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Research article

# Antidepressant and anxiolytic effects of melaleuca cajuputi essential oil on chronic immobilization stress (CIS)-Induced mice model

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## ABSTRACT

The preliminary studies showed that *Melaleuca cajuputi* essential oil (MCEO) contained several phytochemicals of antidepressant and anxiolytic properties. Therefore, the aim of this study is to evaluate the antidepressant and anxiolytic effects of MCEO on chronic immobilization stress (CIS)-induced mice.Eight groups of male *Swiss albino* mice were divided into control, CIS+DMSO, CIS+amitriptyline and CIS+MCEO at different concentrations (1, 2.5, 5, 7.5 and 10% v/v). All mice groups except the control were subjected to a 2-hours CIS procedure for 15 days. All MCEO treatments were administered through the inhalation route, while the amitriptyline was via intraperitoneal from day 16 to 20. The anxiolyticandantidepressant effects of MCEO were evaluated via behavioural tests, histopathological changes and corticotrophin-releasing hormone (CRH) level on day 21. Results were analysed using one-way ANOVA followed by Dunnet's posthoc test with the significant level of $P<0.05$.At its lowest concentration (1% v/v), MCEO has significantly reduced depression-like behaviour, while the anxiolytic effect appeared at concentration 5% v/v. The mice's body weights were significantly increased ($P<0.0001$) at the concentrations of 5, 7.5 and 10% v/v. Meanwhile, at 7.5 and 10% v/v, MCEO able to significantly increase the granular cell layer (GCL) and pyrimidal cell layer (PCL) surface areas ($P<0.05$). For CRH level, it has been reduced from the lowest MCEO concentration. MCEO exhibited antidepressant and anxiolytic effects, probably due to the phytochemical constituents that regulated in the CRH pathway which lead to an increase of the GCL and PCL surface areas.

**Keywords:** Anxiety, Depression, Essential Oil, Inhalation, Melaleuca Cajuput, Swiss Albino

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## INTRODUCTION

Melaleuca cajuputi(MC) which belongs to the Myrtaceae family can be found mainly in humid and hot climate zones in the Asian and Australian regions. In traditional medicine, theMC essential oil (MCEO) also known as 'minyak kayu putih' in Malaysia, was widely used to treat burns, scabies, coughing, stomach aches, cholera, muscular pain and intestinal problems[1, 2]. A previous study also proved that the MCEO possessed several medicinal properties such as anti-dengue, anti-inflammatory, anticonvulsant, antimicrobial and anti-cancer [3]. It was reported that the essential oil (EO) obtained approximately 19 phytochemicals such as α-pinene, β-pinene, α-phellandrene and limonene which contribute to anxiolyticand antidepressant effects [3]. Previously, the total percentage of anxiolytic compounds was 38.45%,while for antidepressant compounds presented in MCEO was 41.67% [3].

Anxiety and depression are the two most common mental health problems which contribute to a higher risk of other diseases and burden to the national economy [4]. According to the WHO reports, about 350 million people globally were suffering from the depression where 7.3% of them are also affected by the anxiety disorder [5].The causative effects of the disorders were thought to be originated from the endocrine disorders, neurotransmitter alteration, and elevation of inflammatory cytokines [6-10]. Apart from that, the reductions on the volume of dentate gyrus (DG) and cornu ammonis 3 (CA3) regions in the hippocampus and high cortisol concentration can also cause to anxiety and depression [11, 12]. Patients were diagnosed and treated based on Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) guidelines through psychological and medical standpoints [7, 13].

The psychological treatments effectiveness would decline when there was limited access to an adequate treatment, mainly due to the practical problems which require much longer time in each session together with the low self-motivation to seek for help and

treatment [14]. In fact, the antidepressants and anxiolytic synthetic agents could cause negative effects such as sexual dysfunction, miscarriage, fatigue, drowsiness, and organ damage to the patients [15, 16]. Thus, this study aims to investigate the anxiolytic and antidepressant effects of MCEO based on the behaviour, hippocampus changes, and CRH level of CIS-mice while determining its toxicity effect in terms of relative organ weight (ROW) and body weight gain. The findings from this experiment can be a door opener to the future research in investigating a potent drug from natural source of the threatening health problems, the anxiety and depression.

## MATERIALS AND METHODS

### *Melaleuca cajupati* Essential Oil (MCEO) Preparation

*Melaleuca cajupati* was collected locally and authenticated by a botanist with an authentication no.: PIIUM 0304. The leaves were subjected to a hydrodistillation process to obtain the MCEO. The EO was stored in a dark room prior to its use to avoid the exposure to the light which might cause the bioactive compounds to degrade.

### Animals

A total of 64 male Swiss albino mice ($n$ = 8 mice per group) at 3 months old weighing around 24-28g were supplied by the Laboratory Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Kelantan, Malaysia.All experimental procedures were conducted in accordance to the USM Guidelines and approved by the Animal Ethics Committee, approval number: USM/IACUC/2019/(116)(969). The sample size of the study was determined based on the previous studies [17, 18].

### Chronic İmmobilization Stress (CIS) in Mice Model

Method to develop the CIS-mice model was according to a previous study with slight modifications [19]. All groups were underwent to CIS procedures by inserting the mice into the cylindrical tank, except mice from the control group. The CIS was performed by putting the mice in the restrainer for 2h per day for 21 days to induce the depression and anxiety-like behaviour that were expected to present after day 15 based on the previous studies [20-22]. The mice body weight were measured and recorded daily.

### Dosages and Treatments

The administration of the treatments (Table 1) started on day 16 after pre-treatment behavioural tests and were continued until day 21. The behavioural tests were carried out 15 min after the drug or MCEO administrations. All of the treatments were administered via inhalation (INH) using nebulizers containing 0.1ml/0.1 kg of MCEO while the amitriptyline-treated mice via the intraperitoneal (i.p.) route. This procedure was carried out based on the standard procedure according to the previous studies with slight modifications [23-25]. Chemicals, dimethyl sulfoxide (DMSO) and amitriptyline were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The control group did not receive any treatments or CIS procedures.

**Table 1:** Experimental management of mice in the study.

| Group | Experimental condition | Concentration |
|---|---|---|
| Control | No CIS, DMSO or saline | - |
| CIS + DMSO (mL/0.1kg, INH) | CIS + 1% DMSO and 0.9% saline | 0.1 |
| CIS + MCEOtreatment (%) | CIS + essential oil + 1% DMSO and 0.9% saline | 1.0<br>2.5<br>5.0<br>7.5<br>10.0 |
| CIS + amitriptyline (mg/kg, i.p.) | CIS + Amitriptyline | 20 |

*n = 8 mice per group*

### Behavioural Test: Force Swimming Test (FST) and Elevated Plus Maze Test (EPMT)

The FST and EPMT were conducted based on a previous study with some modifications [26, 27]. These behaviour studies were conducted on day 0 as a baseline and days 16 to 20. The time for swimming and climbing was taken and recorded for six minutes. For EPMT, the observation took about five minutes, and time spent in the open field was recorded. Later, the time taken was converted to percentages.

### Body Weight and Relative Organ Weight (ROW)

All groups of mice were weighted and recorded every day starting from day 0 until day 21. Then, the average body weight in each group on each day was taken and compared with the CIS+DMSO group to detect any weight changes[28]. After the mice were sacrificed, internal organs such as the spleen, liver, kidney, lung, heart, and testes were collected and weighed. The relative organ weights were calculated by following formula.

ROW (%) = Weight of organ (g)/Body weight (g) × 100

### Histological Analysis

All the brain samples were fixed with 10% of neutral buffered formalin in order to preserve the samples permanently[29]. Then, all of the specimens were subjected to tissue processing and stained with hematoxylin and eosin. The surface areas of the DG granular cell layer (GCL) and CA3 pyramidal cell layer (PCL) surface areas in the hippocampus were observed via a light microscope at 200X magnification and measured by Image Pro Plus software[30].

### Serum Hormonal Assay of Corticotrophin Releasing Hormone (CRH)

After the behavioural tests, the mice were sacrificed and the blood was collected in microcentrifuge tubes. The serums were immediately separated by centrifugation and CRH level was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp.) according to the manufacturer's instructions [31].

### Statistical Analysis

The data was expressed as mean ± SEM, and the results

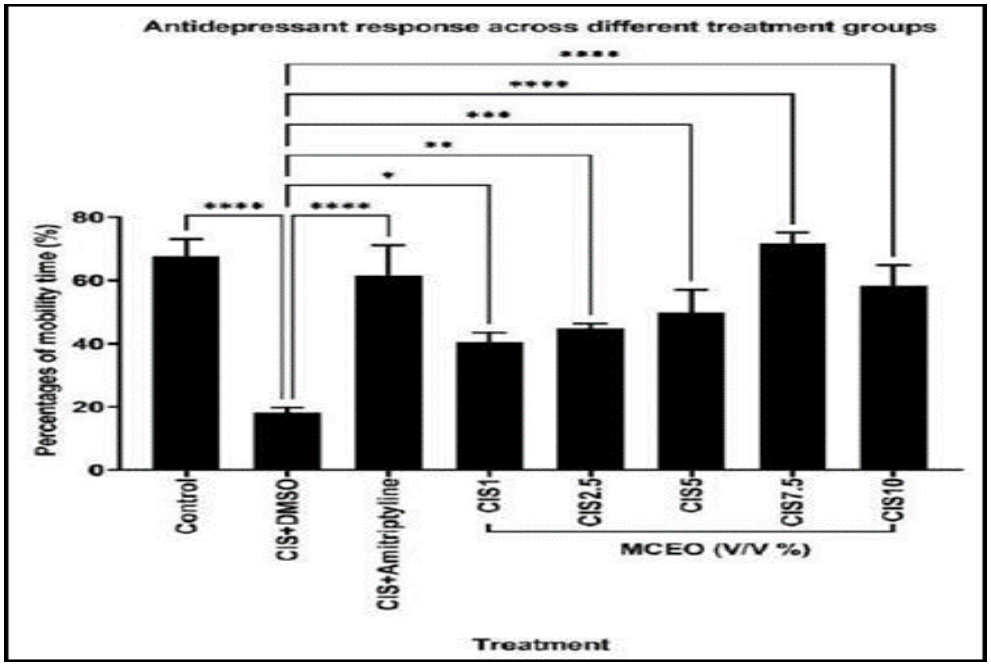
were analyzed with Graphpad Prism, using one-way ANOVA followed by Dunnet's post-hoc test, with p<0.05 set as the significant level.

## RESULTS & DISCUSSION

### Force Swimming Test (FST) for Depression

The CIS-induced mice have gradually demonstrated the depression-like behaviour starting from day 16 until day 21 when the mobility time in the FST showing reduction significantly when control group was compared with CIS+DMSO group. Based on Figure 1 on day 21 the percentage of mobility time of CIS-DMSO mice became significantly different from the control group ($P$<0.0001). However, the administrations of amitriptyline and MCEO at all concentrations were able to significantly increase mobility time among mice which indicated their depression-like behaviours were recovering. From the figure, the MCEO at 7.5% v/v group was the highest to elevate the mobility time of FST while the MCEO at 1% v/v was the lowest. Meanwhile, the MCEO at 10% v/v showed a relatively comparable effect on the time of mobility with the positive group, the CIS+amitriptyline.

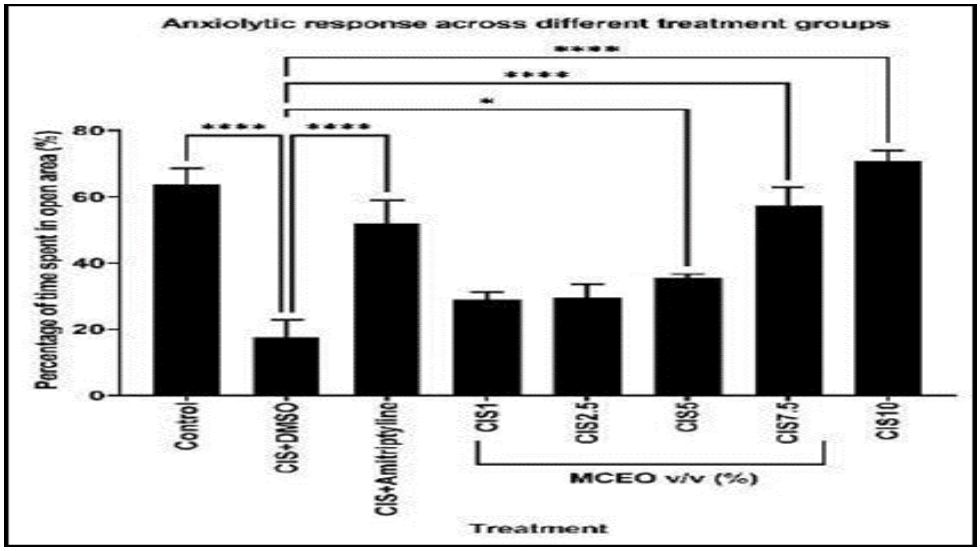
**Figure 1:** Mobility time (%) of mice ($n$ = 8 per group) in FST on day 21. Data was significantly differents if *- P<0.05, **- P<0.01, ***- P<0.001 and ****- P<0.0001, when compared to the CIS+DMSO group.




### Elevated Plus Maze Test (EPMT) for Anxiety

In EPMT, the CIS+DMSO mice showed a reduction of time spent in the open areas compared to the control group starting on day 16, which indicating the presence of anxiety-like behaviour. It appeared to be significantly decreased in the respective group compared to the latter on day 21 ($P$<0.0001). As displayed in the Figure 2, the MCEO at concentrations of 5, 7.5, and 10% v/v and CIS+amitriptyline groups able to significantly increase the percentage of time spent in open areas suggesting that the anxiety-like behaviour

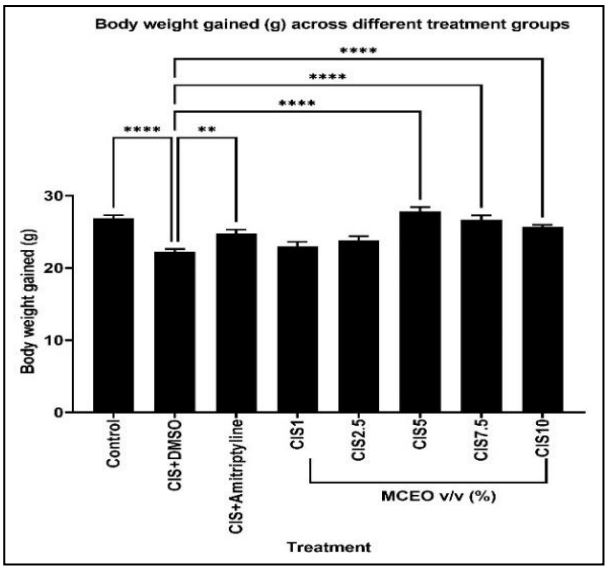
**Figure 2:** Time spent of mice ($n$ =8 per group) in open area (%) in EPMT on day 21. Data was significantly differents if *- $P$<0.05, **- $P$<0.01, ***$P$<0.001 and ****- $P$<0.0001, when compared to the CIS+DMSO group.



reduced in the particular groups ($P$<0.05 and <0.0001 accordingly). The highest contributions to the decreament of anxiety-like behaviour based on the time spent in open areas were the 10 and 7.5% v/v of MCEO ($P$<0.0001). Eventhough the MCEO groups at the concentrations 1 and 2.5% v/v did not show significant effect, yet there was a noticable positive trend on the time spent in the groups compared to CIS+DMSO.

**Effect of MCEO on the Body Weight**

**Figure 3:** Body weight (g) gained of mice ($n$ = 8 per group) after 21 days.




Data was significantly differents if *- $P$<0.05, **- $P$<0.01, ***- $P$<0.001 and ****- $P$<0.0001, when compared to the CIS+DMSO group

A significant weight gain in mice was spotted on day 21 in the control, amitriptyline and MCEO (5%, 7.5 and 10%) v/v groups when compared to the CIS+DMSO ($P$<0.0001) (Figure 3) Mice from the MCEO of 5 and 7.5% v/v gained the highest body weight which were relatively equal with the normal mice body weight in the control group or among the amitriptyline-treated's. On the other hand, the MCEO at 1 and 2.5% v/v did not bring any significant effect to the mice body weight.

**Effect of MCEO on ROW**

Based on the Table 2, all mice did not show any significant changes on the ROW when compared to the CIS+DMSO group. Eventhough no significant effect was determined, the organs of heart, liver, lung and brain in amitriptyline- and MCEO-treated mice disposed higher ROW than the CIS+DMSO where some of the value were on par with the normal mice for instances heart of 10% MCEO (0.65 to 0.64%), liver of amitriptyline, 5 and 10% MCEO (6.85 to 6.81%), lung of 5% MCEO (2.23 to 2.22%) and brain of 1and 7.5% MCEO (2.32 to 2.31%) groups accordingly. Subsequently, the CIS-induced mice derived lower ROW of testes, lung and brain than the normal's.

**Table 2:** ROW of mice in all groups on 21 days

| Group | ROW (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Heart | Liver | Spleen | Kidney | Testes | Lung | Brain |
| **Control** | 0.65 ± 0.03 | 6.85 ± 0.32 | 0.31 ± 0.04 | 1.74 ± 0.04 | 1.81 ± 0.05 | 2.23 ± 0.08 | 2.32 ± 0.20 |
| **CIS+DMSO** | 0.51 ± 0.02 | 5.85 ± 0.17 | 0.24 ± 0.03 | 1.69 ± 0.05 | 1.66 ± 0.06 | 1.98 ± 0.11 | 1.94 ± 0.06 |
| **CIS + Amitriptyline** | 0.61 ± 0.03 | 6.81 ± 0.28 | 0.23 ± 0.03 | 1.82 ± 0.06 | 1.65 ± 0.06 | 2.04 ± 0.07 | 2.24 ± 0.11 |
| **CIS+MCEO (%)** | | | | | | | |
| **1.0** | 0.54 ± 0.03 | 6.11 ± 0.27 | 0.22 ± 0.01 | 1.63 ± 0.04 | 1.60 ± 0.05 | 2.04 ± 0.09 | 2.31 ± 0.18 |
| **2.5** | 0.51 ± 0.02 | 6.32 ± 0.24 | 0.24 ± 0.02 | 1.62 ± 0.04 | 1.62 ± 0.09 | 2.09 ± 0.08 | 2.16 ± 0.11 |
| **5.0** | 0.54 ± 0.02 | 6.81 ± 0.20 | 0.29 ± 0.03 | 1.65 ± 0.02 | 1.69 ± 0.08 | 2.22 ± 0.07 | 2.18 ± 0.14 |
| **7.5** | 0.61 ± 0.06 | 6.52 ± 0.39 | 0.26 ± 0.03 | 1.81 ± 0.07 | 1.57 ± 0.12 | 2.17 ± 0.07 | 2.31 ± 0.08 |
| **10.0** | 0.64 ± 0.07 | 6.81 ± 0.24 | 0.24 ± 0.03 | 1.73 ± 0.09 | 1.54 ± 0.04 | 2.05 ± 0.11 | 2.25 ± 0.09 |

**Granular Cell Layer (GCL) in the Hippocampus**

The GCL which situated in the dentate gyrus (DG) is characterised as packs of polyhedral cells with large vesicular nuclei (Figure 4). The GCL is presented as a bluish-purple colour in a V-shaped. The GCL layer appeared to be clear purplish colour, while other treated groups that underwent the CIS procedure appeared to be dark purplish colour. It was found that the condensed nuclei (black spot) only appeared in all GCL layers of amitriptyline and MCEO treated groups of CIS-mice. The cytoplasm of GCL in depressed and anxious mice was darker than in non-depressed mice and it was accompanied by higher numbers of black spots, the condensed nuclei in the area, indicating an apoptosis process. Based on the current result, the surface areas of GCL were significantly increased and most abundant when treated with amitriptyline and MCEO 10 and 7.5% v/v as presented in the Figure 5. This might indicate that there was a reduction of depression and anxiety like-behaviours in the mice. But at low concentrations of MCEO (1, 2.5 and 5%), no significant results were spotted.

**Pyramidal Cell Layer (PCL) in the Hippocampus**

PCL in cornu ammonis 3 (CA3) is characterised as packs of pyramidal cells with large vesicular nuclei as seen in Figure 6. The PCL is presented as bluish-purple colour in a C-shaped. In the meantime, for control and MCEO 10% v/v, the PCL layer appeared to be a clear purplish colour and other treated groups appeared more darker. The cytoplasm of PCL in depressed and anxious mice was darker than in non-depressed mice and it was accompanied by higher numbers of black spots (condensed nuclei), indicating an apoptosis process. Based on the current result, the highest increment of the

surface area of PCL was amitriptyline, followed by MCEO at 10% v/v and 7.5%, which indicated a reduction of depression and anxiety behaviours.

**Figure 4:** Histological examination images from H&E staining on GCL of mice at 200X magnification. Abbreviation: **CIS*n*%** - Concentration of MCEO given to CIS-induced mice model.

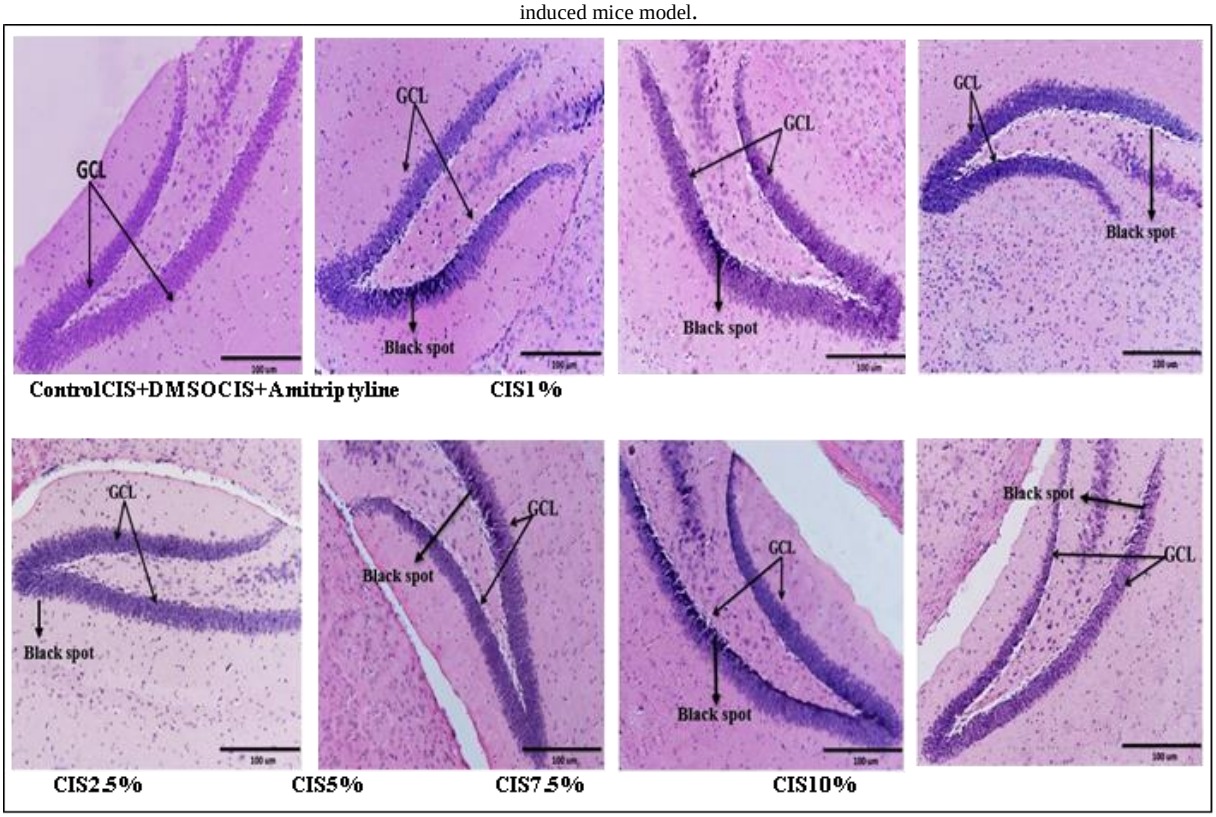
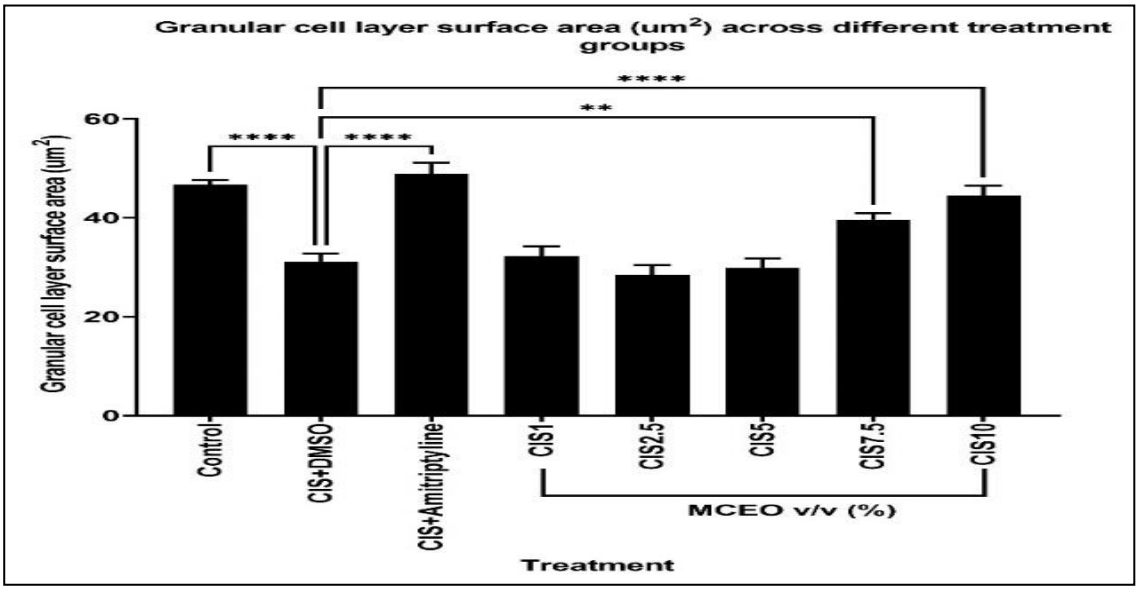



**Figure 5 :** GCL areas (μm²) in mice ($n$ = 8 per group) at different groups on day 21. Data were significantly differents if *- $P$<0.05, **- $P$<0.01, ***- $P$<0.001 and ****- $P$<0.0001, when compared to the CIS+DMSO group.




In the present study, the antidepressant and anxiolytic effects of MCEO were tested on the CIS-mice model through FST and EPMT. The CIS procedure is a reliable animal method which triggerring mental and physical stress and recognized to produce depression- and anxiety-like behaviours[29, 30]. The FST is used to analyze the mobility behaviour of mice in the chamber from their swimming (horizontal movement) or climbing (vertical movement) character[6]. The test evaluated behaviour despair because the rodents would try to escape from their stressful environment either by swimming or climbing or remain immobile, indicating for being hopeless. Concurrently, the EPMT is used to analyze anxiety behavior in which, if the mice spent more time in the enclosed arms, their anxiety

behavior decreased or vice versa. The test reflects the typical instinct of rodents whether to avoid or approach a possible dangerous situation. Occasionally, open arms in EPMT would be considered as dangerous due to their height and open space environment while closed arms are considered less dangerous due to the enclosed surrounding by walls. The depression and anxiety-like behaviours in the CIS-induced mice take about 15 days to be developed. Thus, this study is coherent with the previous studies where both induced behaviours were developedby day 16 [20-22].

**Figure 6:** Histological examination images from H&E staining on PCL of mice at 200X magnification. Abbreviation **CIS*n*%** - Concentration of MCEO given to CIS-induced mice model.

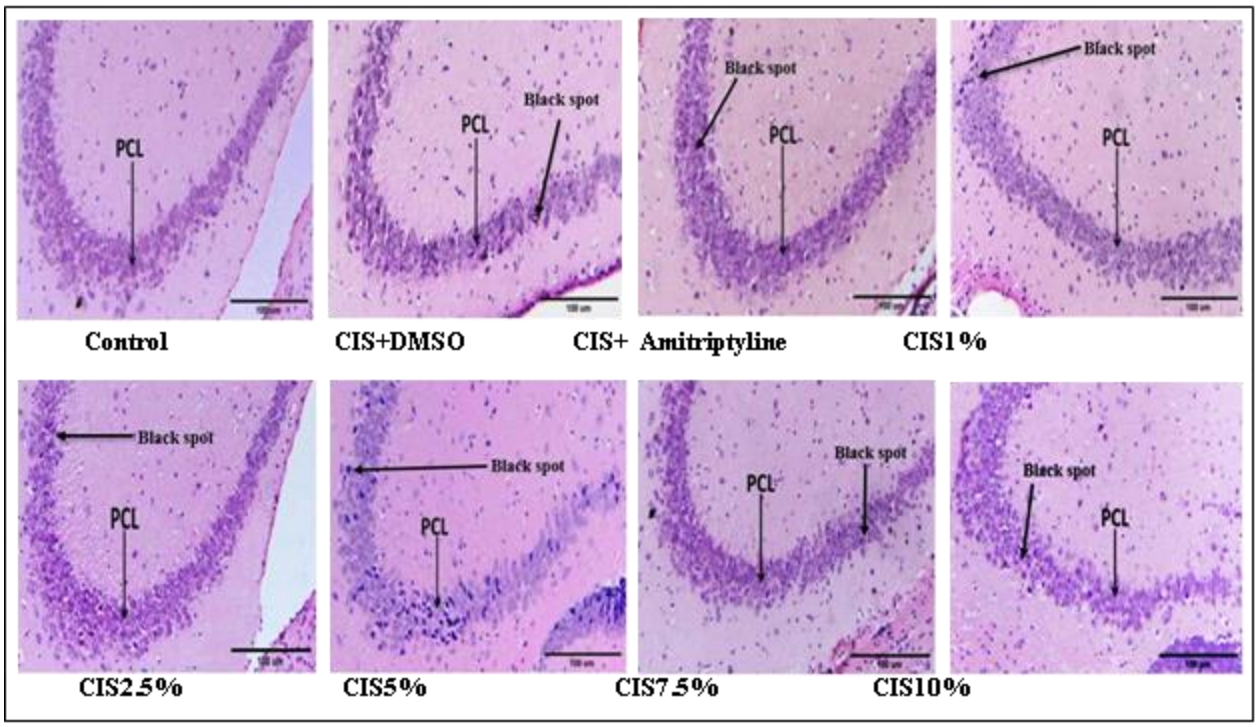



**Figure 7:** PCL surface areas (μm$^2$) in mice ($n$ = 8 per group) at different groups on day 21. Data were significantly differents if *- $P$<0.05, **- $P$<0.01, ***- $P$<0.001 and ****- $P$<0.0001, when compared to the CIS+DMSO group.

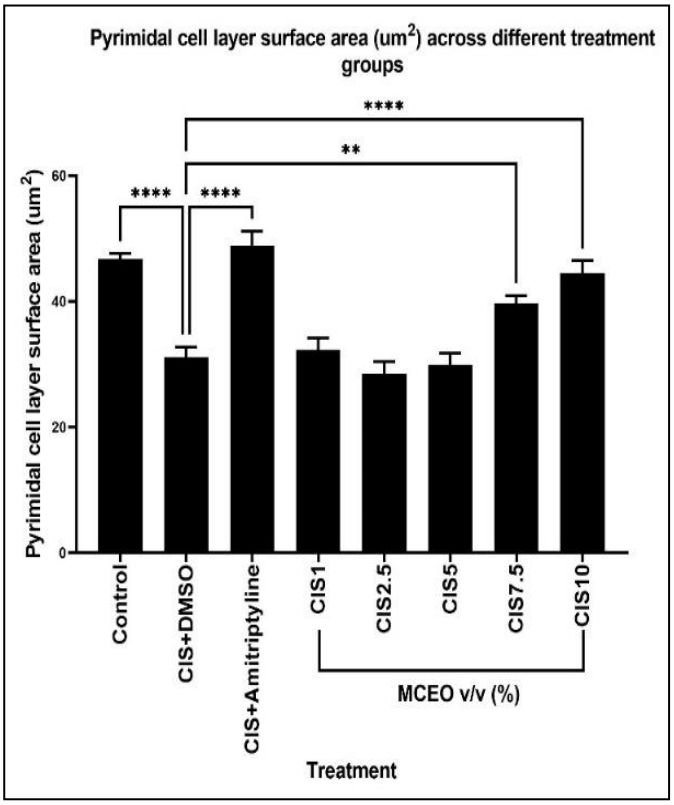
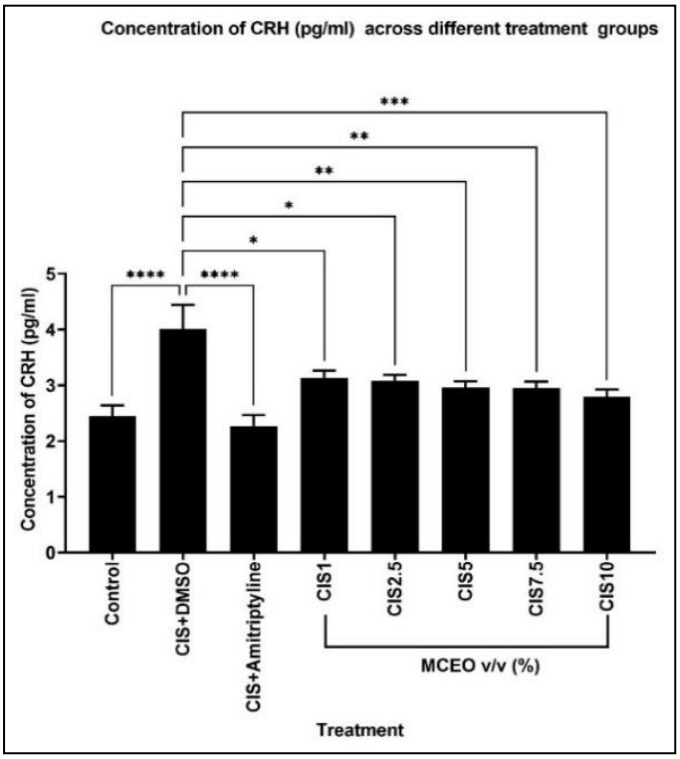



**Figure 8:** Corticotrophin releasing hormoneconcentrtion in mice ($n$ = 8 per group) at different groups on day 21. Data was significantly differents if *- $P$<0.05, **- $P$<0.01, ***- $P$<0.001 and ****- $P$<0.0001, when compared to the CIS+DMSO group.



Based on the Figures 1 and 2, MCEO showed antidepressant and anxiolytic effects at respective concentrations might due to the increase of serotonin and noradrenaline. Previous studies reported that, the increasing of mobility behaviour in FST and increasing of time spent in close space in EPMT were associated with the serotonergic and noradrenergic pathways [32, 33]. A study demonstrated that depression and anxiety were associated with low leptin secretion, body fat, body weight and high ghrelin secretion[ 34]. The increased gherlin secretion would disrupt the HPA axis and prevent serotonin secretion in the hypothalamus [34]. According to current findings (Figure 3), administartion of MCEO and amitriptyline might reverse the depression and anxiety process.Meanwhile, in the Table 2, all of the treatment and control groups did not show any significant changes in the ROW although several organs such as heart, liver, lung, and brain of amitriptyline- and MCEO-treated mice revealed higher ROW than the CIS+DMSO's.This indicated that, the treatment and control groups do not cause any toxicity effect to the internal organs[35, 36]. However, further examination on histopathological of the internal organs need to be conducted to confirm the toxicity effect.

In the meantime, a study reported that the induction of depression and anxiety in the mice would reduce the surface areas of GCL and PCL in the hippocampus [30]. The cytoplasm of GCL and PCL in depressed and anxious mice was darker than in non-depressed mice and it was accompanied by higher numbers of black spots, the condensed nuclei in the area, indicating an apoptosis process (Figures 4 and 6). The reduction of GCL and PCL surface areas and the presence of apoptosis process occurred due to the damage and atrophies of the nerve cells [30, 37]. This eventually leads to the reduction of brain-derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) genes and proteins in the hippocampus [30, 37]. Current finding showed that, the significant increased of surface areas of GCL and PCL as well as reduction of apoptosis processin the treatment groups due to ability of MCEO and amitripytyline to restore the atrophy of nerve cells by up-regulating the BDNF and GR genes and proteins [30, 37]. However, the involvement of BDNF and GR genes and their proteins in the hippocampus need to further investigate using the western blot and polymerase chain reaction techniques.Previous studies reported that prolonged stress can cause HPA axis dysregulation that increased the concentration of cortisol [38]. The elevation of cortisol was associated with the reduction of GR expressions in the hippocampus, especially in DG and CA3 [30, 37, 39]. Based on the results (Figure 8), MCEO might reduce the CRH secretion through a negative feedback mechanism by increasing GR expression in the brain [38]. Further research using immunohistochemistry staining in the anterior pituitary glands is required to investigate the role of GR expression.

## CONCLUSION

From the nutshell, MCEO significantly reduced depression-like behaviours from the lowest concentration, while anxiety-like behaviours were reduced starting from the concentration of 5% v/v. All treatment groups of MCEO showed no toxicity effect in selected internal organs, but weight gain was spotted at different concentrations of the EO (5, 7.5, and 10% v/v). The MCEO is able to increase the GCL and PCL surface areas at concentrations of 7.5% and 10% v/v. The CRH level started to reduce from the lowest concentration of the MCEO. Overall, the most effective concentration of MCEO to be used in the next clinical study based on the overall present findings was 7.5% v/v. This study demonstrated the potential of MCEO as a psychotherapeutic agent in the treatment of depression and anxiety problems.

**Conflict of interest:**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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