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Protective effect of *Piper cubeba* L.f. extract and lignan fraction against cognitive impaired rat models

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Abstract: This study aimed to examine the neuroprotective effect of *Piper cubeba* on cognitive impairment models using two induction methods: electroconvulsive shock (ECS) and scopolamine (SCO). For each model, male Sprague-Dawley rats were divided into five groups: *P. cubeba* 96% ethanol extract (PCE), *P. cubeba* lignan-rich fraction (LRF), citicoline (C; positive control), ECS/SCO control, and normal control (NO). The test substances were administered p.o. for 14 days, after which the rats except those in the NO group, were treated with ECS or SCO, depending on the model. Cognitive function was evaluated using the Morris Water Maze (MWM). Biochemical examinations were performed on the hippocampus and cerebral cortex, including lipid peroxidase inhibition, superoxide dismutase (SOD) and catalase (CAT) activity, and levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The results showed that pretreatment with PCE and LRF improved cognitive function in ECS- and SCO-treated rats, with significantly lower escape latencies than those of the control groups ($p<0.05$). Moreover, PCE and LRF significantly increased hippocampal CAT and SOD activity compared with the ECS or SCO groups ($p<0.05$). PCE and LRF also significantly suppressed inflammatory cytokine levels (TNF-α and IL-1β) in the hippocampus and cerebral cortex, with levels significantly lower than those in the control groups ($p<0.05$). Overall, the extract and lignan fraction of *P. cubeba* improved cognitive function in both models, potentially through antioxidant and anti-inflammatory actions in the hippocampus and cerebral cortex.

Keywords: Cognitive; Inflammation; Oxidative stress

1.0 INTRODUCTION

Cognitive impairment is characterized by difficulty in processing thoughts leading to memory loss, difficulty in making decisions, inability to concentrate, and difficulty in learning new things (Mariani et al., 2007). The etiology of cognitive impairment varies, ranging from

vascular disorders to neuronal degeneration and stroke, with a prevalence of 22 to 76.8% per 1000 people each year (Pais et al., 2020). Cognitive impairment is frequently linked to chronic inflammation, which contributes to elevated oxidative stress levels (Tan & Norhaizan, 2019). Studies have shown that elevated levels of inflammatory markers such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β in older adults are associated with a higher risk of cognitive decline (Dimopoulos et al., 2006). Free radicals and oxidative stress are also responsible for causing neurodegenerative diseases and accelerating the progression of cognitive disorders in the elderlies (Dimopoulos et al., 2006). Therefore, the approach to prevent or treat cognitive disorders could involve antioxidants and anti-inflammatory agents. *P. cubeba* has been studied for its antioxidant and anti-inflammatory effects, the two main mechanisms of potential neuroprotector candidates (Mothana et al., 2017).

Previous studies have shown that *P. cubeba* ethanol extracts can reduce inflammation, comparable to nonsteroidal anti-inflammatory drugs (NSAIDs) (Perazzo et al., 2013). Research also showed that one of the anti-inflammatory mechanisms of *P. cubeba* involved targeting the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway (Qomaladewi et al., 2019). In addition to the ethanol extract, lignan from *P. cubeba* has also been widely studied as an anti-inflammatory agent. For example, cubebin and hinokinin have been tested to inhibit edema in rats (Godoy de Lima et al., 2018; Marcotullio et al., 2014). The lignans of *P. cubeba*, cubebin, and hinokinin have also been investigated as neuroprotective agents in an *in vitro* model (Tarbiat et al., 2023). The traditional use of *P. cubeba* also showed its potential pharmacological effect on the brain, as it relieves headaches (Ibrahim et al., 2024). Although *P. cubeba* has been reported to have anti-inflammatory and antioxidant properties, studies investigating its neuroprotective potential particularly in the context of cognitive impairment, are limited. This study evaluates the neuroprotective effects of its ethanol extract and lignan-rich fraction, validated through two distinct animal models of cognitive dysfunction. We hypothesized that PCE and LRF would mitigate cognitive impairment via antioxidant and anti-inflammatory pathways. This study offers novel insights into its potential as a herbal therapeutic agent for neurodegenerative disorders.

2.0 MATERIALS AND METHODS

2.1 Preparation of PCE and LRF

Dried *Piper cubeba* fruit was obtained from Balai Penelitian Tanaman Rempah dan Obat (BALITRO) (Bogor, Indonesia). The specimen's genus was confirmed by the Indonesian Institute of Science, Research Center for Biology, with the identification number B525/IV/D1.01/4/2021. PCE was prepared from the fruit powder, which was macerated with 96% ethanol (1:10) and then evaporated. Meanwhile, LRF was prepared by extracting the powder with 80% methanol (1:10) under sonication for 1 hour. The extract was subsequently concentrated using a rotary evaporator, and the remaining aqueous portion was fractionated by liquid–liquid extraction with dichloromethane (1:1). The dichloromethane fraction, identified as the lignan-rich fraction (Elfahmi et al., 2007), was then evaporated and dried in an oven. Detailed information on the phytochemical profiles has been previously published (Dwita et al., 2023a; 2023b).

2.2 Animals and treatments

A total of 45 Sprague-Dawley rats (180–200g, 2–3 month-old) were used in this research. A previous study showed that the 200 mg/kg dose of *P. cubeba* extract exhibits the best antioxidant activity in the brain (Dwita et al., 2023a). This dose was selected for use in the present study. The rats were divided into two experiments: electroconvulsive seizure (ECS) and scopolamine (SCO) models. Each experiment was divided into four groups: (1) PCE, 200 mg/kg; (2) LRF, 200 mg/kg; (3) C, 200 mg/kg; (4) ECS/SCO control; and the ninth group was the normal (NO) group. Animals were acclimatized for one week in 12/12h dark/light conditions before the experiment. The ethics committee of Universitas Muhammadiyah Prof. DR HAMKA has approved the experiment with the ethics approval number 02/23.06/02633.

2.3 Electroconvulsive shock (ECS)-induced cognitive impairment

The rats were given the test substance p.o. for 14 days. On the next day, ECS treatment was conducted using a digital electroconvulsimeter (EC-02, Orchid Scientific) via ear clips at an intensity of 200 mA for 0.5 seconds. ECS induced tonic hindlimb extension for approximately one minute. The intensity and duration of ECS were selected based on a preliminary trial to determine the seizure threshold required to induce tonic hindlimb extension without causing respiratory paralysis or mortality. The MWM test was performed 60 minutes after ECS treatment every day for five days. On day 20, a probe test was performed. The next day, the animals were sacrificed, and the brains were isolated (**Figure 1a**).

2.4 Scopolamine (SCO)-induced cognitive impairment

The rats were given the test substance p.o. for 14 days. On the next day, the rats were injected with 20 mg/kg of scopolamine i.p. (Jafarian et al., 2019). Thirty minutes after the injection, the MWM test was performed followed by the sacrifice of the animals (**Figure 1b**).

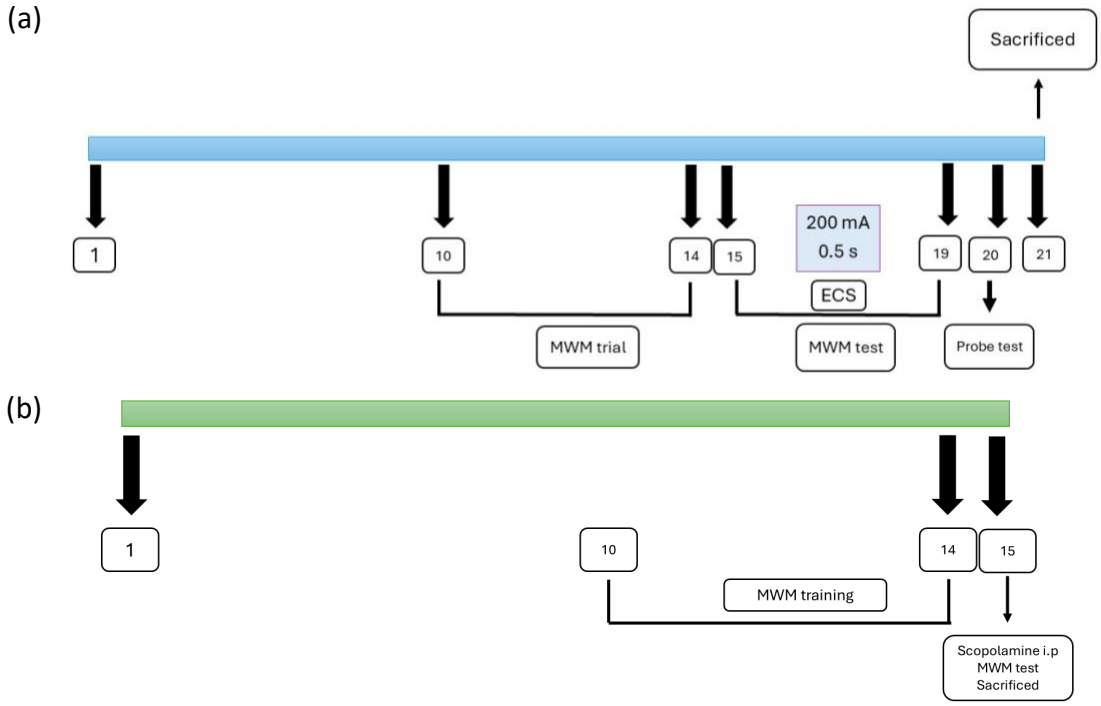

Figure 1: The experimental design for (**a**) electroconvulsive shock (ECS) and (**b**) scopolamine (SCO) models.

2.5 Morris water maze (MWM) test

Training phase: the water maze consisted of a 150 cm diameter pool with a platform of 15 cm diameter, and 50 cm height. The pool was divided into four equal quadrants (**Figure 2**) by imaginary lines and filled with water (25 ± 1°C) to a depth of 2 cm below the platform. The platform was placed in one of the quadrants, and both the platform position and the visual cue (placed on the wall near the platform) kept constant during the experiment. Each rat was allowed to swim for 60 seconds. If the rat did not find the platform within this time, it was guided to the platform and allowed to remain there for 15 seconds. Each rat underwent four trials from different insertion points in each experiment over five days.

Test phase: The procedures were the same as those in the training phase, except that the water was raised to 2 cm above the platform and made opaque with non-toxic white paint. Data was recorded as escape time (time needed to reach the platform), and rats with an escape time exceeding 60 seconds was recorded as 60 seconds. The data were presented as the average escape time from 4 trials at different insertion points for each experiment.

On the final day, a probe test was conducted in which the platform was removed, and the time spent by the rats in the platform quadrant was calculated using the following formula (D'Hooge & De Deyn, 2001):

Time percentage in platform quadrant =

$$\frac{\text{swimming time in platform quadrant (s)}}{\text{total swimming time (s)}} \times 100\%$$

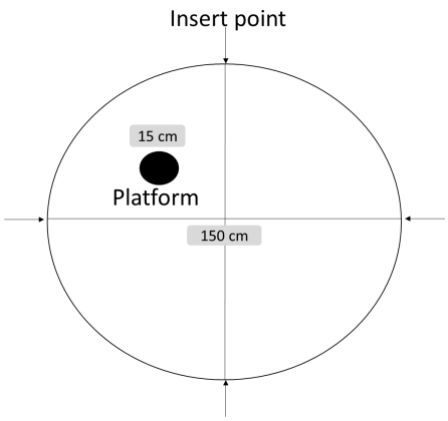

Figure 2: Morris water maze (MWM) test overview.

2.6 Brain homogenate preparation

Immediately after dissection, the rat brain was rinsed with cold phosphate-buffered saline (PBS) of pH 7.4 and dried with filter paper. The hippocampus and the cerebral cortex were isolated on an ice-cold slide. Immediately, each brain region was homogenized in cold PBS containing 0.1M phenylmethylsulphonyl fluoride (PMSF) to prepare a 10% homogenate. The mixtures were centrifuged at 4°C and 8,000 rpm for 15 minutes. The homogenates were stored at -20°C, and the protein comcentration was determined using Bradford's reagent and measured at 595 nm.

2.7 Lipid peroxidation test

The homogenates were mixed with 20% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) in a 1:1:2 ratio and then heated at 95–100°C for 15 minutes. The mixture was then centrifuged, and the absorbance of supernatant was measured at 532 nm, using tetraethoxypropane (TEP) as the standard. Malondialdehyde (MDA) levels were expressed in nm/g of brain tissue.

2.8 CAT activity test

The catalase (CAT) test was carried out following existing research (Hadwan, 2018) with slight modifications. A total of 100 µL of sample was mixed with 200 µL of hydrogen peroxide (H_2O_2), homogenized by vortexing, and incubated at 37°C for 2 minutes. Then, 1200 µL of the working solution [100 µL cobalt(II), 100 µL Graham salt, and 1800 µL sodium carbonate] was added, vortexed, and allowed to stand at room temperature in the dark for 10 minutes. The absorbance was measured at 440 nm. CAT activity was calculated as U/g of brain tissue.

2.9 SOD activity test

A total of 20 µL of diluted homogenate was used. Total superoxide dismutase (SOD) activity was measured using a biochemical assay kit (Elabscience) at 450 nm.

2.10 TNF-α and IL-1β quantitation

A total of 40 µL of diluted homogenate was used. TNF-α and IL-1β levels in the homogenate were measured using an ELISA kit (BT Lab) according to the manufacturer's protocol, at 450 nm.

2.11 Statistical analysis

All data were tested for homogeneity and normality, followed by one-way ANOVA and Tukey's post hoc test, with $p<0.05$ considered statistically significant.

3.0 RESULTS

3.1 *P. cubeba* effects on the MWM test of the cognitively impaired models

In the training phase of the MWM test, all groups showed a drastic decline in escape time from day two, with the decline continuing steadily towards the end of the training phase. There were no significant differences between groups ($p>0.05$), suggesting uniformity in the animals' cognitive function before ECS treatment (**Figure 3a**).

On the other hand, the MWM test phase showed that ECS treatment interfered with spatial learning and memory, as indicated by an increase in escape time compared to the NO control ($p<0.05$) (**Figure 3b**). PCE and LRF significantly reduced the escape time compared to the ECS group starting from day one of the MWM test phase. It is also notable that the most significant difference in escape time between PCE and LRF in the ECS group occurred on day two after ECS treatment. Although the data trend showed a decrease in escape time, the fifth-day data were statistically comparable across all groups, suggesting the disappearance of ECS effects on the test animals.

In the probe test, the ECS group showed worse spatial memory than the other groups, while the group receiving citicoline showed the best performance, followed by the PCE and LRF groups (**Figure 3c**). The SCO model showed a linear result, in which the PCE and LRF groups had significantly lower escape time than the SCO control ($p<0.05$) (**Figure 4**).

3.2 *P. cubeba* effects on hippocampal and cortical biomarkers of the cognitively impaired models

PCE and LRF significantly increased hippocampal CAT activity compared to the ECS group ($p<0.05$). The PCE group showed hippocampal CAT activity comparable to the citicoline group ($p>0.05$), while the LRF group depicted higher CAT activity than the citicoline group. In contrast, LFR showed no significant CAT activity in the cerebral cortex of the ECS model. PCE and LRF also increased CAT activity in the SCO model, especially in the hippocampus, where PCE showed the best result, comparable to the citicoline group (**Figure 5a**).

Moreover, PCE and LRF significantly increased SOD activity in the cerebral cortex compared to the ECS group ($p<0.05$), although they showed no significant effects in the hippocampus. While in the SCO model, PCE and LRF increased SOD activity in both brain regions (**Figure 5b**). **Figure 5c** shows that ECS and SCO treatments resulted in elevated hippocampal and

cortical MDA levels. Meanwhile, the PCE group showed lower MDA levels than the control in both models, especially in the hippocampus. The PCE and LRF groups exhibited significantly lower hippocampal and cortical TNF-α levels than the ECS control. However, in the SCO model, the treatment groups showed lower TNF-α levels only in the hippocampus. In contrast, PCE and LRF affected IL-1β levels only in the cortex of the ECS model. The data also showed that administration of PCE and LRF suppressed IL-1β levels in both the hippocampus and cortex in the SCO model (**Figure 6**).

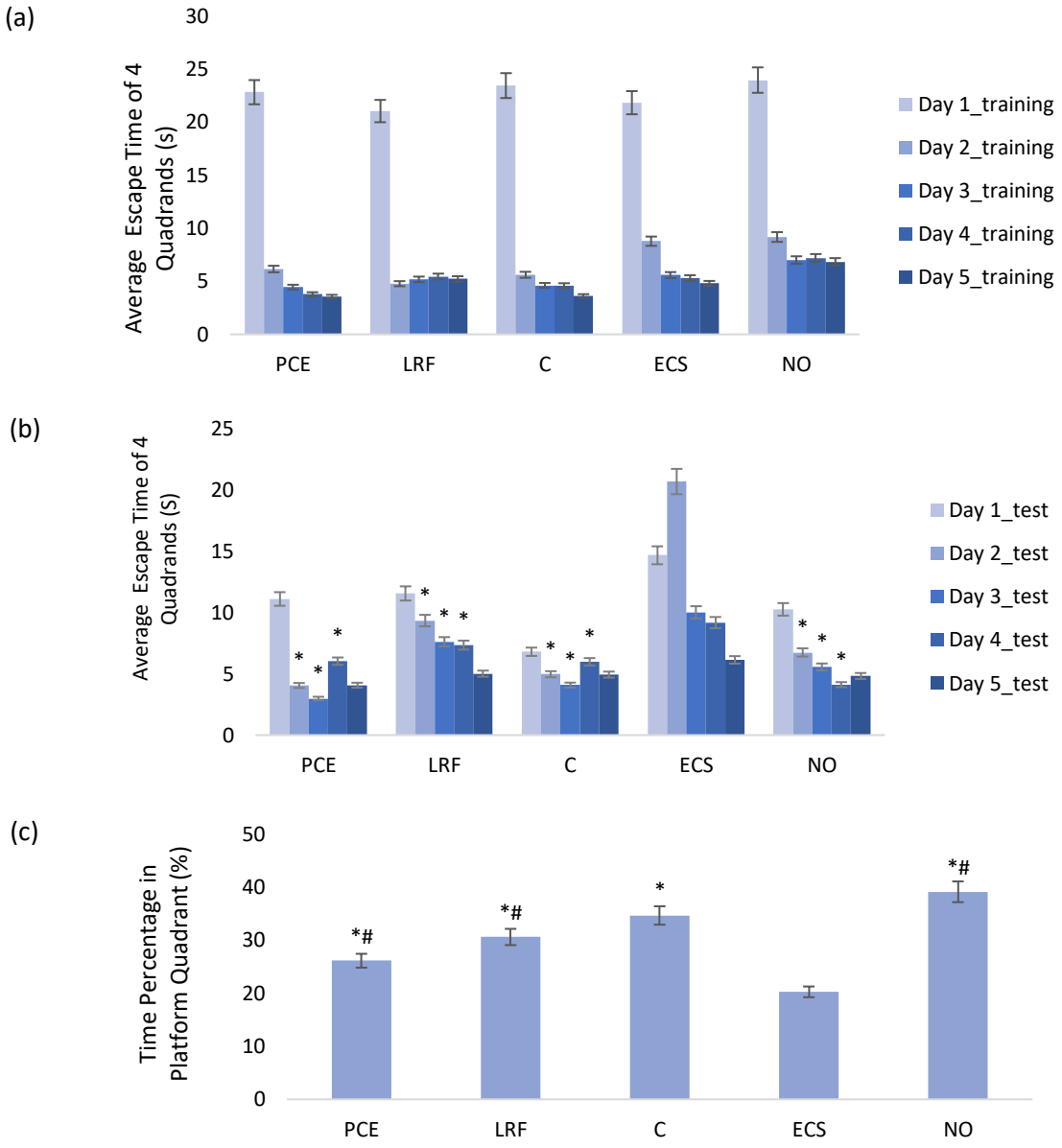

Figure 3: Morris water maze (MWM) test results in **(a)** training phase, **(b)** test phase and **(c)** probe test of *P. cubeba* 's 96% ethanol extract (PCE), lignan-rich fraction (LRF), citicoline (C), electroconvulsive shock (ECS) control, and normal (NO) control. The results are presented as mean ± SEM. * significantly different compared to the ECS group ($p<0.05$); # comparable to C ($p>0.05$).

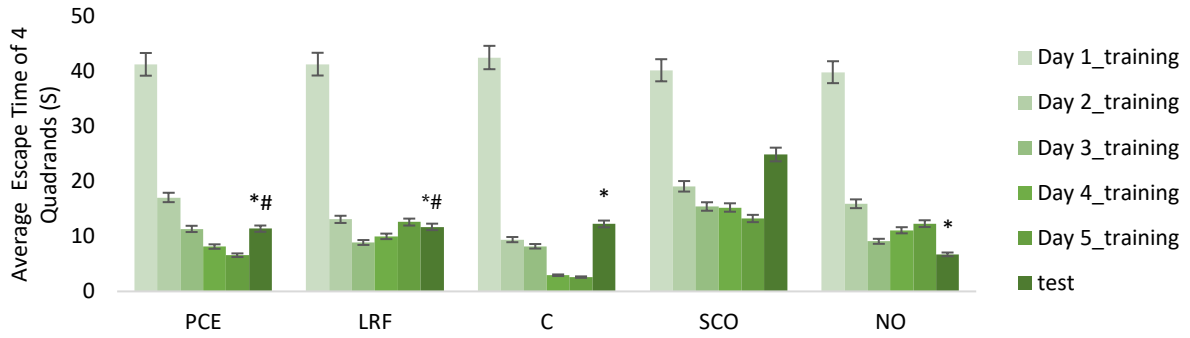

Figure 4: Morris water maze test results of *P. cubeba* 's 96% ethanol extract (PCE), lignan-rich fraction (LRF), citicoline (C), scopolamine (SCO) control, and normal (NO) control. The results are presented as mean ± SEM. * significantly different compared to the SCO group ($p<0.05$); # comparable to C ($p>0.05$).

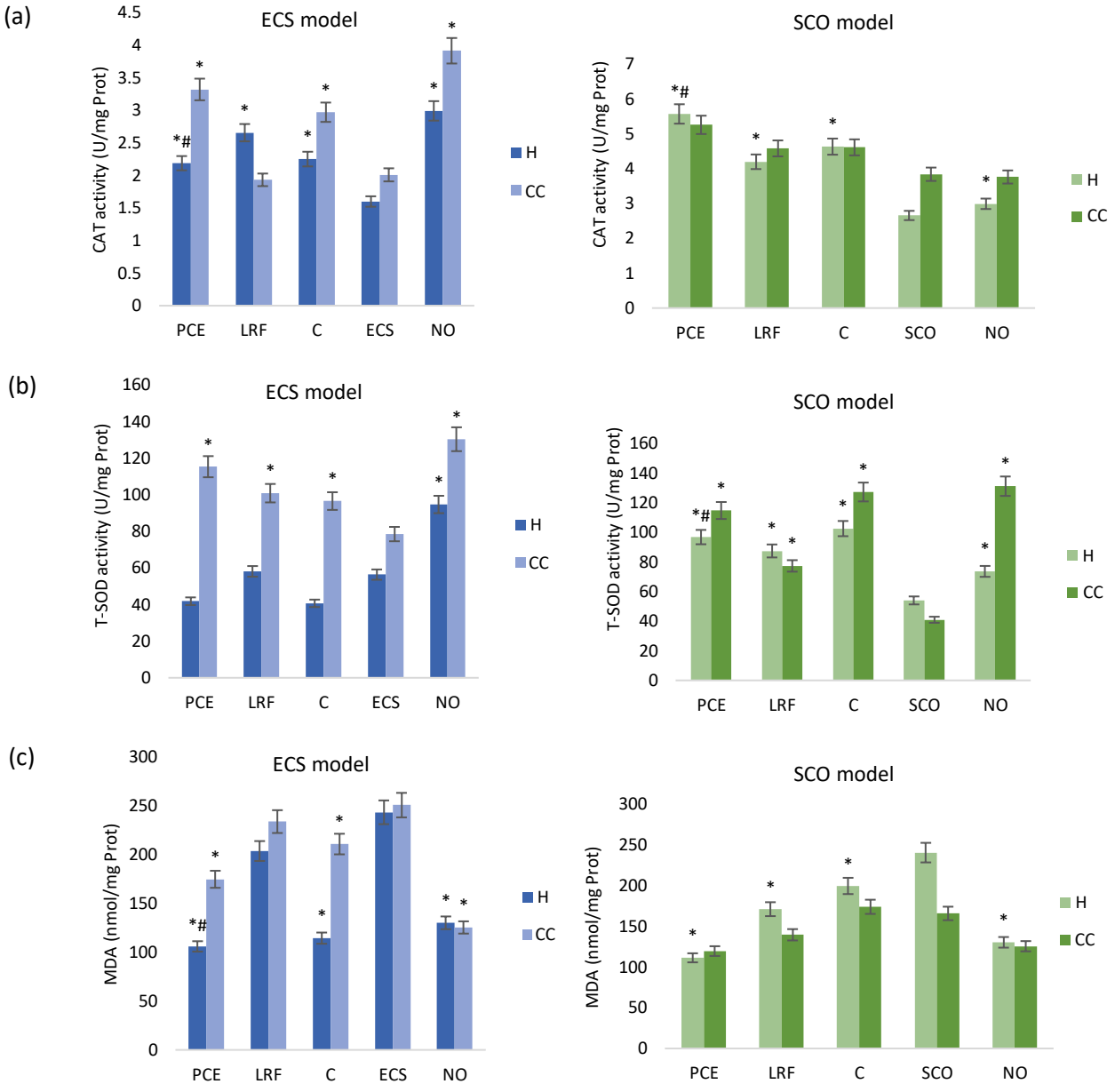

Figure 5: (a) Catalase activity, **(b)** total superoxide dismutase activity and **(c)** lipid peroxidation inhibition in the hippocampus (H) and cerebral cortex (CC) of PCE, LRF, C, ECS/SCO control and NO control groups. The results are presented as mean ± SEM. * significantly different compared to the ECS/SCO group ($p<0.05$); # comparable to C ($p>0.05$).

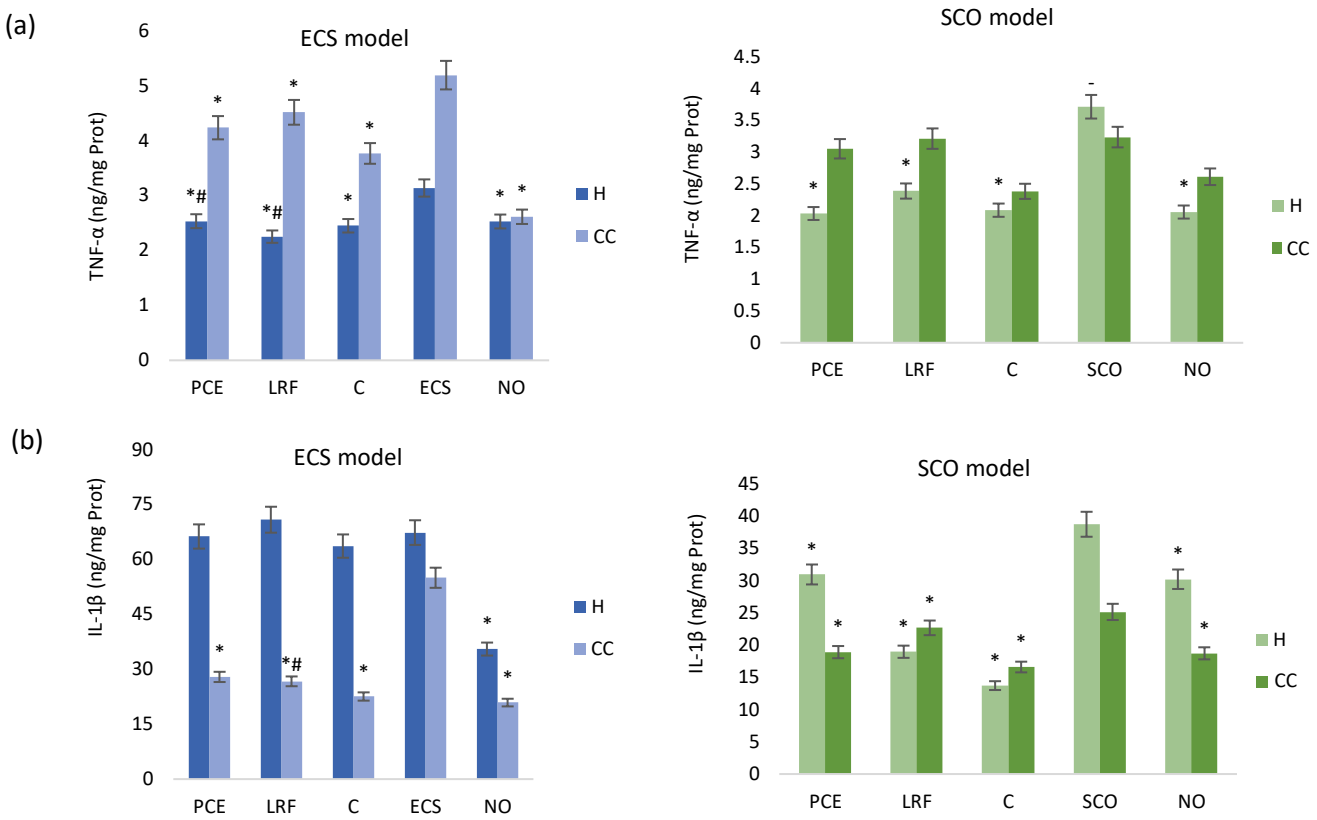

Figure 6: (a) TNF-α and **(b)** IL-1β levels in the hippocampus (H) and cerebral cortex (CC) of of PCE, LRF, C, ECS/SCO control and NO control groups. The results are presented as mean ± SEM. * significantly different compared to the ECS/SCO group ($p<0.05$); # comparable to C ($p>0.05$).

4.0 DISCUSSION & CONCLUSIONS

Piper cubeba 96% ethanol extract (PCE) and the lignan-rich fraction (LRF) improved cognitive performance, as assessed by the Morris water maze (MWM). The MWM assesses spatial memory that relies on environmental cues to find escape platforms (Vorhees & Williams, 2006). Rats treated with PCE and LRF showed lower escape times in both the ECS and SCO models. ECS treatment in animals has been shown to cause cognitive impairment with retrograde amnesia in hippocampal-dependent memory tests (Busnello et al., 2006), while the SCO model induces cognitive deficits through cholinergic disruption, leading to impairments in memory function (Klinkenberg & Blokland, 2010). The results of this study are consistent with the previous findings, where repeated treatment with ECS impairs spatial memory (Svensson et al., 2017). Results showed that the ECS and SCO control groups experienced the greatest cognitive impairment, as indicated by an increase in the escape times. On the other hand, the PCE and LRF groups showed a decrease in escape times, comparable to those observed with citicoline. However, in the ECS model, as the test progressed, the escape times across all groups improved.

The current study showed that ECS and SCO treatments increased oxidative stress, resulting in elevated MDA levels and reduced CAT and SOD activity in the hippocampus and cerebral cortex. In contrast, PCE significantly increased CAT activity in both brain regions. Nevertheless, both groups affected SOD activity only in the cortex and not in the hippocampus. Regarding MDA levels, only the PCE group inhibited lipid peroxidation. Similar trends were observed in the SCO model, where PCE exhibited better antioxidant activity than LRF in both hippocampus and cerebral cortex.

Interestingly, despite showing lower CAT activity in the hippocampus, the PCE group exhibited lower MDA levels than the LRF group, suggesting a divergence in antioxidant mechanisms. This discrepancy might be attributed to non-enzymatic antioxidant constituents in PCE, such as copaene, which are known to inhibit nitric oxide production and reduce lipid peroxidation (Veiga et al., 2007). The hippocampus and cortex differ in metabolic rate, baseline antioxidant levels, and susceptibility to oxidative damage, thereby influencing how each region responds to antioxidant treatments (Halliwell, 2006; Lee et al., 2020).

The elevation of CAT activity in the hippocampus may indicate that H_2O_2 detoxification via CAT is more relevant in this region, potentially due to higher local H_2O_2 production or differences in mitochondrial activity. Additionally, the bioavailability of active compounds in PCE and LRF may differ due to variations in blood-brain barrier permeability. ECS treatment also causes inflammation characterized by increased TNF-α in the hippocampus and IL-1β in the cerebral cortex. While in the SCO model, these cytokines were dominant in the hippocampus. Administration of PCE and LRF significantly reduced these pro-inflammatory cytokine, although with varying results across the two models of cognitive impairment.

In neurodegenerative diseases, reactive oxygen species (ROS) overproduction is often associated with inflammation in the brain (Lalkovičová & Danielisová, 2016). One part of the brain that is commonly affected is the hippocampus. This brain region plays a vital role in processing spatial and temporal memories, and hippocampal dysfunction is closely related to cognitive decline (Sweatt, 2004). The prefrontal cortex also plays an essential role in working memory and reward processing (Ma et al., 2023). In addition, research shows that chronic oxidative stress will decrease cell proliferation in the cerebral cortex (Banasr et al., 2007). The neuroprotective effects of *P. cubeba* in the ECS and SCO models can be attributed to its dual action of reducing oxidative stress and suppressing neuroinflammation.

P. cubeba contains various lignans, each having individual pharmacological activity (Godoy de Lima et al., 2018). In this study, a lignan-rich fraction was used to grasp the effects of all lignans in *P. cubeba*. This research showed that the effect of PCE, which contains multiple components such as flavonoids, alkaloids, steroids, and tannins, was comparable to that of LRF in terms of cognitive enhancement. As an antioxidant, PCE showed better results than LRF, while both substances showed a comparable effect as anti-inflammatories. Although PCE and LRF exhibited neuroprotective effects, PCE consistently outperformed LRF in several behavioral and biochemical assays. This difference might be attributed to the multicomponent nature of PCE, including lignans, flavonoids, and terpenoids with known antioxidant, anti-inflammatory, and cholinergic activities (Ibrahim et al., 2024). PCE also contains essential oils such as eugenol, copaene, and β-caryophyllene, which have been individually shown to exert neuroprotective (Bos et al., 2007). In contrast, LRF may lack synergistic compounds that contribute to the crude extract's full pharmacological potential.

In this study, citicoline was used as a comparator. This supplement is known to have comprehensive neuroprotective effects, which could increase neurotransmitters such as acetylcholine, dopamine, and norepinephrine, and sirtuin-1 (SIRT1) in the brain, and inhibit inflammation by blocking phospholipase A2 and ROS formation, thereby preventing neural damage. This study confirmed citicoline's antioxidant and anti-inflammatory effects on ECS and SCO-induced rats. The anti-inflammatory findings are consistent with previous studies, where citicoline only affected the brain TNF-α but not IL-1β (Bogdanov et al., 2018). Interestingly, this result was also observed with PCE and LRF. The connection between antioxidants and brain protection is also shown in other plants. For example, *Datura innoxia* aqueous extracts show an anti-amnesia effect associated with increased SOD and CAT activities in the brain (Kinda et al., 2020).

In addition to antioxidant and anti-inflammatory pathways, the cognitive enhancement observed with PCE administration may also involve modulation of the cholinergic system. A previous study confirmed the effect of cubebin on inhibiting acetylcholinesterase (AChE) activity (Somani et al., 2017), which is assumed to play a role in cognitive enhancement by *P. cubeba* in this study. This mechanism is particularly relevant in the context of scopolamine-induced cognitive impairment, which is primarily cholinergic. Therefore, the neuroprotective effects of *P. cubeba* involve not only suppression of oxidative stress and neuroinflammation but also enhancement of cholinergic neurotransmission.

The limitation of this study is that it focuses only on the two main mechanisms that cause cognitive impairment: oxidative stress and inflammation. While there may be an effect through other mechanisms, further research is needed. Given that lignans have been reported to modulate inflammatory signaling pathways particularly through the NF-κB pathway (Godoy de Lima et al., 2018), future studies should explore the involvement of this pathway in the observed neuroprotective effects of *P. cubeba*. Specifically, the expression levels of the NF-κB subunit p65, IκB degradation, and the nuclear translocation of NF-κB could be assessed to determine whether these compounds act through classical or alternative NF-κB pathways. However, the relatively small sample size, although meeting standard statistical guidelines, may limit the power to detect subtle effects

and should be considered when interpreting the findings.

Additionally, other limitations include the absence of dose-response experiments, the exclusive use of male animals (which may overlook potential gender-specific effects), and the lack of long-term outcome assessments. Addressing these aspects in future research would strengthen the translational relevance of the findings. Furthermore, expanding the analysis to include clinical biomarkers, pharmacokinetics, and bioavailability studies could facilitate the development of *P. cubeba*-derived compounds as potential therapeutic agents for neurodegenerative diseases.

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Conflict of Interest: The authors declare no conflict of interest.

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