**Protocol for investigating neuroprotective effect of the methanol leaf extract of *Mallotus oppositifolius* in lipopolysaccharide neuro-inflammation-associated depression in mice.**

**Kennedy Kwami Edem Kukuia1, Ferka Yaw Takyi1, Patrick Amoateng2, Frimpong Appiah3, Kevin Kofi Adutwum-Ofosu4**

¹Department of Medical Pharmacology, University of Ghana Medical School, College of Health Sciences, University of Ghana, Accra, Ghana. [KKEK: [kkekukuia@ug.edu.gh](mailto:kkekukuia@ug.edu.gh), FYT: [takyiyawferka@gmail.com](mailto:tyferka@st.ug.edu.gh)]

2Department of Pharmacology and Toxicology, School of Pharmacy, College of Health Sciences, University of Ghana, Accra, Ghana. [PA: [pamoateng@ug.edu.gh](mailto:pamoateng@ug.edu.gh)]

3Department of Community Health and Medicine, School of Food and Health Sciences, Anglican University College of Technology, Nkoranza, Ghana [FA: fappiah@angutech.edu.gh]

4Department of Anatomy, University of Ghana Medical School, College of Health Sciences, University of Ghana, Accra, Ghana. [[KKA: kadutwum-ofosu@ug.edu.gh](mailto:KKA:%20kadutwum-ofosu@ug.edu.gh)]

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# **1.0 Background**

Depression is associated with significant morbidity and mortality. Despite the availability of effective antidepressants, more than a third of patients with depression do not benefit for current medications which mainly act by altering monoaminergic systems in the brain (Wiles *et. al*., 2014). Furthermore, the current medications are associated with intolerable side effects that further aggravate the patients’ condition. Also, the incomplete understanding of the neurobiology of depression militates against the discovery of more effective medications. However, the discovery that neuro-inflammation contributes to depression pathogenesis and that markers of neuro-inflammation could be putative targets for treatment-resistant depression is an indication that there is light at the end of the tunnel for those suffering from depression (Feltes *et al*., 2017).

Globally, there is a high dependence on medicinal plants for the treatment and prevention of chronic diseases such as depression. The predilection towards medicinal plants may be due to their ready availability, affordability, perceived safety and efficacy (Kukuia *et al*., 2012). *Mallotus oppositifolius* is one of such plants that have received considerable attention in the management of diseases (Kukuia *et. al*., 2014a). Traditionally, the leaves of *Mallotus oppositifolius* is used to treat convulsions, epilepsy, pain, infections, etc. Our laboratory and others have validated some of the traditional uses of this plant. For instance, our works have confirmed the anticonvulsant, acute antidepressant-like, rapid onset antidepressant and anti-aggressive effects of the leaves of *Mallotus oppositifolius* (Kukuia *et. al*., 2014b, 2016a, 2016b, 2022a). In these studies, it was shown that the observed effects were likely mediated through mechanisms involving serotoninergic and GABAergic enhancement, inhibition of glycine/NMDA glutamatergic effects as well as increased dendritic spine density in the prefrontal cortex. Aside these, anti-inflammatory effects of the leaves of *Mallotus oppositifolius* have been reported (Nwaehujor *et. al*., 2014).

Collectively, these effects seem to suggest that the plant may possess neuroprotective effect. The present work therefore provides a protocol for investigating possible neuroprotective effects of *Mallotus oppositifolius* in neuro-inflammation models of depression in mice.

**Aim**

To evaluate the potential neuroprotective effect of the methanol leaf extract of *Mallotus oppositifolius* in lipopolysaccharide neuro-inflammation-associated depression model in mice.

**Objectives**

1. To obtain a methanol extract from theleaves of *Mallotus oppositifolius* (MOE).
2. To evaluate antidepressant-like effects of a 7-day daily treatment of MOE in acute antidepressant models such as forced swim (FST) and tail suspension (TST) tests.
3. To measure the antidepressant-like effect of an 11-day MOE pretreatment against LPS neuro-inflammation associated depression in the FST and TST.
4. To investigate the antidepressant effect of MOE in a chronic depression model such as the open space swim test.
5. To evaluate the effect of MOE on locomotor activity.
6. To determine the effect of MOE on mouse activated and resting microglia.

# **2.0 Method**

## 2.1 Plant collection and extraction

### *2.1.1 Plant nomenclature and collection*

The plant, *Mallotus oppositifolius* (Geiseler) Müll. Arg (Family: Euphorbiaceae), is known locally as ‘Sroti’ in Ewe, ‘Anyanforowa’ in Asante and ‘Sratadua’ in Fanti in Ghana.

1. The leaves were collected from the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, Ghana (5°55′05.6″N,0°08′04.9″W),
2. The leaves were authenticated at the same Centre (Voucher Specimen Number: CPMR 4977).
3. The plant name (Figure 1) was checked at [http://www.theplantlist.org](http://www.theplantlist.org/).

 

Figure 1: *Mallotus oppositifolius* plant (retrieved from [www.westafricanplants.senckenberg.de](http://www.westafricanplants.senckenberg.de)

and [www.iNaturalist.org](http://www.iNaturalist.org)).

### *2.1.2 Plant extraction*

1. The leaves were washed with clean water, air-dried for 7 days before being ground with a hammer mill.
2. The constituents of the powder were extracted for 72 hours using the cold maceration method with absolute methanol. Considering the plant's traditional use, the benefits of the leaves appear to reside in the polar extracts. As a result, methanol was used as a solvent to extract the polar constituents in the leaves. This will guarantee that the extracted substance is quite similar to the conventional aqueous form.
3. In the cold maceration method, the powdered leaves (1 kg) were poured into a glass jar with a cock stopper at the tip of the base. After adding about 2 L of methanol to the plant material, the glass jar was covered with a lid and then allowed to stand for 72 hours with little shaking occasionally.
4. The crude extract was subsequently drained into a clean container by removing the cock stopper. The solid material in the glass jar was pressed to the mark to ensure that any liquid residue in the powdered material was recovered.
5. The crude extract was concentrated into a syrupy mass on a rotary evaporator at 60 °C and low pressure. The syrupy mass was dried in a water bath to a dark brown semisolid mass and stored in a desiccator for later use.
6. The final yield (2.81%) was referred to as *M. oppositifolius* extract (MOE).

**Figure 2: Schematic diagram showing the protocol for plant collection, milling and extract**

## 2.2 Animal husbandry

Healthy experimentally naïve Institute of Cancer Research (ICR)mice (*Mus musculus* of the family Muridae) of both sexes weighing between 20-30 g and aged 6 weeks old (young adult) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana and transported to the Department of Medical Microbiology, University of Ghana Medical School, University of Ghana. The animals were housed in groups of eight in stainless steel cages (34 × 47 × 18 cm3) with soft wood shavings as bedding, and were fed with normal commercial pellet diet (GAFCO, Tema) and water *ad libitum*. The mice were maintained at room temperature (26 ± 2), relative humidity between 45 - 65% with a 12:12 h light/dark cycle. They were acclimatized for two weeks prior to start of experiment. All efforts were made to ensure minimal stress to the animals. The forced swimming and tail suspension test did not involve any invasive technique or electrical shocks that could result in pain. After the forced swimming test, animals were allowed to dry off before being returned to their cages to prevent hypothermia. Similar precautions were taken during the open space swim test (OSST). In the OSST, the water temperature was monitored constantly with a digital thermometer and where the temperature falls below 30 °C, the experiment was paused and the water was either changed or reheated till a temperature of approximately 32±2°C was achieved.

## 2.3 Place and Time of Experiment

The experiment was performed at Neuropsychopharmacology Research Laboratory, Department of Medical Pharmacology, University of Ghana Medical School, Korle-Bu. All procedures and techniques used in this studies were done in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NRC,1996) and ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments). Ethical clearance for this research was obtained from the College of Health Sciences Ethical and Protocol Review Committee, University of Ghana on November 4, 2019 (CHS-Et/M2-4.11/2019-2020).

## 2.4 Study design

Mice arriving from Noguchi Memorial Institute for Medical Research, Ghana were weighed and randomly divided into 6 groups (n = 8). Sample size used was chosen based on our previous studies where similar numbers gave reliable results (Kukuia *et. al*., 2014, 2016a). From these previous studies, it was realized that a minimum of about 7 or 8 can give consistent and reliable results. Randomization was done through minimization. Briefly, each group had equal number of males and females and it was ensured that animals of the same weight were not kept in the same group. This was done in order to attain some uniformity in weight distribution between the groups. Mice were allocated to various treatments and were subjected to behavioral tests as described in Table 1. Testing was carried out between 8 am to 3 pm daily. The recorder was blinded to the treatments given. No other special procedure was followed in determining the order of treatment or allocation of cage numbers. Mice from the behavioral studies were sacrificed 24 hours after the last experiment for histological investigation of their microglia. In total, 48 mice were used for the acute studies without the lipopolysaccharide (LPS) treatment, 48 mice for the studies involving the LPS and another 48 mice for the open space swimming test.

**Table 1: Experimental protocol for behavioral studies**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Animal model** | **Treatment group, n=8** | **Duration** |
| **Acute behavioral**  **experiments** | OFT (without LPS)  FST (without LPS)  TST (without LPS) | **Group I**: VEH (normal saline, *p.o*. 10 ml/kg)  **Groups II, III, IV**: MOE (10, 30 and 100 mg/kg *p.o.*)  **Group V**: fluoxetine (20 mg/kg *p.o.*)  **Group VI**: minocycline (50 mg/kg *p.o*.). | 7 days |
| LPS-induced neuroinflammation  Followed by FST & TST | **Group I**: vehicle (normal saline, *p.o*. 10 ml/kg)  **Group II**: LPS (1 mg/kg, i.p.)  **Group III, IV, V**: LPS + MOE (10, 30 and 100 mg/kg *p.o*.)  **Group VI**: LPS + minocycline (50 mg/kg *p.o*.) | 11 days of extract or minocycline pretreatment followed by LPS injection.  FST and TST conducted 6 hr & 24 hr post LPS injection |
| **Chronic behavioral**  **experiments** | OSST | **Group I**: vehicle (normal saline, *p.o*. 10 ml/kg)  **Groups II, III, IV**: MOE (10, 30 and 100 mg/kg *p.o.*)  **Group V**: fluoxetine (20 mg/kg *p.o.*)  **Group VI**: minocycline (50 mg/kg *p.o*.). | 18 days |

**KEY**: OFT= open field test; LPS= lipopolysaccharide; FST=forced swimming test; TST=tail suspension test; OSST= open space swimming test; *p.o.*= per os (oral), VEH= vehicle.

## 2.5 Forced swim test

Forced swim test (FST) was performed according to the procedure described by (Amoateng *et. al*., 2018) to evaluate effect of MOE on depressive behavior. A video recorder (Sony 4K Handy Cam, FDR-A×100E) supported by a tripod stand was placed above transparent cylindrical tanks (30 × 20 cm2) filled with tap water to a height of 20 cm at a temperature of 25 ± 1°C.

1. The mice were brought to the testing room and allowed to acclimatize for an hour prior to oral drug administration.
2. One hour after drug administration, they were gently placed in the cylindrical tank and their behavior was recorded for 5 minutes.
3. At the end of the experiment, the mice were removed from the water by their tails, dried using a towel and placed in recovery cages for 5 minutes before returning them to their home cages.
4. The experiment was repeated daily for 7 days.
5. The immobility behavior of the mice was analyzed by an observer blinded to the experimental conditions in accordance with the method described by (Kudryashov et. al., 2017). The mouse was judged to be immobile when it remained remained floating passively in the water and the only movement it makes was one necessary for breathing.

## 2.6 Tail suspension test

Tail suspension test (TST) was done as described by (Steru *et al*., 1985) with modifications described by (Adongo *et. al*., 2015). This test was used to confirm the acute behavioral effects of MOE on depressive behavior.

1. Briefly, mice were hung by their tails from a horizontal tube raised 40 cm above the floor and a video recorder was used to record mouse behavior for 5 minutes.
2. Each mouse was tested only once in a day for 7 consecutive days
3. The immobility behavior of the mice was analyzed by an observer blinded to the experimental conditions.
4. Animals that climbed their tails were removed gently and suspended again for the test to continue. However, if they continued climbing their tails, they were excluded from the experiment.

## 2.7 Open-space swim test

Open-space swim test (OSST), a chronic depression model, was performed as previously described (Stone and Lin, 2011). The stress involved in the OSST, produces depressive and inflammatory features in the brain that mimics human depression more than the FST and TST, hence this model was used to evaluate the effect of MOE on chronic depression.

1. Tubs with dimensions of 24 × 43 × 23 cm3 (w × h × l) were filled with water to a depth of 13 cm at room temperature.
2. Mice were gently placed in the water for 15 min/day whiles being recorded using a video recorder (Sony 4K Handy Cam, FDR-A×100E).
3. Mice swam for the first four days to induce a depressive state without any drug treatment.
4. Twenty-four hours after the fourth round of swimming, mice were treated with oral minocycline (50 mg/kg), MOE (10- 100 mg/kg) or saline (vehicle, 10 ml/kg) daily and tested an hour post-treatment.
5. The effect of treatment was evaluated on days 5, 7, 10, 14 and 18. At the end of each swimming session, the mice were wrapped and rubbed in towel to dry them and placed in their recovery cages for 5 minutes before being returned to home cages.
6. The behavior of the mice was analyzed by an observer blinded to the experimental conditions.

## 2.8 Open-field test

Open-field test (OFT) was performed to measure the locomotor activity of mice as well as anxiety behavior following the method used by (Kukuia *et. al*., 2022b).

1. In brief, open-field apparatus was built using plywood and painted white to heighten the natural aversion of mice for bright or lit areas.
2. It measured 60 cm × 60 cm × 25 cm (*l* × *b* × *h*). Visible lines were drawn on a white background. The floor was divided into 16 squares.
3. A central square of 15 cm × 15 cm was drawn in the middle of the open field. Mice were transported to the testing room to acclimatize for one hour before the test was performed.
4. A video recorder (Sony 4K Handy Cam, FDR-A×100E) was put above the setup.
5. The open field was cleaned with alcohol to wipe any residual smell before the start of the test and after testing each mouse.
6. The video recorder was turned on before a mouse was placed in the center of the open field. The activity of the mouse in the open field was recorded for a period of 5 minutes during which outside disturbances and movements were limited.
7. At the end of the 5 minutes, the mouse was removed and placed in its home cage.
8. The number of lines crossed, which refers to distance moved and activities in the central compartment, were analyzed by an observer blinded to the experimental conditions.

## 2.9 LPS-induced neuro-inflammation model

1. In this acute neuroinflammation experiment, mice received vehicle (normal saline, 10 ml/kg, *p.o*.), minocycline (50 mg/kg, *p.o*.) or MOE (10, 30, 100 mg/kg, *p.o*.) for 11 consecutive days before lipopolysaccharide (LPS) injection.
2. LPS was dissolved in sterile normal saline and injected (1 mg/kg, s.c) into all groups of mice (except the vehicle-treated group) after 1 hour MOE or minocycline administration on the 11th day. The vehicle-treated group served as negative control.
3. FST and TST were used to assess the effect of treatment at 6- and 24-hours following injection of saline (vehicle) or LPS.

## 2.10 Histological evaluation of the volume of neurons in the brain

### *2.10.1 Golgi-Cox solution, tissue collection and preservation*

Golgi-Cox stain was used as previously described (Das et. al., 2013) with modifications.

1. The Golgi-Cox solution was prepared by mixing 5% w/v solutions of potassium dichromate (K2Cr2O₇), mercuric chloride (HgCl2) and potassium chromate (K2CrO₄) in a ratio of 5:5:4 respectively.
2. Mice were sacrificed and brain tissues were then extracted immediately after the animals were perfused transcardially with 0.9 % saline. Briefly, mice were anesthetized with diethyl ether (volume not exceeding 5 mL via the respiratory route for approximately 2 minutes in a transparent jar) and brain carefully removed from the skull of mouse.
3. Though the intention was to use ketamine and xylazine, at the time of euthanasia, diethyl ether was the agent readily available.
4. It has been observed that ether could be irritating to the eyes of the animals, we ensured that the quantity used did not exceed the 5 mL threshold and duration did not exceed 2 minutes.
5. The brain tissues were divided into two transverse halves and post-fixed in a 40 mL bottle containing Golgi-Cox solution (a mixture of potassium dichromate, mercuric chloride and potassium chromate) for 24 hours.
6. After 24 hours, the Golgi-Cox solution was replaced with a freshly prepared one and the brain samples were stored in the dark for 14 days.
7. Next, the brain samples were removed from the Golgi-Cox solution, slightly blotted with tissue paper and transferred into a 30% w/v sucrose solution for storage in the refrigerator until sectioning was done at 50 μm using a microtome.

### *2.10.2 Tissue processing, sectioning and color development*

1. Upon removal from the sucrose solution, each brain sample was cut into three coronal sections, placed in histological cassettes (Rotilabor embedding cassettes; K114.1, Carl Roth GmbH, Germany) and passed through increasing order of ethanol series of 70% for 1 hour, 95 % for 1.5 hours and 100 % twice for 2 hours each.
2. The processed tissues were placed in molten paraffin wax for 3 hours after which they were embedded in molten paraffin wax and placed in the refrigerator at 4 °C until sectioning.
3. The refrigerated tissue blocks of all groups of brain tissue samples were sectioned at 50 with a microtome (Leica RM 2235) and placed on water at 60°C.
4. The floating sections were picked with a brush and mounted on the gelatin-coated slides. The sections were blotted with tissue paper and direct, downward moderate pressure was applied with the heel of the palm (Gibb and Kolb, 1998) so that the sections were firmly glued to the gelatin slides and dried in the dark for 3 days.
5. The tissue sections were dewaxed by passing them through xylene two times for 2 minutes each, and through 100% ethanol twice for 2 minutes each and finally through 50% ethanol for 5 minutes before being placed in a 3:1 ammonia solution for 8 minutes in the dark at room temperature.
6. The sections were washed two times with distilled water for 5 minutes each and then immersed in 1% sodium thiosulfate solution to fix the stain for 5 minutes at room temperature in the dark.
7. Next, the tissue slides were washed two times with distilled water for 1 minute each followed by incubation in 5% Mallory stain C as a counter stain for 1 minute and then dehydrated bypassing them through an increasing order of ethanol series of 70%, 95% and 100% (twice) for 5 minutes and fresh xylene for 2 minutes in the dark.
8. Finally, the tissue sections were mounted with DPX (mixture of distyrene, a plasticizer, and xylene) and allowed to dry in the fume hood for 3 days before examining under light microscope.

### *2.10.3 Neuronal and microglial count*

1. The tissue sections were observed under a light microscope (Leica Galen III-1154XV) at low (×100) magnification and images were captured with a camera (Lenovo Q350 USB PC) attached to the microscope.
2. The microscope stage was moved from around the tissue at 2 and 3 microscope stage unit intervals on the x- and y-axes respectively. Snapshots of the cell bodies within the field of view were captured (×100) onto a computer (HP Compaq dx2300 Microtower) with the eyepiece.
3. This was done until the whole area of tissue was covered. A total of 2160 micrographs were randomly selected across all treated groups using ImageJ Software (version 1.52a) to quantify neurons while a total of 1020 micrographs was used to count the number of resting and activated microglia at ×100 magnification as described by (Gibb and Kolb, 1998).

## 2.11 Statistical analysis

[GraphPad Prism](https://www.graphpad.com/scientific-software/prism/) for Windows version 8.0.2 (GraphPad Software, San Diego, CA, USA) was used for all data and statistical analyses. *P* < 0.05 between groups was considered statistically significant. SigmaPlot version 15.0 (Systat software Inc., USA) could have been used in place of the GraphPad Prism. The time-course curves plotted were subjected to two-way(treatment × time) analysis of variance (ANOVA) with Bonferroni’s post-hoctest. Area under curves were analyzed by one-way ANOVA followed by Tukey’s post-hoc test.

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