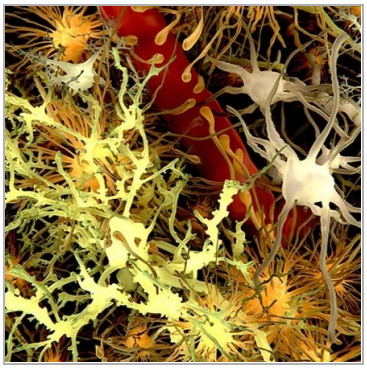

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Protocol for investigating neuroprotective effect of the methanol leaf extract of Mallotus oppositifolius in lipopolysaccharide neuro-inflammation-associated depression in mice.

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ABSTRACT

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. Furthermore, the current medications are associated with intolerable side effects that further aggravate the patients' condition. Neuro-inflammation contributes to depression pathogenesis and markers of neuro-inflammation could be putative targets for treatment-resistant depression is an indication. *Mallotus oppositifolius* is used to treat convulsions, epilepsy, pain, infections, etc. Our works have confirmed the anticonvulsant, acute antidepressant-like, rapid onset antidepressant and anti-aggressive effects of the leaves of *Mallotus oppositifolius.* Aside these, anti-inflammatory effects of the leaves of *Mallotus oppositifolius* have been reported. Collectively, these effects seem to suggest that the plant may possess neuroprotective effect. However, no study has been conducted to assess the neuroprotective effect of the leaves of *Mallotus oppositifolius*. The present work therefore provides a protocol for investigating possible neuroprotective effects of *Mallotus oppositifolius* in neuro-inflammation models of depression in mice.

PROTOCOL integer ID:
75472

Keywords: Depression, Neuro-inflammation, Microglia, Golgi-Cox, Mallotus oppositifolius

ATTACHMENTS

Protocol for Mallotus_LPS Neuroinflammation Study.docx

IMAGE ATTRIBUTION

Image credit: ©iStock.com

GUIDELINES

Collection of *Mallous oppositifolius* leaves

Milling and extraction

Behavioral tests without LPS

Behavioral tests with LPS

Golgi-Cox staining technique

MATERIALS

Chemicals

Fluoxetine hydrochloride
Sodium thiosulfate
Mallory stain C
Diethyl ether
Distyrene
Xylene
Potassium dichromate ($K_2Cr_2O_7$)
Mercuric chloride ($HgCl_2$)
Potassium chromate (K_2CrO_4)
Lipopolysaccharide (Escherichia coli 0111:B)
Methanol
Gelatin

Equipment

Rotary evaporator
Light Microscope
Digital thermometer
Bath tub for open space swim test
Microtome

BEFORE START INSTRUCTIONS

If you are allergic to animal fur, make sure to wear nose masks and take necessary precautions. It is important for those who usually have asthmatic attacks due to expose to animal fur to have their medications near them.

Read through each protocol and understand the steps to take before beginning.

Chemicals for preparing the Golgi-Cox solution can be carcinogenic and so should be handled with care. Wear nose mask, gloves and work in fume chamber anytime you are working with such chemicals.

Keep your lab note near you to record observations (both expected and unexpected ones).

Go over your calculations a number of times before beginning. If possible allow a second person to cross-check.

COLLECTION OF MALLOTUS OPPOSITIFOLIUS LEAVES

1 *Mallotus oppositifolius* plant is common shrub. However, for this work, we collected the plant material from Centre for Plant Medicine Research, Akuapem-Mampong in the Eastern Region of Ghana. The geographical coordinates have been provided in the attached document.
Some of previous works reported findings on plant materials collected from the Botanic gardens of Kwame Nkrumah University of Science and Technology, Kumasi-Ghana.

It is important that the plant is collected and identified by a botanist or a Pharmacognosist before proceeding with any research study.

We dried the leaves after they were washed with clean water for 7 days. Air-drying method was used.

MILLING AND EXTRACTION OF PLANT MATERIALS

2 Mill dried leaves with a harmer mill

Pour powdered leaves into a glass container with a cock stopper before adding absolute methanol.

Aside minimal shaking, allow the methanol to extract the constituents of the leaves for 7 hours

Drain the extract.

Concentrate in a rotary evaporator.

Dry on a water bath till you obtain a dark brown paste and then store in a desicator.

BEHAVIORAL TESTS WITHOUT LIPOPOLYSACCHARIDE (LPS)..

3 OPEN FIELD TEST

The open field test is a behavioral test for evaluating locomotor activity and anxiety-like behavior in rodents. In our study, we used it to assess the effect of treatment on locomotor activity.

A. Weigh mice and assign them randomly to groups (n=8).

B. Administer drugs orally (usually dissolved in normal saline or the appropriate solvent). Dose of drugs are based on the weight of the mouse. Wait for about 60 minutes before beginning.

C. Set up the camcorder above the brightly illuminated open field. Start the recorder and capture the group to be recorded before placing the mouse in the open field. Dimensions for the open field can be found in the attached document.

D. Place each mouse in the centre of the open field.

E. Allow the mouse to explore freely for 6 minutes without being seen.

F. The blinded observer evaluates the number of line crossings to determine locomotor activity

G. If interested in anxiety-like behavior, the time spent in the central compartment can be measured as well.

4 TAIL SUSPENSION TEST (TST)

The tail suspension test evaluates the ability of drugs to exert antidepressant-like effects in rodents. Mice are hanged by the tail for 5 to 6 minutes. The time spent in the immobile state gives an indication of depressive behavior.

A. Weigh the mice to determine doses to administer. ***In this study, same animals used for TST are used for FST. There is no need to reweigh them during FST.***

B. Randomly assign mice into groups based on the treatment (n=8). Each animal must be identified by indelible non-poisonous ink or by by color codes to ensure there is no mix up.

C. Administer drugs or extracts by oral route unless otherwise specified.

D. Wait for about 1 hour if drug/ extract was administered by the oral route. The time must be consistent throughout.

E. While waiting, set up the metal rods on which the mouse tail will be suspended.

F. Set up the camcorder in front of these rods so it can capture the behavior of each animal hanging on the rod.

G. Before hanging the animal on the rod, start the camcorder and record the group to be tested in the video before hanging the animal by the tail. The group should be coded so that it is not clear to

the observer who will do the tracking the type of treatment given.
H. Allow the animals to hang for 6 minutes without being seen or making noise. Repeat this procedure for all animals.
I. After waiting for about 5 minutes, the animals that went through the TST, can undergo the FST (described in the next step).
J. Repeat steps C-J daily for 7 days.
K. The recorded videos are given to a blinded observer who tracks the animal behavior and analyzes the immobility score of each animal. The blinded observer does not know the treatment given except the behavior to measure.

5 FORCED SWIM TEST (FST)

This is another acute behavioral test used to assess antidepressant-like effects of drugs. Mice are allowed to swim for 5 or 6 minutes per day after they have been given treatments. The experiment was repeated daily for 7 days. How long one waits after drug administration before beginning the FST depends on the nature of drug given and the route of administration. Generally, one can wait for about 15 to 30 minutes before beginning the FST if drugs were given by intraperitoneal, subcutaneous or intramuscular route to mice. For oral route, one can wait for about 30 to 60 minutes. Immobility behavior is measured as an index of depression.

A. Weigh the mice to determine doses to administer. **B.** Randomly assign mice into groups based on the treatment (n=8). Each animal must be identified by indelible non-poisonous ink or by by color codes to ensure there is no mix up. **C.** Administer drugs or extracts by oral route unless otherwise specified. **D.** Wait for about 1 hour if drug/ extract was administered by the oral route. The time must be consistent throughout.
NOTE: STEPS A TO D are not necessary if TST was done before the FST. However, in case FST is done first, then those steps are important.

E. While waiting, fill the transparent glass or plastic container with water to the height of 13 cm.
F. Set up the camcorder above these containers so it can capture the behavior of each animal placed in the containers.
G. Before placing the animal in the water individually, start the camcorder and record the group to be tested in the video before placing the animal in the water. The group should be coded so that it is not clear to the observer who will do the tracking the type of treatment given.
H. Allow the animals to swim for 6 minutes without being seen making noise.
I. Repeat this procedure for all animals.
J. Dry animals with paper towel after each swim session before returning it to the home cage. This will prevent hypothermia in the animals
K. Repeat steps C-J daily for 7 days.
L. The recorded videos are given to a blinded observer who tracks the animal behavior and analyzes the immobility score of each animal. The blinded observer does not know the treatment given except the behavior to measure.

6 OPEN SPACE SWIM TEST (OSST)

The open space swim test is a chronic depression model that mimics human depression. It involves a depression induction phase and then a treatment phase.

A. Weigh mice and randomly group them (n=8- 10).
B. Switch on camcorder to capture the group (group should be coded) to be swum. The blinded observer must not know the real identity of each group.
C. Depression induction phase: Swim each mouse for 15 minutes daily for 4 days in a bath tub (dimensions are in the attached document). The bath tub should be marked into four quadrants prior to this stage. It is expected that after the 4th day, mice will exhibit signs of depression by showing increased immobility state.
C. Treatment phase: Treatment with drug/extract begins on the 5th day till the 18th day. On each day, administer the specified dose orally (dose is calculated based on the recorded weight of the mouse) and wait for about an hour before testing.
D. Though treatment is done daily, the swimming is done on day 5, 8, 11, 15 and 18.
E. Since the immobility behavior is the most consistent index for measuring antidepressant effect, it is what the blinded observer evaluates from the videos. However, the total distance travelled by the mouse can also be measured. This can be done by tracking the number of quadrants the mouse crosses. Alternatively, one can use softwares such as behavioral tracker or anymaze that measure distance travelled directly.

BEHAVIORAL TESTS WITH LIPOPOLYSACCHARIDE (LPS) PRE..

7 **A.** Mice are weighed and randomly assigned to groups (n=8).
B. The are given pre-calculated doses of extract or drugs for 11 days.
C. On the 11th day, LPS (1 mg/kg. i.p) is administered to the all groups of mice except one group.
D. About 6 hours post LPS treatment, FST and TST are carried out as described above.
E. Step D is repeated after 24 hours post LPS treatment.

GOLGI-COX STAINING

8 **A.** After the behavioral tests, mice were euthanized with diethyl ether (not more than 2 mL for 5 minutes).
B. The brains were removed and sectioned for the Golgi-Cox staining technique.
C. Step by step details to be found in the attached document.
D. The effect of treatment of treatment on activated and resting microglia are assessed as described in the attached document.