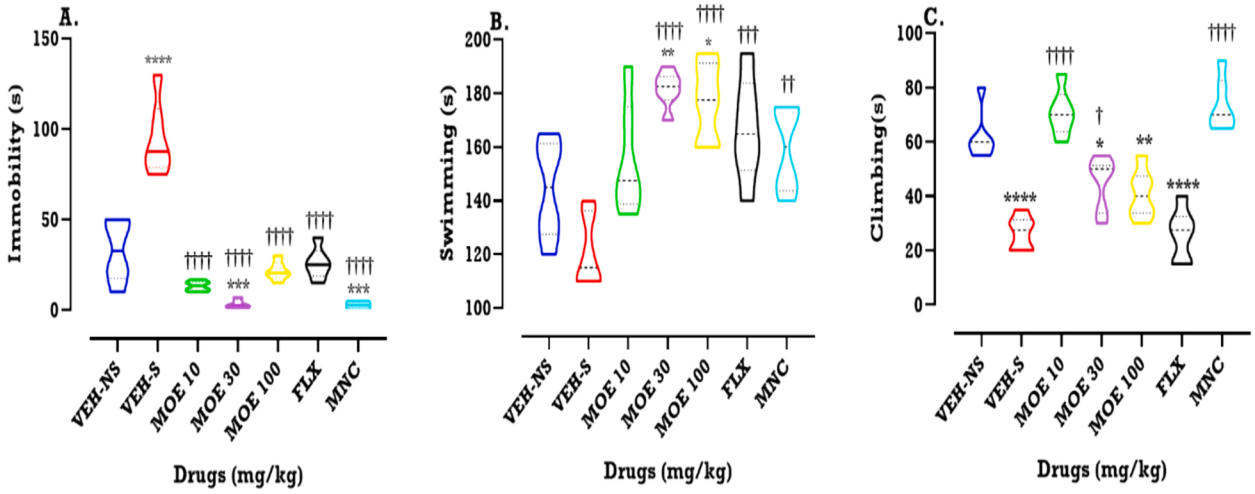


Fig. 3. Effects of *Mallotus oppositifolius* extract (MOE 10, 30 and 100 mg/kg), fluoxetine (FLX 12 mg/kg) and minocycline (MNC 40 mg/kg) treatment on A; Duration of Immobility B; Duration of Swimming and C; Duration of Climbing in the FST of CUMS- induced mice. Data presented as mean ± SEM (n = 6–8) *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001 compared to no-CUMS control, †P<0.05, ††P<0.01, †††P<0.001, ††††P<0.0001 compared to CUMS-only control (One-way ANOVA followed by Tukey's multiple comparison test).

taxonomic levels, specific taxa exhibited dissimilar relative abundances.

As shown in Fig. 5A, the dominant microbial groups within the mouse gut microbiota belonged to four primary phyla: *Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria*. The abundances of *Firmicutes and Proteobacteria* were decreased while that of *Actinobacteria* was increased in the VEH-S group when compared to the VEH-NS group. In contrast, the abundances of *Firmicutes, Proteobacteria* were increased *Actinobacteria* declined in the MOE, FLX and MNC groups when compared to the VEH-S. MOE 30 mg/kg decreased the abundance of *Bacteroidetes* in comparison to the CUMS-only group.

In Fig. 5B, exposure to the CUMS procedure decreased the abundances of *Bacilli Gammaproteobacteria, Bacteroidia* and *Clostridia* in the VEH-S group when compared to the VEH-NS group. Following treatment of CUMS-exposed mice with MOE and the reference drugs, the abundances of *Bacilli, Gammaproteobacteria, Bacteroidia* and *Clostridia* increased in comparison to the VEH-S group.

In Fig. 5C, exposure to the CUMS procedure decreased the abundances of *Bacteriodales, Lactobacillales, Bacillales,* and *Enterobacterales* but *Clostridiales* and *Bifidobacteriales* increased in the VEH-S group when compared to the VEH-NS group. Following treatment with MOE and the reference drugs, the abundances of *Lactobacillales,* and *Enterobacterales* increased in comparison to the VEH-S group while *Bifidobacteriales*

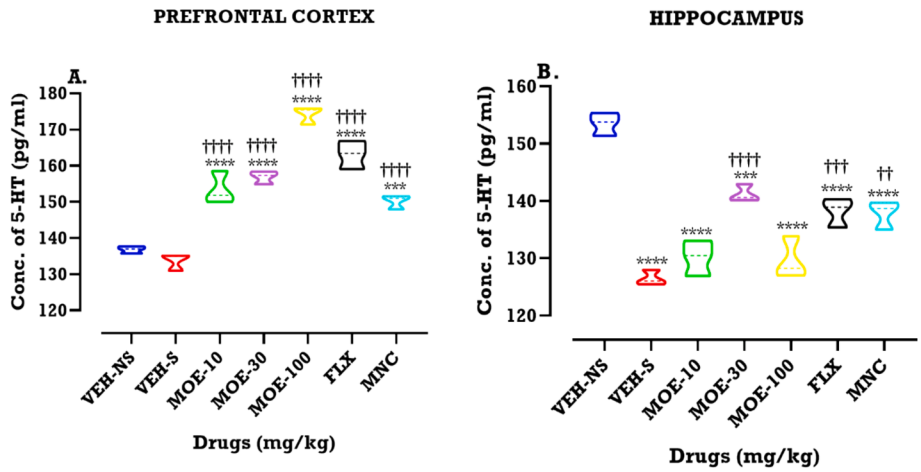


Fig. 4. Effect of *Mallotus oppositifolius* extract (MOE 10, 30 and 100 mg/kg), fluoxetine (FLX 12 mg/kg) and minocycline (MNC 40 mg/kg) treatments on concentration of serotonin (5-HT) in A; Prefrontal cortex and B; Hippocampus (A) ***P<0.005, ****P<0.0001 when compared to no-CUMS control, ††††P<0.0001 when compared to the CUMS-only control (B) ***P<0.001, ****P<0.0001when compared to no-CUMS control, ††P<0.01, †††P<0.005, ††††P<0.0001 when compared to CUMS-only control (For both A and B; One-way ANOVA followed by Tukey's multiple comparison test)..

decreased. Though MOE 10 mg/kg increased the abundance of *Bacteriodales*, MOE 10, 30 and MNC increased the abundance of *Bacillales* while MOE 30 mg/kg, 100 mg/kg and FLX increased the abundance of *Clostridiales* in comparison to the VEH-S group.

In Fig. 5D, exposure to the CUMS procedure decreased the abundances of *Lactobacillaceae, Staphylococcaceae, Bacillaceae, Bacteriodaceae* and *Prevotellaceae* while *Clostridiaceae* and *Bifidobacteriaceae* increased in the VEH-S group when compared to the VEH-NS group. Following treatment of CUMS-exposed mice with MOE and the reference drugs, the abundances *of Lactobacillaceae, Bacillaceae,* and *Bacteriodaceae* increased in comparison to the VEH-S group with *Bifidobacteriaceae* decreasing. However, MOE 10 and 100 mg/kg, just as the reference drugs, increased the abundance of *Prevotellaceae* with MOE 10 mg/kg and MNC increasing the abundance of *Staphylococcaceae,* and FLX increasing the abundance of *Clostridiaceae* in comparison to the VEH-S group.

As illustrated in Fig. 5E, exposure to the CUMS procedure decreased the abundances of *Lactobacillus, Prevotella, Staphylococcus and Bacteriodes* but *Bifidobacterium* increased in the VEH-S group when compared to the VEH-NS group. MOE and the reference drugs increased the abundances of *Lactobacillus and Bacteriodes* in comparison to the VEH-S group while *Bifidobacterium* decreased. However, MOE 10 and 100 mg/kg, as well as the reference drugs increased the abundance of *Prevotella* with MOE 10 mg/kg and MNC increasing the abundance of *Staphylococcus* in comparison to the VEH-S group.

Alpha diversity analysis

Alpha diversity analysis was performed to determine the diversity (the microbial richness and evenness) of microbial species within each test sample. The Shannon index and the Simpson index are two commonly used measures of alpha diversity in microbial communities, including gut microbiota. The Shannon index considers both the number of species present and the relative abundance of each species. The Simpson index on the other hand was used to calculate the probability that two randomly selected microorganisms in the sample belong to the same species. The alpha diversity analysis conducted revealed that there was no significant variation in the total composition of microbial communities between the groups. As shown in Fig. 6 (A, B) below, the Shannon index (P=0.2498) and the Simpson's index (P=0.2814) revealed no significant difference in the alpha diversity indices of the test groups compared with the VEH-S group.

Bray-Curtis dissimilarity

Bray-Curtis dissimilarity was carried out to measure the dissimilarity or variability in the microbial community structure among samples by taking into account the abundances of microbial species present in each sample and how they differ between the samples. Visual inspection the Principal Coordinates of Analysis (PCoA) plot for the Bray-Curtis dissimilarity among the test groups showed that administration of MOE clustered the plots of the gut microbiota of CUMS-exposed mice samples distinctly from the vehicle-stressed sample plots in a similar manner as the reference drug groups and the VEH-NS group (Fig. 7).

Linear discriminant of effect size (LEfSe) analysis

LEfSe analysis was conducted to determine the significant taxa for each test group (Linear discriminant analysis (LDA) > 2.0, P<0.05). The significant taxa at the genus level for the VEH-NS group were *Draconibacterium, Corynebacterium, Odoribacter* and *Bacillus*; MOE 10 mg/kg group were *Yersinia, Anaerostipes, Olleya, Marinitoga*; MOE 30 mg/kg group were *Mycoplasma, Porphyromonas, Yersinia, Enterobacter, Olleya, Klebsiella, Kibdelosporangium, Clostridioides, Coraliomagarita, Pelosinus, Tannerella, Teredinibacter, Verminephrobacter, Spiroplasma, Ethanoligenens, Paenibacillus, Candidatus_Arthromitus, Desulfovibrio, Parabacteroides, Lactobacillus*; MOE 100 mg/kg group were *Mycoplasma, Yersinia, Enterobacter, Burkholderia, Toxoplasma, Pelosinus. Draconibacterium, Salinivirga, Tenacibaculum, Odoribacter*; FLX group were *Anaerostipes, Klebsiella, Blautia, Photobacterium, Chryseobacterium*, and *Treponema, Bibersteinia, Tannerella, Prochlorococcus, Megasphaera, Marinilactibacillus, Spiroplasma, Candidatus_Cardinium, Clostridium;* and MNC group were *Desulfovibrio, Enterobacter, Helicobacter, Erwinia, Blautia, Candidatus_Cardinium, Megasphaera, Salinivirga, Vitreoscilla, Teredinibacter, Enterococcus, Odoribacter, Bacillus, Lactobacillus* (Fig. 8).

Correlation between significant taxa, behavioral tests of depression and 5-HT levels

Spearman correlation analysis was used to investigate the correlations between the effects MOE 10, 30 and 100 mg/kg had on the significant genera identified in the LEfSe analysis, depression-like behaviors, and 5-HT levels in the prefrontal cortex (PFC) and hippocampus (HPC).

For MOE 10 mg/kg (Fig. 9A), we compared the relationship between the abundances of significant taxa at the genus level, *Yersinia,*

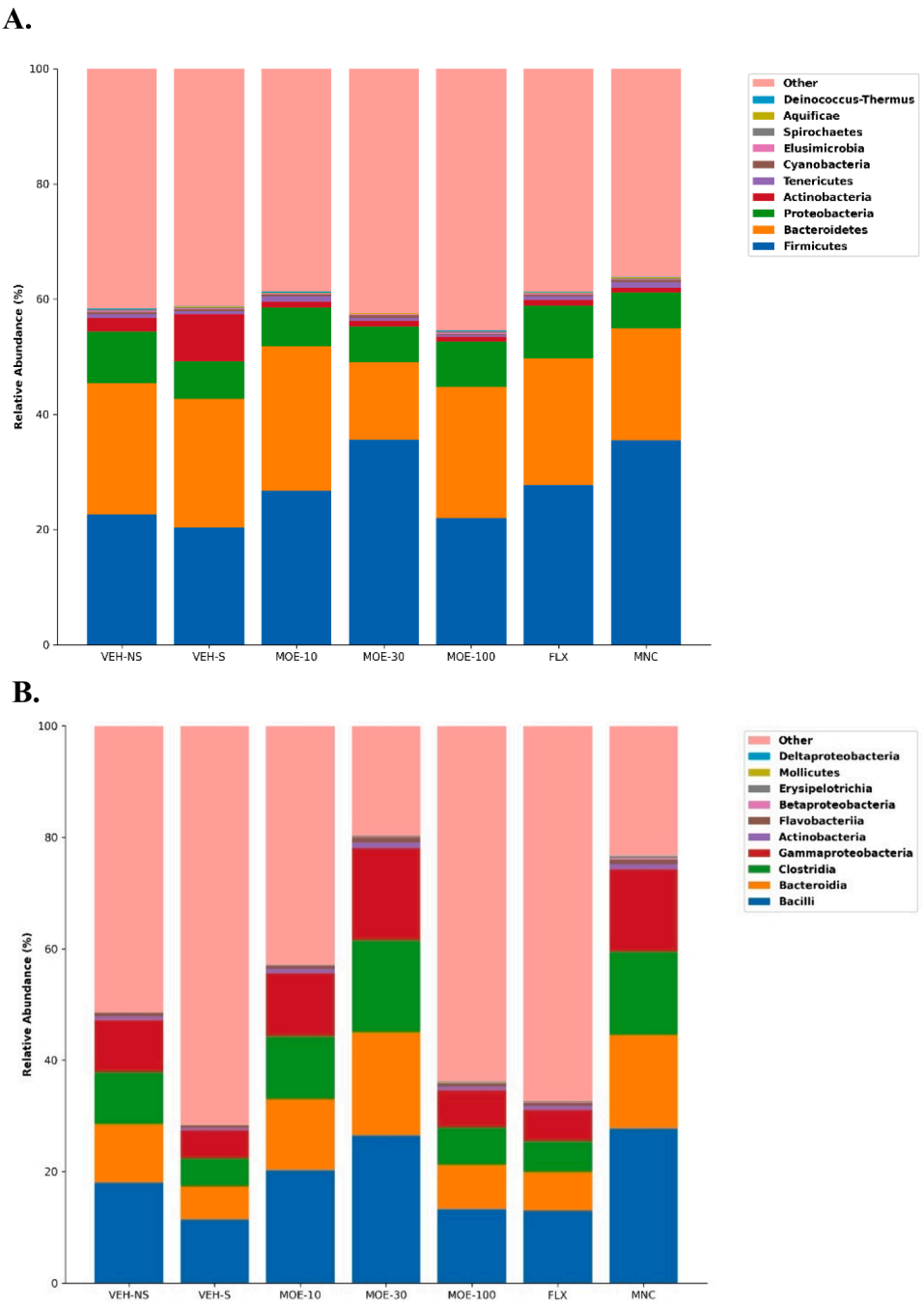
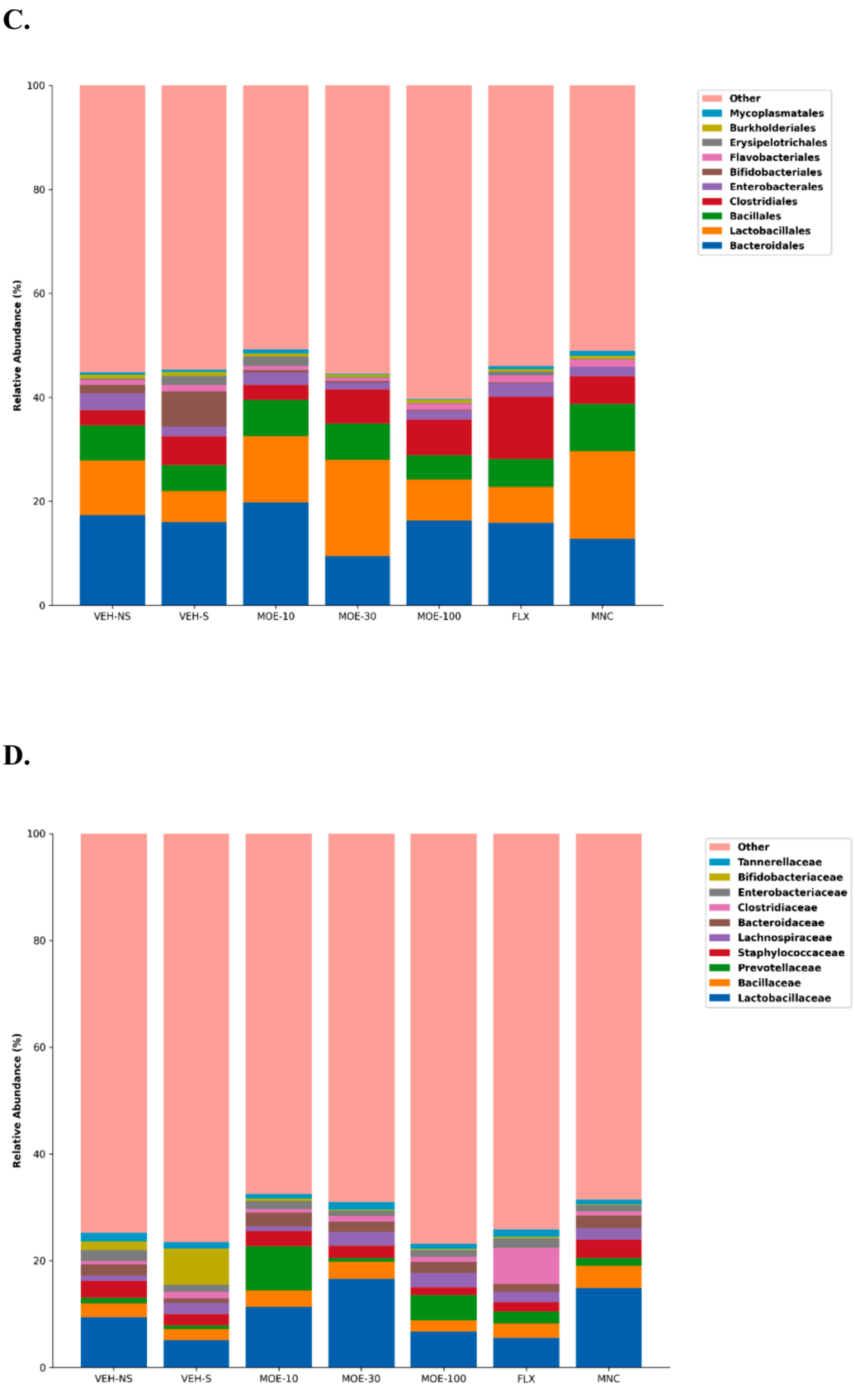


Fig. 5. Graph showing the relative abundances for top 10 phyla, classes, orders, families, genera found across samples for *Mallotus oppositifolius* extract (MOE 10, 30 and 100 mg/kg), fluoxetine (FLX 12 mg/kg) and minocycline (MNC 40 mg/kg) treatments to depict the taxonomic classification.

Anaerostipes, Olleya, and *Marinitoga* with the antidepressant-like effects in the behavioral tests of depression and concentration of the 5-HT. We found that *Anaerostipes* and *Olleya* were positively correlated with the reduction in immobility time while *Yersinia* and *Marinitoga* were negatively correlated. Also, *Yersinia, Anaerostipes, Olleya,* and *Marinitoga* were negatively correlated with swimming time in the FST. In contrast, *Yersinia, Anaerostipes, Olleya,* and *Marinitoga* were positively correlated with climbing time in the FST. *Anaerostipes* and *Marinitoga* were positively correlated with SPT results while *Yersinia* and *Olleya* were negatively correlated. *Anaerostipes* and *Marinitoga* showed positive correlation with prefrontal cortex 5-HT levels; *Yersinia* exhibited negative correlation whereas *Olleya* showed no correlation. *Yersinia* and *Marinitoga* were positively correlated with hippocampal 5-HT levels with *Olleya* and *Anaerostipes* showing no correlation.

For MOE 30 mg/kg (Fig. 9B), we assessed the relationship between the abundances of significant taxa at the genus level, *Lactobacillus, Parabacteroides, Desulfovibrio, Candidatus_Arthromitus, Ethanoligenens* and *Verminiphrobacter* with the antidepressant-like effects in the behavioral tests of depression and concentration of the 5-HT. The results show that *Lactobacillus, Candidatus_Arthromitus, Ethanoligenens* and *Verminiphrobacter* were positively correlated with immobility time in the FST with *Parabacteroides* and *Desulfovibrio* demonstrating a negative correlation. *Parabacteroides and Desulfovibrio* were positively correlated with swimming time in the FST with *Lactobacillus,*



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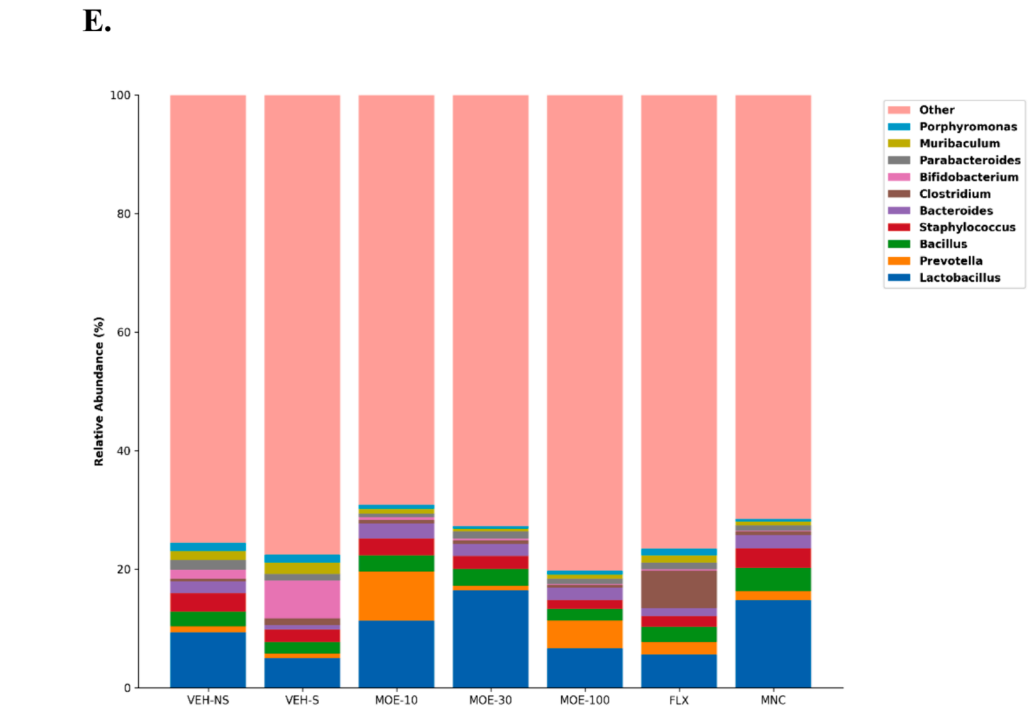
Candidatus_Arthromitus, Ethanoligenens and *Verminiphrobacter* being negatively correlated. *Lactobacillus, Desulfovibrio, Candidatus_Arthromitus, Ethanoligenens* and *Verminiphrobacter* were negatively correlated with climbing time in the FST with *Parabacteroides* being positively correlated. *Lactobacillus, Candidatus_Arthromitus, Ethanoligenens* and *Verminiphrobacter* were positively correlated with SPT results



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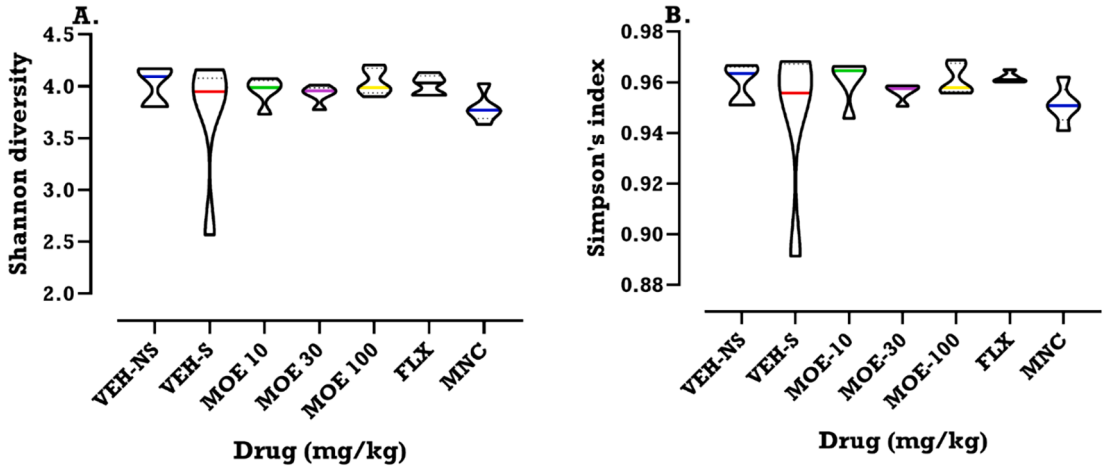


Fig. 6. Violin plots illustrating alpha diversity indices (A) Shannon diversity and (B) Simpson index in the gut microbiota across samples for *Mallotus oppositifolius* extract (MOE 10, 30 and 100 mg/kg), fluoxetine (FLX 12 mg/kg) and minocycline (MNC 40 mg/kg) treatments.

while *Parabacteroides* and *Desulfovibrio* were negatively correlated. *Lactobacillus, Desulfovibrio, Candidatus_Arthromitus, Ethanoligenens* and *Verminiphrobacter* were negatively correlated with prefrontal cortex 5-HT levels while *Parabacteroides* and *Desulfovibrio* were positively correlated. *Lactobacillus, Desulfovibrio, Candidatus_Arthromitus, Ethanoligenens* and *Verminiphrobacter* were positively correlated with hippocampal 5-HT levels with *Parabacteroides* being negatively correlated.

In the case of MOE 100 mg/kg (Fig. 9C), we correlated the abundances of significant taxa at the genus level *Draconibacterium, Salinivirga, Tenacibaculum,* and *Odoribacter* with the antidepressant-like effects in the behavioral tests of depression and concentration of the 5-HT. *Salinivirga* and *Tenacibaculum* were positively correlated with immobility time in the FST with *Draconibacterium* and *Odoribacter* demonstrating a negative correlation. *Tenacibaculum* was positively correlated with swimming time in the FST with *Draconibacterium, Salinivirga* and *Odoribacter* being negatively correlated. *Tenacibaculum* and *Odoribacter* were positively correlated with climbing time in the FST with *Salinivirga* and *Draconibacterium* demonstrating a negative correlation. *Salinivirga* and *Draconibacterium* were positively correlated with SPT results in the FST with *Tenacibaculum* and *Odoribacter* demonstrating a negative correlation. *Draconibacterium* and *Odoribacter* were negatively correlated with prefrontal cortex 5-HT levels while *Salinivirga* and *Tenacibaculum* were positively correlated. *Draconibacterium* and *Tenacibaculum* were positively correlated with hippocampal 5-HT levels with *Salinivirga* and *Odoribacter* being negatively correlated.

Discussion

To assess the role of the gut microbiota in the antidepressant-like effect of hydro-ethanolic leaf extract of *M. oppositifolius* (MOE), we used behavioral tests, regional brain serotonin concentrations and the 16S rRNA Oxford Nanopore sequencing of the fecal DNA samples from

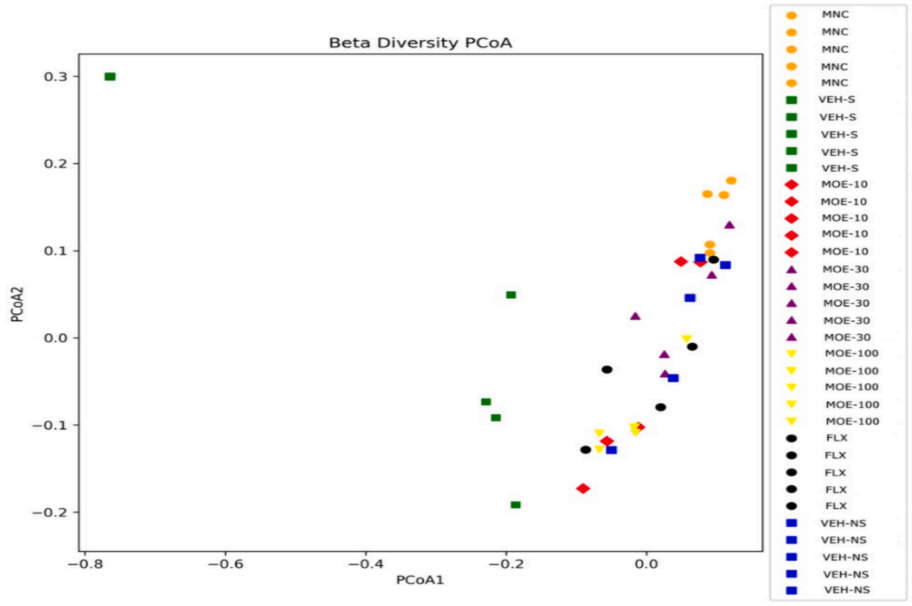


Fig. 7. Principal Coordinate of Analysis (PCoA) plot using distance matrices for Bray-Curtis dissimilarity of the gut microbiota across samples for *Mallotus oppositifolius* extract (MOE 10, 30 and 100 mg/kg), fluoxetine (FLX 12 mg/kg) and minocycline (MNC 40 mg/kg) treatments.

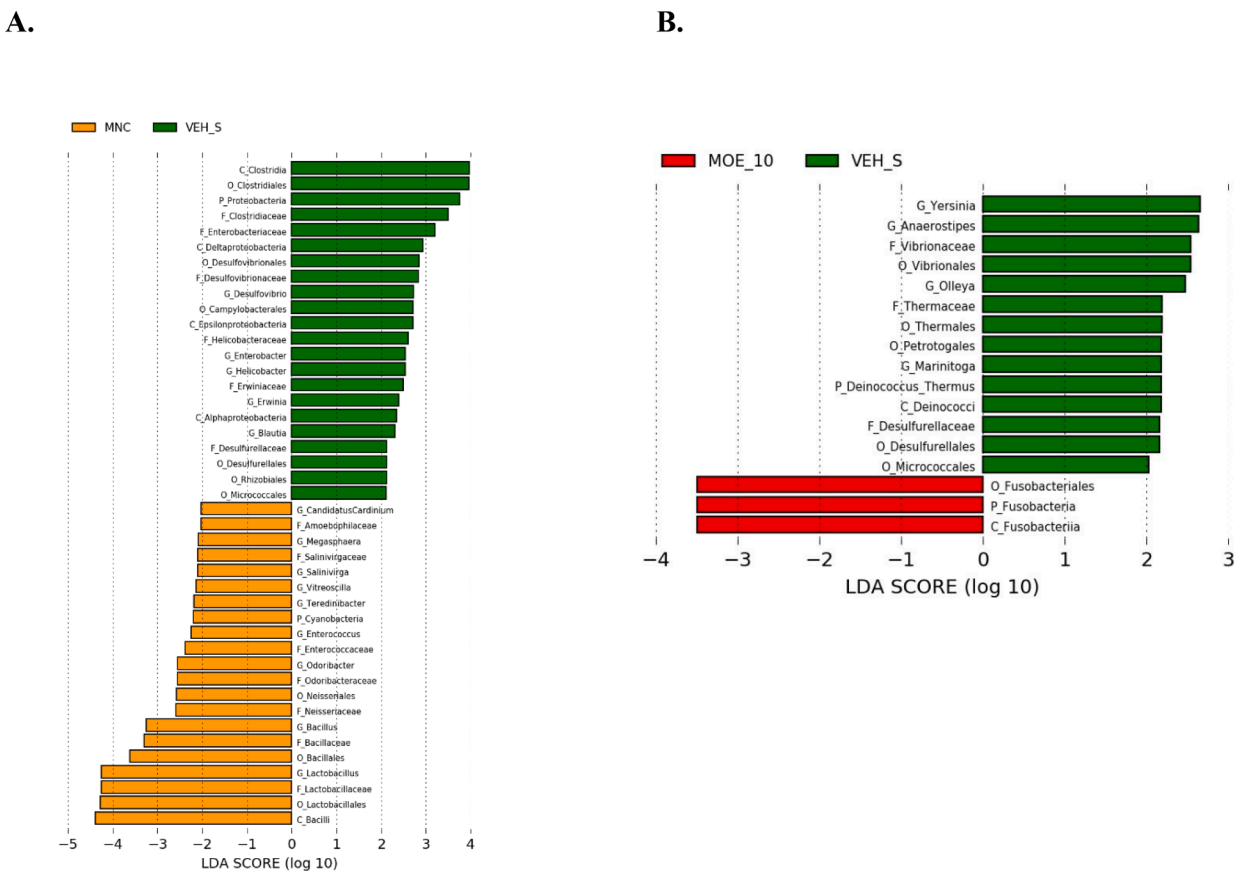
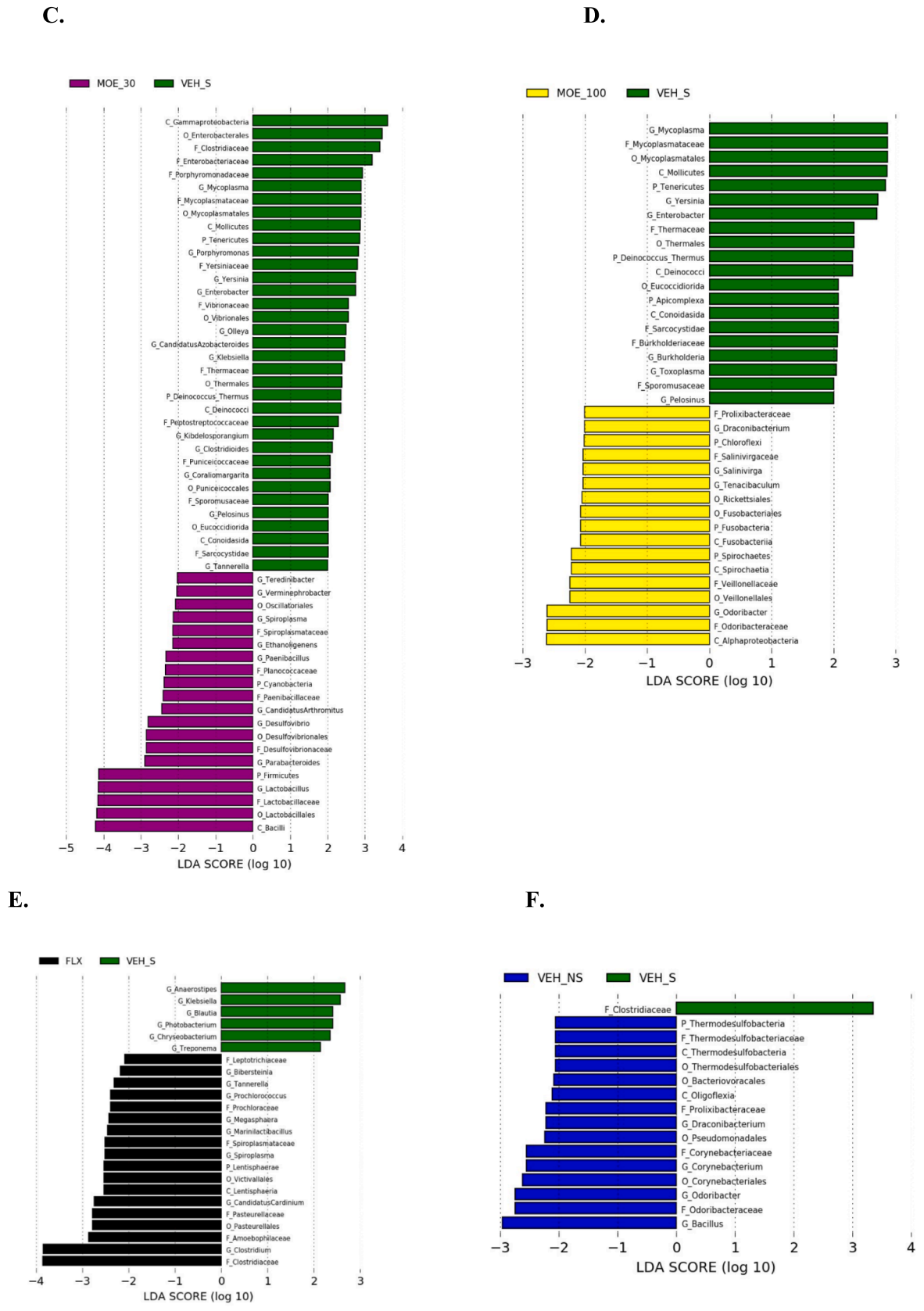


Fig. 8. Results of LEfSe (LDA score > 2) showing significantly abundant taxa between; (A) VEH-S and MNC; (B) VEH-S and MOE 10; (C) VEH-S and MOE 30; (D) VEH-S and MOE 100; (E) VEH-S and FLX; (F) VEH-S and VEH-NS.

mice. In this present study, we show that the antidepressant-like effect of MOE is influenced by changes in the gut microbiota and increased concentrations of serotonin in the hippocampus and prefrontal cortex. Importantly, MOE increased the abundances of *Lactobacilli* and other organisms, which is implicated in the improvement of mood disorders such as depression.



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To confirm antidepressant-like effect of MOE, we combined the chronic unpredictable mild stress (CUMS) test with the sucrose preference test (SPT). We employed the SPT during the CUMS procedure to evaluate anhedonia, a classical symptom in depression (He et al., 2020). The induction of mild stress in this study had a negative impact on the reward-related behavior across the various stressed groups. This

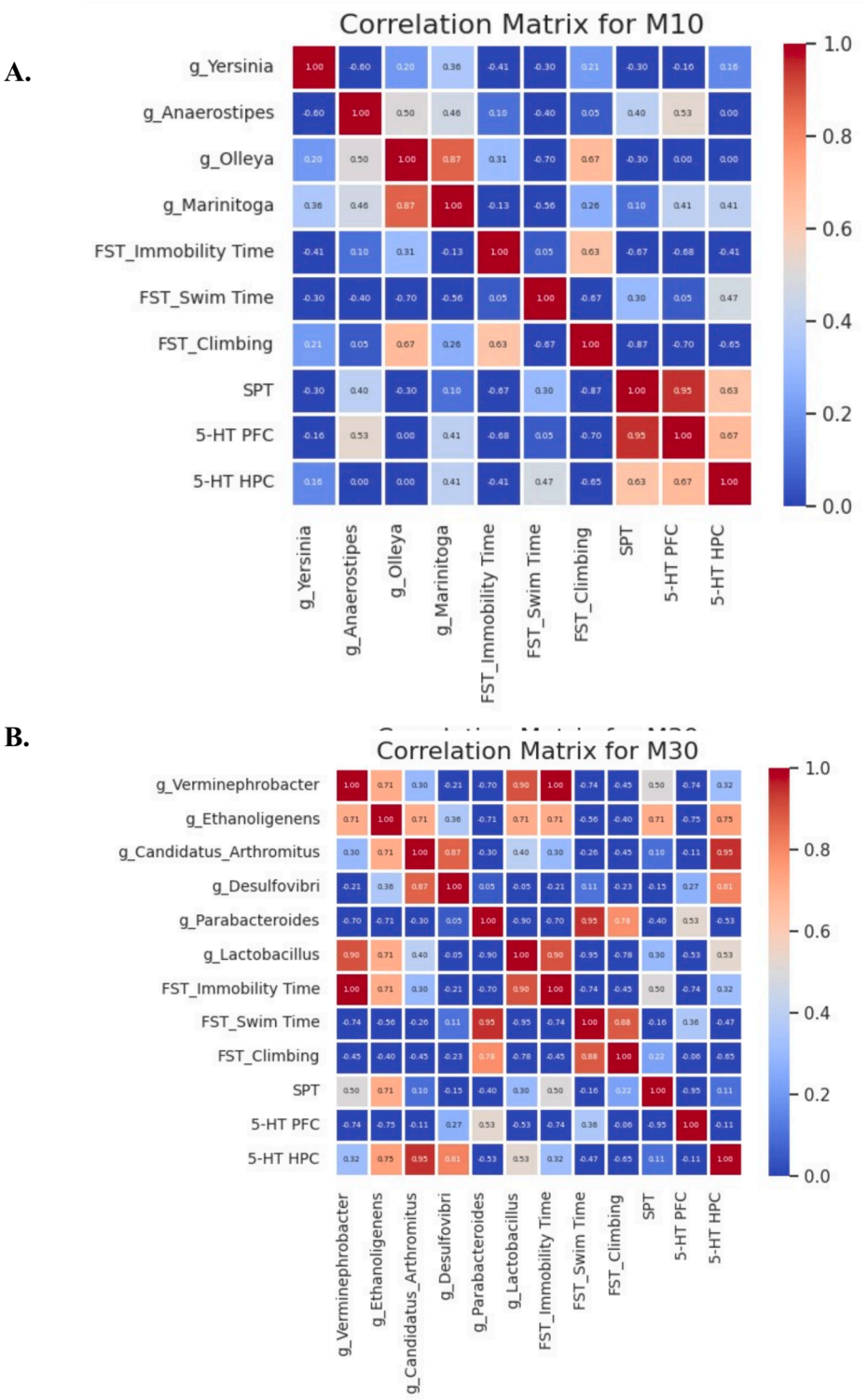
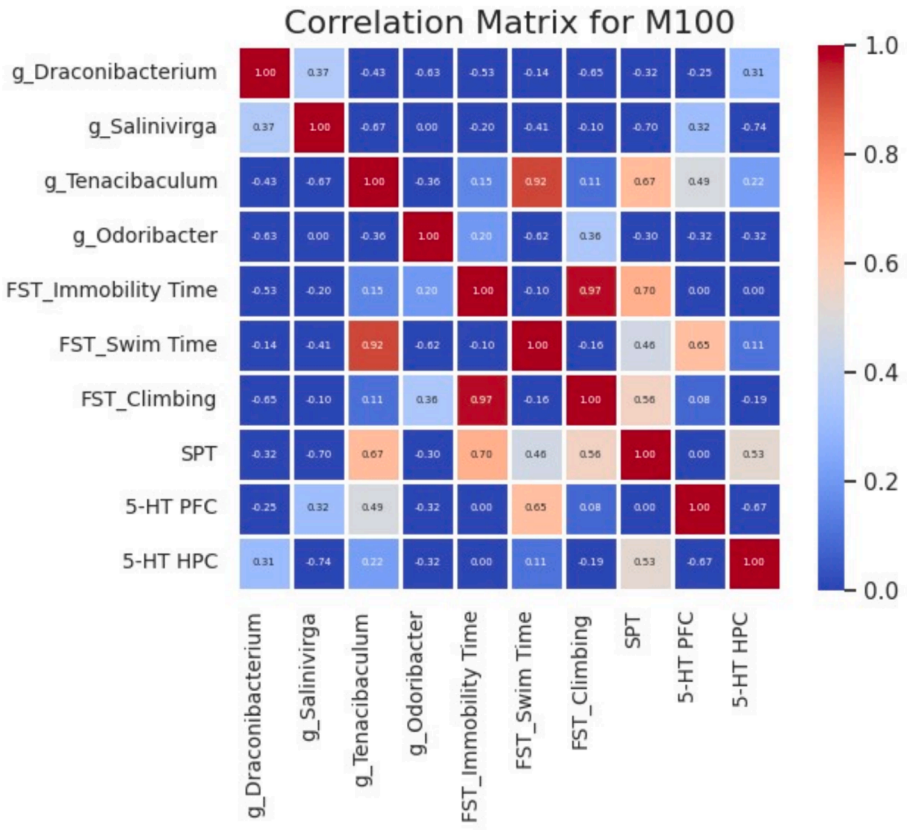


Fig. 9. Analysis of Spearman's correlation of significant depression-related gut microbiota genera, forced swim test results, sucrose preference results and 5-HT concentrations for (A) MOE10 mg/kg; (B) MOE 30 mg/kg; (C) MOE 100 mg/kg.

depression-related behavior was evidenced by the reduction in sucrose consumption, a result which is consistent with other previous findings (Chai et al., 2019; Chevalier et al., 2020; Sharma et al., 2022; Xie et al., 2022). Administration of MOE and the reference drugs, fluoxetine and minocycline, produced a significant reversal of the reduced sucrose preference (anhedonia) in the stressed mice. This reversal of the anhedonia by the extract to near-baseline levels suggests that it may have the potential of reversing depressive symptoms to pre-depression state. The consistent decrease in sucrose preference in the stressed untreated mice (VEH-S) in comparison to the no-CUMS group (VEH-NS) lends support to theories that suggest stress is a contributory factor to depression and the absence of stress does the opposite. In support of a previous study that showed that MOE produces sustained antidepressant-like effect after the open space swim test, the current work showed that MOE was able to

C.



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produce sustained antidepressant-like effect in the FST 24 h post treatment (Kukuia et al., 2016a). Additionally, the administration of *M. oppositifolius* augmented active behavior such as swimming and climbing in the FST when compared to the CUMS-induced control (VEH-S). The increase in swimming and climbing behavior are consistent with increased monoaminergic neurotransmission (Bogdanova et al., 2013). These behavioral results corroborate findings which showed that the antidepressant-like effect of *M. oppositifolius* may be mediated *via* enhancement of monoaminergic neurotransmission (Kukuia et al., 2022; Kukuia et al., 2014). Since the previous studies did not use the CUMS procedure, the effect of MOE on the levels of serotonin (5-HT) in the prefrontal cortex (PFC) and hippocampus (HPC) during the CUMS procedure was evaluated using the enzyme-linked immunosorbent assay (ELISA) technique. The study showed that MOE increased regional levels of 5-HT in the PFC and hippocampus. This present finding seems to suggest that MOE may contain compounds that increase the synthesis of serotonin and/or inhibit the reuptake of serotonin. Future studies would focus on elucidating the exact mechanism.

One of the overarching objectives of this study was to assess whether the antidepressant effect of the extract was dependent on its influence on the gut microbiota. To evaluate the effect of MOE on the gut microbiota of CUMS-exposed mice, the composition and structure of gut microbiota were analyzed using Oxford Nanopore 16S rRNA gene sequencing. Subsequent analysis of sequencing data involved the determination of the dominant taxa at the various levels of taxonomic classification, determination of alpha and beta diversity indices as well as the significant taxa associated with each group in this research. Assessment of the Simpson and Shannon indices of alpha diversity across the seven groups in this study showed no significant difference between the groups. There have been several studies investigating the effects of chronic unpredictable mild stress (CUMS) on the alpha diversity indices of the gut microbiota in mice, but the results have been equivocal. Some studies have reported a decrease in alpha diversity indices such as the Shannon index and the Simpson index in mice subjected to CUMS (Sun et al., 2019). However, other studies found that while CUMS had a significant effect on the overall structure of the gut microbiota in mice, it did not significantly affect alpha diversity indices (total number of species present) such as the Shannon index (Chevalier et al., 2020; Naseribafrouei et al., 2014; Zhu et al., 2019). Thus, the results from this study are consistent with these findings. It is worth noting that the specific details of the CUMS procedure, such as the duration, intensity, and frequency of the stressors, can vary between studies and may contribute to the variability in results. Additionally, other factors such as diet, genetics, and housing conditions may also influence the gut microbiota and its response to stress (Lozupone et al., 2012; Samuthpongtorn et al., 2021). It may be necessary to evaluate the impact of these confounding factors on the results in future studies. The Principal Coordinates of Analysis (PCoA) plot for the Bray-Curtis dissimilarity among the test groups showed the plots for the VEH-S group clustered separately from those of *M. oppositifolius*, VEH-NS, fluoxetine and minocycline. This indicates that the beta diversity of the vehicle stressed control (the CUMS-only control) was clearly distinct from the treated groups and the vehicle unstressed control. It can be deduced that *M. oppositifolius* elicited an effect on the gut microbiota of CUMS-induced mice like that of the reference drugs, fluoxetine and minocycline.

A more thorough examination of the gut microbiota community makeup at the various levels of taxonomy was performed using the Linear Discriminant of Effect Size (LEfSe) analysis. The success of the CUMS procedure in shifting the gut microbiota of non-depressed mice

after exposure was evidenced by the modification of significant taxa between the vehicle stressed and vehicle non-stressed groups. The CUMS procedure caused a shift in the gut microbiota of mice, resulting in a decrease in the relative abundances of beneficial bacteria such as the genus *Bacillus* and *Corynebacterium*. This finding was congruent with numerous studies which have established the effect of the CUMS procedure on the relative abundances of the gut microbiota. The significant taxa however differ among studies (Okuyama et al., 2022; Wu et al., 2021; Xie et al., 2022; Zhang et al., 2021; Zhu et al., 2019). It is worth noting that results of the LEfSe analysis provide adequate evidence that the administration of MOE reversed the effect of stress on the gut microbiome of mice, with peak effect observed at 30 mg/kg of MOE where the abundances of over fifty-five taxa were affected. Even though the actual mechanism by which MOE elicits this effect on the gut microbiome was not studied, the functions of some of the significant taxa affected could provide a hint. For instance, MOE caused an increase in the abundance of the *genus Lactobacillus, Desulfovibrio and Parabacteroides* which are recognized for its beneficial impact on the central nervous system and ability to regulate depression (Bravo et al., 2011; Li et al., 2019). Interestingly, stress application has been shown to reduce *Lactobacillus* levels within the gut while oral consumption of this probiotic can improve behavior induced by stress and alleviate mild depression (Hashikawa-Hobara et al., 2022; Ouwehand et al., 2002). The effect of *Lactobacillus* has been attributed to its anti-inflammatory activity (Archer et al., 2015). Earlier research has also discovered that *Lactobacillus* in the gut microbiome controls the expression of GABA receptors through the vagus nerve, which are implicated in antidepressant activity of some drugs (Bravo et al., 2011). It is plausible that the anti-inflammatory effect of MOE and its impact GABAergic neurotransmission may be associated with the increase in the genus *Lactobacillus* (Kukuia et al., 2016a; Nwaehujor et al., 2014).

Finally, Spearman's correlation analysis was conducted to assess the extent of correlation between the relative abundances of depression-related genera from the gut microbiota, the behavioral test results and the prefrontal cortex and hippocampal levels of 5-HT determined for the various extract treatment groups. An assessment of the heatmaps of the correlation matrices indicated that MOE at 30 mg/kg showed the strongest correlation between the relative abundances of depression-related bacteria taxa from the gut microbiota analysis, behavioral test results and the levels of 5-HT as compared to the 10 mg/kg and 100 mg/kg doses. At 30 mg/kg, MOE showed a positive correlation between the relative abundances of depression-related bacteria taxa and levels of 5-HT in the HPC and a negative correlation between depression-related bacteria taxa and levels of 5-HT in the PFC. This may suggest that the mechanism through which the effect of the extract on the gut microbiota elevates 5-HT to ameliorate depression may be more pronounced in the HPC than the PFC. The neurochemical mechanism through which the effect of *M. oppositifolius* contributes to its antidepressant activity may also be mediated through the modulation of other neurochemical substances aside 5-HT. Though this statistical correlation has been established, it is necessary to include that correlation does not equal causation. Hence, more research is needed to completely understand how the extract's influence on the gut microbiota affects specific MDD-related neurochemical indicators.

As important as the findings of this research are, it should be emphasized that this study has some limitations. Although Oxford Nanopore sequencing can identify levels of taxa within the gut microbiota, it is incapable of evaluating the distinct functions of these individual taxa. Moreover, there have been limited research endeavors directed towards exploring the correlation between gut microbiota and *M. oppositifolius*. The present work only represents an initial confirmation regarding the impact *M. oppositifolius* has on depression-like behavior and gut microbiota, which necessitates further explanation concerning how this plant's antidepressant mechanism operates along with the microbiota-gut-brain axis in detail. Furthermore, this study does not specifically address the role of the vagus nerve in the mediation of the anti-depressant like effect of the extract via the gut-brain axis. Previous research has demonstrated that gut microbiota changes require vagus nerve integrity to promote depressive-like behaviors in mice, indicating that the vagus nerve plays a crucial role in gut-brain communication (Siopi et al., 2023). To demonstrate the contribution of the vagus nerve to the antidepressant-associated changes in the gut microbiota, vagotomy would be required. This represents a limitation in our study and recommend that future studies consider investigating the role of vagotomy in the behavioral effect seen and how it influences the gut-brai axis. Also, though this study measured the levels of 5-HT and sought to establish a statistical correlation between the levels of 5-HT and effect of the extract on the gut microbiota, there may be a need to explore this effect in relation to other neurochemical markers such as BDNF and endocannabinoids. Finally, it may be worth studying the association between the antidepressant properties of the hydro-ethanolic leaf extract of *Mallotus oppositifolius* and its previously reported effect on the glycine/NMDA receptor and opioidergic pathways (Kukuia et al., 2014).

Our study shows for the first time that the hydro-ethanolic extract of *Mallotus oppositifolius* ameliorates CUMS-induced depressive symptoms by modifying the gut microbiota and increasing brain 5-HT levels, with peak effect at 30 mg/kg. The antidepressant-like effect of *Mallotus oppositifolius* was mediated through modulating the levels of important microbiota constituents at the genus level such as *Lactobacillus*, *Desulfovibrio and Parabacteroides*. The findings from our research provide insight into how *Mallotus oppositifolius* treats depression and could be useful for further exploration into the pathogenesis of this condition.

Ethical approval

Animal work was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996. Approval was granted by the College of Health Sciences Ethical and Protocol Review Committee (EPRC) and assigned identification number: CHS-Et/M.11-P 4.4 / 2021–2022.

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CRediT authorship contribution statement

Blay Kwofie: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Philip Debrah:** Supervision, Resources, Methodology, Funding acquisition. **Patrick Amoateng:** Writing – review & editing. **Donatus Wewura Adongo:** Writing – review & editing. **Selorme Adukpo:** Writing – review & editing, Resources, Methodology. **Kennedy Kwami Edem Kukuia:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors Contribution

Blay Kwofie: Conceptualization, Methodology, Writing - Original Draft Preparation, Investigation, Formal analysis, Data Curation **Philip Debrah**: Resources, Methodology, Fund Acquisition, Supervision **Patrick Amoateng**: Writing - Review & Editing Preparation, **Donatus Wewura Adongo**: Writing - Review & Editing Preparation **Selorme Adukpo**: Methodology, Writing - Review & Editing Preparation, Resources **Kennedy Kwami Edem Kukuia**: Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Resources.

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