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Original Article

Neuroprotective potential of lignan-rich fraction of *Piper cubeba* L. by improving antioxidant capacity in the rat's brain

Potencial neuroprotetor da fração rica em lignana de *Piper cubeba* L. melhorando a capacidade antioxidante no cérebro do rato

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Abstract

Piper cubeba contains various types of lignans. These compounds have been found to have potential pharmacological activities, one being a neuroprotector through an antioxidant mechanism, especially in the brain. This study examined the antioxidant activity of the lignan-rich fraction of *P. cubeba* (LF) in rat brains. The rats were given LF (200 and 400 mg/kg), Vitamin C (200 mg/kg), and a carrier as the control group for one-week p.o. The following day, rat brains were collected for antioxidant tests, including examining lipid peroxide inhibition, superoxide dismutase and catalase activity, and determination of nitric oxide (NO) concentration. The phytochemical compounds were analyzed with thin-layer chromatography (TLC), ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS), and gas chromatography-mass spectrometry (GC-MS). Test results show that the LF of both doses of 200 and 400 mg/kg could significantly increase antioxidant activity in the brain by inhibiting lipid peroxidation. LF could also increase catalase, despite the decrease in superoxide dismutase activity. Reduction in NO only occurred in the LF-200 group, while LF-400 showed insignificant results compared to the control group. In conclusion, LF showed potential as an antioxidant in the brain and could be beneficial for treating neurological diseases.

Keywords: brain, antioxidant, lignan.

Resumo

Piper cubeba contém vários tipos de lignanas. Descobriu-se que esses compostos possuem atividades farmacológicas potenciais, sendo uma delas um neuroprotetor por meio de um mecanismo antioxidante, principalmente no cérebro. Este estudo examinou a atividade antioxidante da fração rica em lignana de *P. cubeba* (LF) em cérebros de ratos. Os ratos receberam LF (200 e 400 mg/kg), Vitamina C (200 mg/kg) e um transportador como grupo de controle por uma semana p.o. No dia seguinte, os cérebros de ratos foram coletados para testes antioxidantes, incluindo o exame da inibição do peróxido lipídico, a atividade da superóxido dismutase e catalase e determinação da concentração de óxido nítrico. Os compostos fitoquímicos foram analisados por cromatografia em camada delgada (TLC), cromatografia líquida de ultra-alta eficiência-espectrometria de massas em tandem (UPLC-MS) e cromatografia gasosa-espectrometria de massas (GC-MS). Os resultados dos testes mostram que o LF de ambas as doses de 200 e 400 mg/kg pode aumentar significativamente a atividade antioxidante no cérebro, inibindo a peroxidação lipídica. O LF também pode aumentar a catalase, apesar da diminuição da atividade da superóxido dismutase. A redução do óxido nítrico ocorreu apenas no grupo LF-200, enquanto o LF-400 apresentou resultados insignificantes em relação ao grupo controle. Em conclusão, LF mostrou potencial como antioxidante no cérebro e pode ser benéfico para o tratamento de doenças neurológicas.

Palavras-chave: cérebro, antioxidante, lignana.

1. Introduction

Neuroprotective agents inhibit brain injury during or after exposure to ischemia in cellular, biochemical and metabolic processes (Ovbiagele et al., 2003), which could prevent neural cell death in conditions such as Parkinson's, traumatic brain injury, and ischemic stroke (Goenka et al., 2019). Neuroprotective agents can be derived from natural materials with multi-mechanisms. Several activities of natural substances known as neuroprotectors

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are through suppression of oxidative stress, suppressing neuroinflammation, modulating microglia polarization, regulating metabolism, and maintaining the integrity of the blood-brain barrier (Tao et al., 2020). Among the natural compounds, lignan is one of the components responsible for neuroprotective activity. Lignans are secondary metabolites of the oxidative dimerization of phenylpropanoids and have been studied for their various biological activities (Barker, 2019). Many studies have confirmed the neuroprotective effects of lignan or lignan rich-extract, such as lignans from *Eucommia ulmoides* (Han et al., 2022), lignans from *Crataegus piatifida* (Xin et al., 2022), and lignans form *Pteris laeta* (Zhang et al., 2022). In addition, lignans have been clinically proven as a potential antioxidant. For example, sesamin, honokiol, and schisandrin have been studied as antioxidants with various mechanisms, including suppressing the expression of SIRT3 (Sirtuin-3), decreasing reactive oxygen species (ROS) generation, inhibiting lipid peroxidase, and increasing the amount of glutathione (GSH) (Soleymani et al., 2020).

Studies show that *P. cubeba* fruits contain a variety of lignans, such as yatein, hinokinin, and cubebin (Elfahmi et al., 2007). Cubebin is one of the active lignans of *P.cubeba* that has been studied as neuroprotective in animal models of dementia (Sathaye et al., 2017). Cubebin has also been studied as an anti-inflammatory (Godoy de Lima et al., 2018; Silva et al., 2005) and antibacterial (Rezende et al., 2016). Other *P.cubeba's* major lignan, hinokinin, also possesses potent antioxidant and anti-inflammatory activities (Lima et al., 2017). Several studies have shown the effect of *P. cubeba* fruits as an anti-inflammatory in various animal models (Choi and Hwang, 2003, 2005; Godoy de Lima et al., 2018; Perazzo et al., 2013; Souza et al., 2004). The anti-inflammatory mechanism of this plant has been demonstrated in vitro through the inhibition of COX-1, COX-2, and 5-LOX. (Yam et al., 2008), also through inhibition of NF-κB, TNF-α and IL-1β (Deme et al., 2019; Qomaladewi et al., 2019). Empirically, people have also used *P. cubeba* seeds to relieve headaches (Abolhasanzadeh et al., 2017). Other than anti-inflammation, *P. cubeba* also exhibits a strong antioxidant effect (Alminderej et al., 2020; Andriana et al., 2019). Moreover, the ethanol extract from the fruits has also been studied to prevent lipofuscinogenesis, which plays a role in dementia and Alzheimer's disease (Muchandi and Dhawale, 2018). Based on existing studies, *P.cubeba* showed potential as a neuroprotective agent due to the presence of lignans in this plant. However, the exploration of lignan-rich *P.cubeba* for this activity is limited. Therefore, this study examined the antioxidant potential of the lignan-rich fraction of *P. cubeba* in rat brains as a candidate for neurodegenerative disease therapy.

2. Material and Methods

2.1. Plant material and lignan-rich fraction preparation

P. cubeba fruits used were from Balai Penelitian Tanaman Rempah dan Obat (Balitro), Bogor, Indonesia. A kilogram of dry *P. cubeba* fruit powder was extracted with 80% methanol (1:10) under sonification for one hour. The extract was then evaporated, and the remaining liquid extract was fractionated using a separating funnel with dichloromethane (CH_2Cl_2) in a 1:1 ratio. Finally, the dichloromethane layer was separated and evaporated into a thick extract (Elfahmi et al., 2007).

2.2. Total phenolic determination

The LF solution (1000 ppm in methanol) and gallic acid solution (as standard) were added with 10% Folin-Ciocalteu reagent (1:10). Then, 4 mL of 1 M sodium carbonate was added and incubated for 15 minutes at room temperature. The samples were read at 765 nm.

2.3. Total flavonoid examinations

First, 0.5 mL of 1000 ppm LF and quercetin solution (as standard) in methanol was added to 1.5 mL of methanol. Then, 2.8 mL of distilled water was added, followed by 0.1 mL of 10% aluminum chloride ($AlCl_{3)}$. Finally, 0.1 mL of 1 M sodium acetate was added to the mixtures, incubated for 30 minutes at room temperature, and measured at 415 nm.

2.4. TLC analysis

TLC analysis was performed using silica gel 60 F_{254} as the stationary phase and toluene: acetone (7:3) as the mobile phase. First, the chamber was saturated with mobile phase, and then the LF was spotted in the silica gel and developed. The plate was then sprayed with 10% sulfuric acid and heated to develop colors. Cubebin (ChemFaces, Wuhan) was used as a comparator.

2.5. UPLC-MS conditions

The system consists of an autosampler and a binary pump (Waters, Milford, MA) equipped with a 10 µL loop. The compounds were separated on an Acquity BEH C18 (2.1 mm × 50 mm, 1.7 µm. Waters, Milford, MA) analytical column at 40 °C. A gradient elution was achieved using two solvents: 0.1% (v/v) formic acid water (A) and 0.1% (v/v) formic acid acetonitrile (B) at a flow rate of 0.3 mL/min. The gradient program consisted of 10% to 20% B over 2 min, 40% B over 5 min, and then linear from 40% to 60% B over 2 min, followed by an increase of 100% B over 6 min, maintained for 2 min, then return to the initial condition over 5 min with a sample injection volume of 5 µL. The UPLC system was the operating conditions for the electrospray ionization source interface (ESI) coupled with continuous switching polarity between positive and negative modes. For full scan ESI–MS analysis, the spectra covered the range from m/z 150 to 600. Analyst software package (MassLynx) used for instrument control and data acquisition.

2.6. GC-MS analysis

The instrument used was Agilent Technologies 7890 Gas Chromatograph with autosampler, 5975 mass selective detector, and chemstation data system. The experiment used the HP INNOWAX column. The initial temperature was 60 °C for 0 minutes, then 2 °C/min to 150 °C for 1 minute,

and finally 20 °C/min to 210 °C for 10 minutes. The injection temperature was 250 °C, the ion source temperature was 230 °C, the interface temperature was 280 °C, and the quadrupole temperature was 140 °C. The carrier gas was Helium, with a column flow of 0.6 mL/min and an injection volume of 1 mL.

2.7. Animal groups and treatments

This study used 2-3 months old Wistar rats (175-200 g), and all animals were acclimatized in a cage at 23 ± 2 °C for one week. The protocol was approved by the Health Research Ethics Commission of Muhammadiyah University, Prof. DR HAMKA (KEPK-UHAMKA), Jakarta, No: 03 / 21.08 / 02172. A total of 20 rats were divided into four groups: LF 200 and 400 mg/kg, Vitamin C (Vit-C) 200 mg/kg, and normal control (N; given carrier). The test substances were given orally for a week, and the brains were collected the following day. During the experiment, the animals were given standard feed and drink.

2.8. Brain homogenate preparation

The brain homogenate (10%) was prepared in a mixture of phosphate-buffered saline (PBS) at pH 7.5 and phenylmethylsulphonyl fluoride (PMSF) 0,1 M (MedChemExpress, USA). The brain samples were then homogenized on an ice bath, centrifuged for 20 minutes at 4 °C, 4000 x g, and stored at -20 °C. The protein content of the homogenate was determined using Bradford's reagent and measured at 595 nm.

2.9. SOD activity test

A total of 20 µL of diluted samples were used to determine total SOD activity using the SOD assay kit (Elabscience, USA). The procedures were completed following the kit instructions, where 50% SOD inhibition is equivalent to 1 SOD activity unit (U).

2.10. CAT activity test

The procedure to determine CAT activity followed the method outlined by Hadwan (2018) with slight modifications. First, 100 µL of the sample was added with 200 µL of H_2O_2, homogenized, and incubated for 2 minutes at 37 °C. The mixture was then added with 1.2 mL working solution, consisting of 10 mL cobalt (II) solution (Pudak scientific, Indonesia), 10 mL sodium hexametaphosphate solution (Loba Chemie PVT.LTD, India), and 180 mL sodium bicarbonate solution (Pudak scientific, Indonesia). The mixture was then homogenized and left in the dark at room temperature for 10 minutes. The absorbance was measured at 440 nm.

2.11. NO determination

NO is an unstable compound and difficult to determine directly. Therefore, the standard $NaNO_2$ was used in this study because it can represent the actual NO levels (Termin et al., 1992). The procedure was carried out with a slight modification. First, 200 µL of the sample was added to 200 µL of 2N $KMNO_4$. After 30 minutes, 800 µL of 5% (v/v) salicylic acid in H_2SO_4 (Mallinckrodt, Ireland) was added and allowed to stand for 20 minutes. Next, 8 N NaOH (Merck, Germany) was added to 10 mL and filtered. The absorbances were measured at 414 nm.

2.12. Lipid peroxidation test

A total of 250 µL samples and TEP (Merck, Germany) solution (as standard) were added to 250 µL of 20% TCA (Merck, Germany), followed by 0.5 mL 0,67% TBA (Merck, Germany). The mixtures were heated to 95-100 °C for 15 minutes until the color changed and then cooled and centrifuged. The absorbance of the supernatant was read at 532 nm.

2.13. Statistical analysis

The data were analyzed for normality and homogeneity and continued with the Analysis of Variance (ANOVA) test.

3. Results

3.1. Phytochemical profile

The TLC analysis of LF showed separated colored bands corresponded to lignans, one of which was cubebin with Rf 0.4 (Figure 1). The UPLC-MS analysis showed the presence of compounds presumed to be cubebin, hinokinin, and yatein (Figure 2), and these results were confirmed by GC-MS analysis, where hinokinin showed the highest area percentage (Table 1). The LF yield was 5.35%, and the phytochemical screening also showed the presence of flavonoids, tannins, quinones, steroids/triterpenoids, and alkaloids. The phenol content of LF was 134.62±2.83 (mg GAE/g extract), while the flavonoid content was 152.58±15.38 (mg QE/ g extract).

3.2. Antioxidant activity of LF on rats' brain

Figure 3 showed that LF inhibits lipid peroxidation, shown by lower MDA levels comparable to Vit-C, and

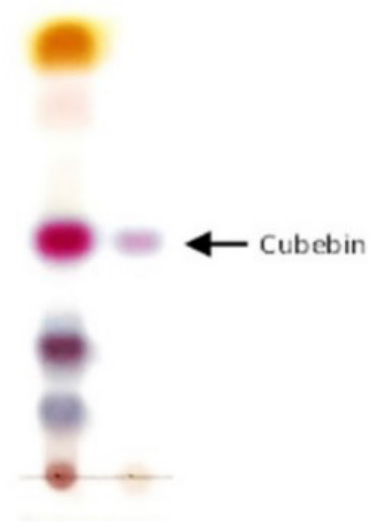

Figure 1. TLC analysis result of LF showed the presence of cubebin and other lignans.

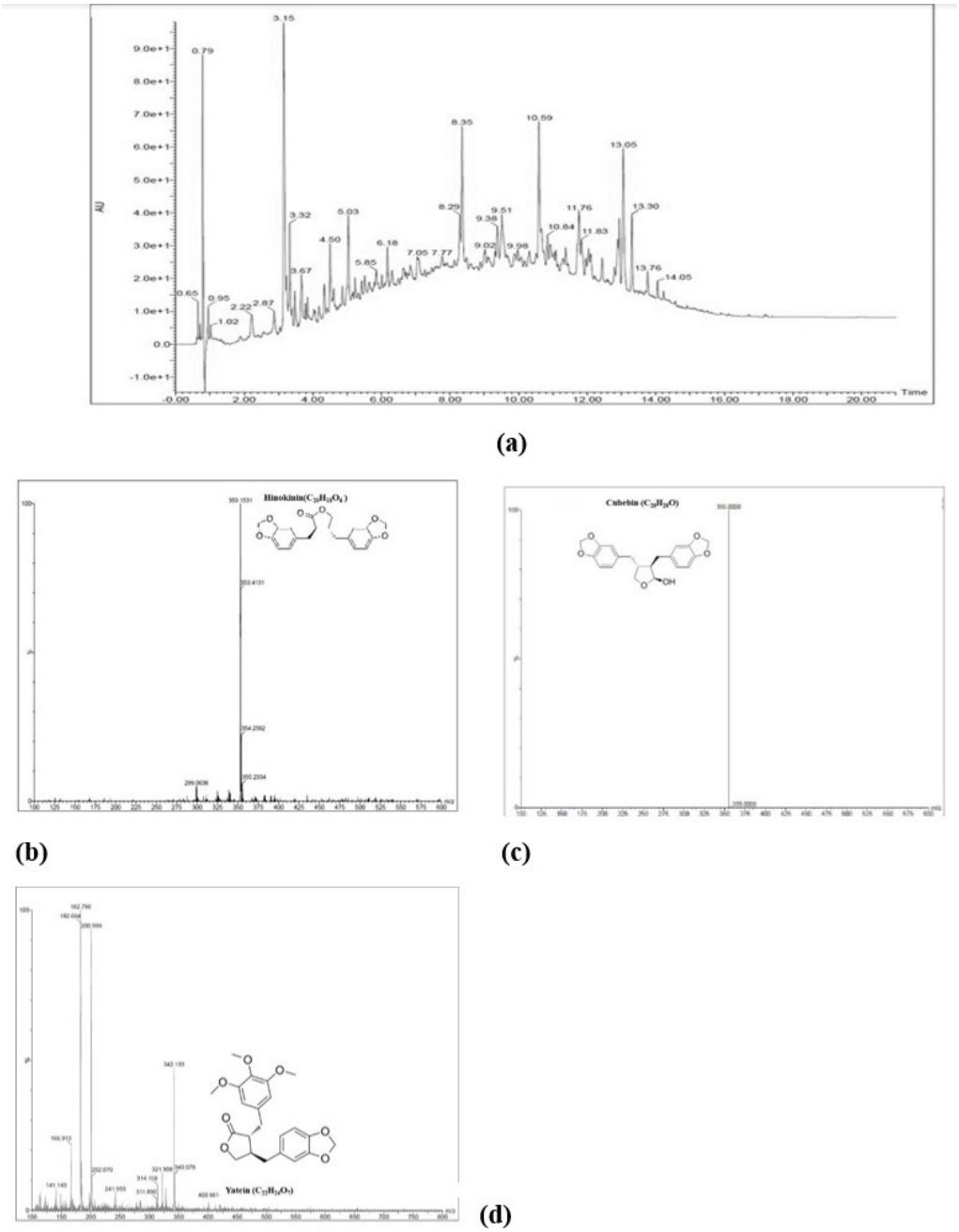

Figure 2. LF Chromatogram (a) and the mass spectrum of presumed (b) Hinokinin, (c) Cubebin, (d) Yatein by UPLC-MS.

Table 1. Lignans in LF by GC-MS analysis.

Molecular formula	Molecular weight	Name of compound	Retention time	Area (%)
$C_{21}H_{20}O_7$	384.2	5-Methoxyhinokinin	38.9	6.12
$C_{20}H_{20}O_6$	356.2	Cubebin	39.7	3.93
$C_{20}H_{18}O_6$	354.3	Hinokinin	41.4	11.4
$C_{23}H_{26}O_8$	430.3	Cubebinone	41.6	2.73
$C_{22}H_{26}O_7$	402.2	Clusin	43	1.52
$C_{27}H_{28}O_3$	400.2	Yatein	44.2	3.49

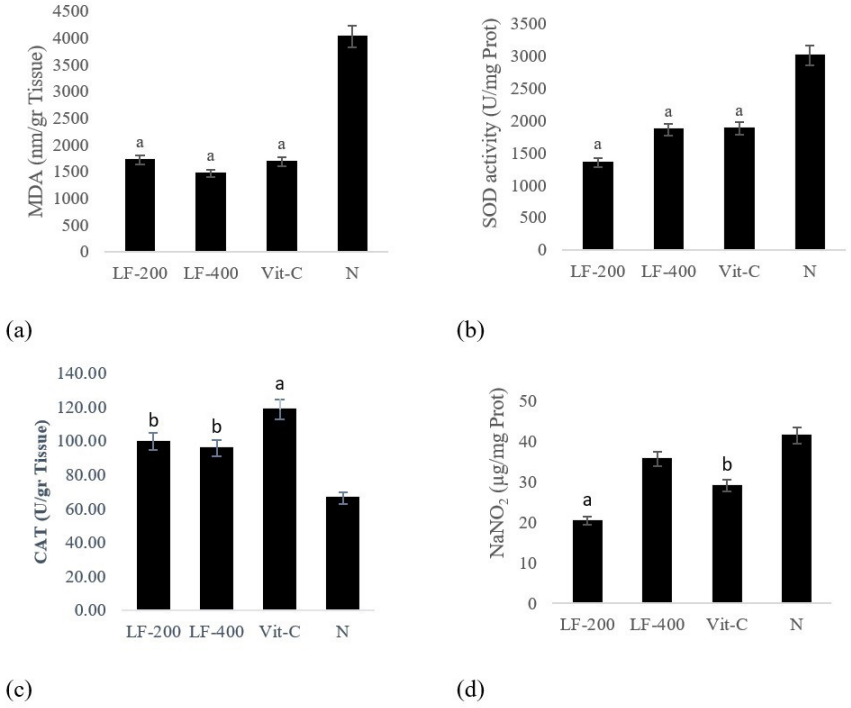

Figure 3. Antioxidant activities on rat's brain based on activities of lipid peroxidation inhibition (a), SOD (b) and CAT (c) activities, and NO concentration (d) of lignan-rich fraction of *P.cubeba* 200 mg/kg (LF-200), 400 mg/kg (LF-400), Vitamin C 200 mg/kg (Vit-C), and normal control (N). The results are present as the mean ± SEM, [a]significantly different compared to the N group ($p<0.01$), [b]significantly different compared to the N group ($p<0.05$).

there was no significant difference between the lower and higher dose of LF. The linear result was shown in CAT activity, where both LF doses could increase this antioxidant enzyme significantly compared to N control ($p<0.05$), despite decreased SOD activity. Finally, LF could inhibit NO production, especially at 200 mg/kg ($p<0.01$).

4. Discussion

The central nervous system is very susceptible to oxidative stress due to high lipids, low enzymatic antioxidants, and high oxygen consumption (Prasad et al., 2018). Oxidative stress plays a significant role in neurological impairment due to the vulnerability of the nerve systems to reactive oxygen species (ROS). Under normal conditions, the level of lipid metabolism in the brain is the highest in the body, and one of the primary producers of ROS in resting brain cells is the high metabolism in mitochondria (Angelova et al., 2021), which could lead to cell death through apoptosis or necrosis (Desai et al., 2014). The imbalance of antioxidants and oxidative stress in the brain could further lead to various degenerative diseases (Lee et al., 2020).

The current study showed that LF significantly suppressed lipid peroxidation, increased CAT activity, and reduced NO levels in rat brains, showing LF's potential to develop as a neuroprotective agent. ROS peroxidase polyunsaturated fatty acid (PUFA) generates MDA, which becomes the marker of lipid peroxidation (Tsikas, 2017), and the current study revealed that LF significantly suppressed lipid peroxidation as indicated by lower MDA levels than the control. Both doses of LF inhibited lipid peroxidation comparable to Vit-C, and there was no difference between the two doses of LF.

In addition to lipid peroxidase, this study evaluated SOD and CAT activity. SOD works as an enzymatic antioxidant by metabolizing superoxide to hydrogen peroxide (H_2O_2). Simultaneously, catalase breaks down H_2O_2 into water and oxygen, protecting the cells against H_2O_2 damage (Hopkins and Li, 2020). These two enzymes could work synergistically to produce antioxidant effects. In this study, LF in both doses increased CAT activity significantly compared to N but decreased the SOD activity. The same trend was found in the Vit-C group, possibly due to the inhibition of the SOD expression (Kao et al., 2003).

In addition, LF could suppress NO production. NO is one of the prooxidative species, which is known as a toxic gas with a dual identity (neuroprotector and neurotoxin). In ischemic conditions, NO in the brain is synthesized mainly by 3 NO synthases (NOS) subtypes, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Both nNOS and iNOS could cause nerve damage, while eNOS produces a neuroprotective effect. Further, NO produced by nNOS shortly after reperfusion causes nitrosative stress and increases apoptosis through the downregulation of caspase-3 (Chen et al., 2017). A decrease

in NO concentration indicates low oxidative stress. In this study, LF-400 did not affect the NO level, as shown by a comparable result to N, while LF-200 caused a significant decrease in NO production compared to N, even better than Vit-C. However, further research needs to be done to determine which subtype of NOS is affected by LF.

The TLC analysis of LF confirmed the presence of lignans, which showed that separated colored bands corresponded to lignans. TLC results also confirm that LF contained cubebin, one of the active compounds and the marker of *P. cubeba*. UPLC-MS analysis showed that LF contains cubebin, hinokinin, and yatein, which GC-MS confirmed. GC-MS analysis showed that LF contains 5-methoxyhinokinin, cubebin, hinokinin, cubebinone, clusin, and yatein. These lignans have various pharmacological activities and have been studied to reduce the risk of various chronic diseases. Cubebin and hinokinin have shown anti-inflammatory, antibacterial, and antitumor effects in various studies (Godoy de Lima et al., 2018). Previous research also showed that the dichloromethane fraction of *P. nigrum* and *P. longum,* other piper species containing piperine, piperglin, and hinokinin, had a significant neuroprotective effect through antioxidant activity (Hua et al., 2019; Wang et al., 2017). Besides lignans, flavonoids and other phenol are also responsible for the antioxidant activity in plants (Tungmunnithum et al., 2018), and both contents were detected in high amounts in LF. The results of this study indicate that the lignan-rich fraction of *P. cubeba* has antioxidant potential in the brain. Since oxidative stress is critical in aggravating neurological disorders, the lignan-rich fraction of *P.cubeba* could benefit these conditions.

5. Conclusion

The lignan-rich fraction of *P. cubeba* revealed an antioxidant property in the brain by inhibiting lipid peroxidase, increasing catalase, and inhibiting NO production, especially at 200 mg/kg. Thus, this fraction has the potential to be further developed for research on neurodegenerative diseases.

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