ORIGINAL ARTICLE

Phytochemical, *in vitro* radical scavenging and *in vivo* oxidative stress analysis of peppermint (*Mentha piperita* L.) leaves extract

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ABSTRACT

This current work aims to determine phytochemicals, *in vitro* radical scavenging, and *in vivo* oxidative stress reduction activities of peppermint (*Mentha piperita* L.) ethanolic extract (PEE). The Clule method was used to determine the phytochemical content. An *in vitro* antioxidant with radical scavenging activity was measured using 2,2-diphenyl-1-picrylhydrazyl. An *in vivo* antioxidant with oxidative stress reduction was carried out for 10 days on 25 male Sprague–Dawley rats (divided into five groups). Every day, each group was given positive control, negative control, 5, 10, and 20 mg/200 gr of body weight (BW) of the extract. The blood plasma was taken for malondialdehyde analysis. A phytochemical identification of PEE revealed more compounds, such as flavonoids, alkaloids, steroids, essential oils, and tannin. PEE exhibits significant *in vitro* radical scavenging activity, with an IC_{50} value of 126.695 μg/mL. In the *in vivo* antioxidant with oxidative stress reduction experiments, 5 mg/200 gr BW was the most effective dose, as evidenced by a considerable drop in malondialdehyde level (0.312 nmol/mL) after and before treatment. In conclusion, PPE has the potential to be developed as a herbal antioxidant based on *in vitro* and *in vivo* test results.

Key words: Antioxidant, fifty percent inhibition concentration, malondialdehyde level, peppermint (Mentha piperita L.) extract, phytochemical

INTRODUCTION

Degenerative diseases are still the most prevalent cause of death in Indonesia. In 2014, the World Health Organization (WHO) mentioned that the total deaths in Indonesia were 1.551.000 and that about 71% of them were because of degenerative diseases or noncommunicable diseases (NCDs). The most considerable percentage of NCDs was cardiovascular diseases with 37%, higher than other communicable, maternal, perinatal, and nutritional conditions with 22%. The rest of the causes are cancers, other NCDs, diabetes, and chronic respiratory diseases, with a percentage of 13%, 10%, 6%, and 5%, respectively.[1]

The main problems in degenerative diseases are damaged healthy cells in our bodies, caused by free radicals. These damaged cells make them unable to work correctly as their function and structure. These free radicals originate from reactive oxygen species (ROS), reactive oxygen atoms with an unstable electrical charge or

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those with an unpaired electron, thus finding another electron but making them unstable. When ROS attacks the normal functional cells, causing them to be damaged, this condition is known as oxidative stress. Oxidative stress is a shift in the balance between prooxidants and antioxidants inside our bodies.[2]

Consuming antioxidants to counteract pro-oxidants can be gained by endogenous antioxidants and exogenous antioxidants (dietary antioxidants).[3] One of the most considerable resources for those antioxidants are plants, among which peppermint (*Mentha piperita* L.) (PEE) can be gained quickly in Indonesia. Therefore, research is needed to prove the antioxidant effect of peppermint leaves in Indonesia.

The antioxidant substances are polyphenols and volatile phenolic oils, without including the *in vivo* assay on animal models.[4] Thus, it raises a question about whether the antioxidant substances present in peppermint can neutralize free radicals based on the *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and compensate for the *in vivo* oxidative stress using Sprague–Dawley rats at its most effective dosage. It is hypothesized that peppermint has an exogenous antioxidant effect on *in vitro* free radicals (IC_{50} value) and *in vivo* oxidative stress (malondialdehyde level).

The present study examines the effect of peppermint leaves as an herbal antioxidant and how effective it is *in vivo* and *in vitro*. This research is expected to guide and reference future clinical practice regarding the health problems caused by free radicals.

MATERIALS AND METHODS

Preparation of peppermint (*mentha piperita* L.) ethanol extract

The leave of peppermint sample was achieved from Balai Penelitian Tanaman Rempah dan Obat (Balittro) with specimen voucher No. 056/BPTRO-RR/FK-UI/VIII/2019, Bogor, West Java, Indonesia, in August 2019. The sample specimen was authenticated and identified at the Indonesian Institute of Sciences – Cibinong, West Java, Indonesia. The extraction method used maceration at room temperature using 70% ethanol. The extract solution was evaporated to obtain a thick extract (50 rpm, 30°C–40°C).[5]

Phytochemical identification

A phytochemical identification was conducted to identify secondary metabolites (mainly the antioxidant compound) in PEE.[6] The aforementioned concentrated extract was separated using three polarity solvents (n-hexane, ethyl acetate, and 70% ethanol). The qualitative phytochemical constituent analysis was used in the standard procedures described by Ambarwati *et al*.[7]

In vitro activity assay of 2,2-diphenyl-1-picrylhydrazyl radical scavenging

The DPPH radical scavenging test is one of the most widely utilized techniques to assess the antioxidant activity of certain substances. The *in vitro* test is based on the Yen and Chen method[8] to measure the IC_{50}. Then, to calculate the IC_{50}, linear regression analysis should be done by converting the result of inhibition percentage into a linear equation. As a result, the formula (y = ax + b) is formed with the y-axis as the inhibition percentage and the x-axis as the extract concentration.[9] We used quercetin as a positive control.

In vivo malondialdehyde level-oxidative stress reduction activity assay

This study is an analytical experimental approved (the ethical approval number is 0070/UN2. F1/ETIK/2018) by the ethical committee of the Faculty of Medicine, Universitas Indonesia. This study employed 25 mature male Sprague-Dawley rats (weighing 180–200 g). Some different dosages of extract were used, including 5, 10, and 20 mg/200 g BW/day, Vitamin C as a positive control (2 mg/200 g BW/day). They were given for 10 days after the first blood puncture. A blood sample and a malondialdehyde test (500 µL) were taken with a capillary pipe in the sinus orbitalis canthus and placed in a tube with heparin at two moments, before and after the rat was given both physical exercise and PEE. The blood samples were centrifuged to get the plasma. The clear filtrate at the top, the plasma, was transferred to another tube and kept at-20°C.

Malondialdehyde determination was performed based on the literature.[10] Firstly, 250 µL blood plasma was mixed with 100 µL of 8.1% sodium dodecyl, 750 µL of thiobarbiturates (20 M), and 750 µL of HCl (0,5 M), and 125 µL of aquabidest. Then, it was mixed using a vortex. After that, the mixture was heated at a temperature of 90°C for 15 min. Then, it settled and cooled down for 10 min. The next step was to add 500 µL aquabidest and 2.5 mL n-butanol to the mixture, vortex it, and then centrifugated at 3000 rpm. Finally, the filtrate was placed in a fluorometer, and the absorbance at 520 nm was measured. To compute the malondialdehyde level, 1,1,3,3-tetra-ethoxy-propane (with some concentration of 5, 10, and 20 nmol/mL) was employed in conjunction with the malondialdehyde standard curve (linear equation) by comparing malondialdehyde concentration in the y-axis with absorbance in the x-axis.[10]

Data analysis and processing plan

The *in vitro* (IC_{50} value) and *in vivo* antioxidant activity experiments were evaluated using linear regression analysis with IBM SPSS statistic 24 software (IBM Corp., USA). A paired *t*-test and a one-way ANOVA test were both performed. To compare the Malondialdehyde levels after and before the physical and antioxidant activity, the paired *t*-test was utilized. The one-way ANOVA test was performed to examine the differences and efficiency of the PEE and Vitamin C as positive controls. The Shapiro-Wilk test was

used to determine a normal data distribution. The *post hoc* Bonferroni test was used for homogeneity of variances.

RESULTS

Phytochemical identification

The phytochemical identification result of fractions from PEE was demonstrated in Table 1.

In vitro 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of PEE

As shown in Table 2, the higher the concentration of quercetin and the sample, the better the activity exerted. The linear regression equation for quercetin was y = 1.4331x + 29.686; R^2 = 0.99165. While the R^2 value was used to determine an adequate correlation between quercetin concentration and DPPH inhibition. The IC_{50} value was calculated by inserting 50 as the value of "y," resulting in 14.17 µg/mL of quercetin concentration needed to inhibit 50% of the DPPH radical. Moreover, antioxidant activity of PEE was calculated with the equation as follows y = 0.3983× +0.4567; R^2 = 0.99834. After calculating, the IC_{50} of PEE was 126.695 µg/mL.

In vivo malondialdehyde level-oxidative stress reduction activity of PEE

The difference in malondialdehyde level was indicated in Table 3, where all groups, including the positive control, saw a substantial drop in malondialdehyde level following treatment.

Table 4 shows the outcome of the paired *t*-test, which indicated the relationship between malondialdehyde levels before and after therapy. The $P < 0.05$ suggests that there is a statistically significant difference between at least two data groups.

The one-way ANOVA test [Table 5] was employed to determine a statistically significant amount of variation of two or more unconnected elements. It also determines the most effective PEE dose for reducing oxidative stress.

The following test was the *post hoc* test with Bonferroni Correction [in Table 6], where each component was compared to other components in the same group. There were several significant comparison values ($P < 0.05$) between components in the group, including rats given 5 mg of extract and a positive control group with 0.029, meaning that it was more significant when applying 5 mg rather than the positive control. PEE (5 mg) with a negative control group with 0.006, meaning that it was more significant when applying 5 mg rather than the negative control.

DISCUSSION

Phytochemical identification was made by fractionating the extract with ethanol, ethyl acetate, and n-hexane through several chemical reactions to determine the content of PEE as an antioxidant. Most of the antioxidant compounds, saponin, alkaloid, flavonoid, essential oil, tannin, and triterpenoid/steroid, are present in the ethanol fraction due to the polar solvent. However, because hexane was a nonpolar solvent, it did not contain any phytochemical compounds. In contrast, ethyl acetate was a semi-polar solvent that contained saponin, alkaloids, flavonoids, essential oils, and tannin.

Previous studies about the phytochemical profile of peppermint have been conducted by Ji *et al.*, who stated that the major constituents of the antioxidant substance were essential oil and terpenoid.[11] Based on a study conducted

Table 1: The phytochemical identification of some fraction from peppermint (*Mentha piperita* L.) ethanolic extract

Secondary metabolite	n-Hexane fraction	Ethyl acetate fraction	Ethanol fraction
Tannin	–	+	+
Saponin	–	+	–
Terpenoid/steroid	–	–	+
Alkaloid	–	+	+
Glycosides	–	–	–
Flavonoid	–	+	+
Essential oil	–	+	+

+: Positive, -: Negative

Table 2: IC_{50} value of peppermint (*Mentha piperita* L.) ethanolic extract, based on 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

Sample	Concentratio (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Quercetin	1	4.72	14.17
	5	39.23	
	10	73.68	
	50	96.70	
PEE	10	2.85	126.695
	50	18.99	
	100	41.36	
	200	78.36	

DPPH: 2,2-diphenyl-1-picrylhydrazyl, PPE: Peppermint (*Mentha piperita* L.) ethanolic extract, IC_{50}: fifty percentage inhibition concentration

Table 3: Malondialdehyde level

Groups	Before treatment (nmol/mL)	After treatment (nmol/mL)	Differences (nmol/mL)
PEE dose of 5 mg	1.228	0.735	−0.493
PEE dose 10 mg	1.272	0.874	−0.398
PEE dose 20 mg	1.012	0.772	−0.241
Negative control	0.823	0.974	+0.151
Positive control	0.889	0.678	−0.210

PPE: Peppermint (*Mentha piperita* L.) ethanolic extract

Table 4: The paired *t*-test result of malondialdehyde level

Malondialdehyde level of groups	Total subjects	Mean (nmol/mL)±SD	Mean difference (nmol/mL)±SD	95% CI of the difference	*P*
Before treatment	25	1.075±0.182	0.299±0.177	0.226–0.372	0.000
After treatment	25	0.776±0.101			

SD: Standard deviation, CI: Confidence interval

Table 5: The one-way ANOVA result of malondialdehyde level

Group (total subject)	Mean (nmol/mL)±SD	*P*
PEE dose of 5 mg (5)	0.493±0.072	0.003
PEE dose of 10 mg (5)	0.398±0.119	
PEE dose of 20 mg (5)	0.241±0.134	
Positive control (5)	0.210±0.416	
Negative control (5)	0.151±0.219	

SD: Standard deviation, PPE: Peppermint (*Mentha piperita* L.) ethanolic extract

Table 6: The *post hoc* test result

Groups 1	Groups 2	Significance
PEE dose of 20 mg	PEE dose of 10 mg	0.739
	PEE dose of 5 mg	0.067
	Positive control	1.000
	Negative control	1.000
PEE dose of 10 mg	PEE dose of 20 mg	0.739
	PEE dose of 5 mg	1.000
	Positive control	0.358
	Negative control	0.077
PEE dose of 5 mg	PEE dose of 20 mg	0.067
	PEE dose of 10 mg	1.000
	Positive control	0.029
	Negative control	0.006
Positive control	PEE dose of 20 mg	1.000
	PEE dose of 10 mg	0.358
	PEE dose of 5 mg	0.029
	Negative control	1.000
Negative control	PEE dose of 20 mg	1.000
	PEE dose of 10 mg	0.077
	PEE dose of 5 mg	0.006
	Positive control	1.000

PPE: Peppermint (*Mentha piperita* L.) ethanolic extract

by Lisawati and Sulianti (2002), the main antioxidant constituents are flavonoids and essential oils.[12] The other study by Thantsin *et al*. showed that flavonoids, terpenoids, and essential oils are the major antioxidant substances in peppermint.[13] According to the aforementioned references, the most probable antioxidant substances that exert antioxidant activity are flavonoids, essential oils, and terpenoids.

In order to obtain the data, the antioxidant activity of PEE was compared with quercetin (positive control). Then, DPPH radical scavenging was given to evaluate the IC_{50} value, the extract concentration needed to decrease the oxidative stress level by 50%. A small value of the IC_{50} level indicates intense antioxidant activity.[14,15,16] Based on the comparison of antioxidant activity, antioxidant intensity was categorised such as strong (IC_{50} <50 μg/mL), active (IC_{50} 50–100 μg/mL), moderate (IC_{50} 101–250 μg/mL), weak (IC_{50} 251–500 μg/mL), and inactive (IC_{50} >500 μg/mL).[17,18] From the result mentioned above, the IC_{50} value of PEE is categorized as moderate antioxidant intensity.

Malondialdehyde was defined as the lipid peroxidation product in the long chain of unsaturated fatty acids in the lipid membrane. Lipid peroxidation happens when the oxidative stress level increases. Thus, this indicator was used to determine the antioxidant activity, which, in this case, was PEE, to decrease the oxidative stress level.[19] The paired *t*-test was used to assess the comparison. The $P = 0.005$, which was <0.05, showing a significant change in malondialdehyde levels before and after treatment.[20]

CONCLUSION

The phytochemical compounds of PEE were saponin, flavonoids, alkaloids, terpenoids, essential oils, and tannin. The effect of PEE in compensating for oxidative stress was evident *in vitro*, as shown by the IC_{50} value of 126.695 μg/mL, which is categorized as moderate antioxidant intensity. By comparing the extract concentration with the malondialdehyde level differences before and after treatment, the most effective dosage was 5 mg/200 gr BW, which decreased the malondialdehyde level by 0.492 nmol/mL.

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Conflicts of interest

There are no conflicts of interest.

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