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Research Article

Phytochemicals and Antioxidant Activities Evaluation of *Origanum vulgare* (L.) Stem Bark Extracts

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ABSTRACT

The present study aimed to evaluate phytochemical and antioxidant activity (*in vitro* and *in vivo*) of *Origanum vulgare* (L.) ethanolic extract. The phytochemical test was assessed using the Clule method in ethanol, ethyl acetate, and hexane. *In vitro* evaluation of antioxidant activity was determined by radical scavenging assay using DPPH (2,2-diphenyl-1-picrylhydrazyl) as an artificial free radical activity. *In vivo* test was conducted to evaluate the effect of malondialdehyde (MDA) level in blood plasma during maximum physical activity treatment. *In vivo* test was done using 25 male Sprague Dawley rats in pre and post-test control group design. The phytochemical test of *O. vulgare* ethanol extract was showed some compounds, such as a flavonoid, alkaloid, triterpenoid/steroid, essential oil, and tannin, then in ethyl acetate and hexane. *In vitro* assay showed that *O. vulgare* extract has strong antioxidant activity with an IC_{50} value of 133.47 µg/mL. While in the *in vivo* test, the most effective dosage is 20 mg/200 gr B.W., represented by a significant decrease of MDA level (0.509 nmol/mL) before and after treatment. So, the ethanolic extract of clove has potency as an herbal antioxidant because of the low level of IC_{50} and can decrease the MDA level.

Key Words: 2,2-diphenyl-1-picrylhydrazyl, Antioxidant activity, Malondialdehyde, *Origanum vulgare* (L.), Phytochemical.

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INTRODUCTION

The most common cause of death in Indonesia is a degenerative disease.[1] The three main causes of death in Indonesia in 2016 were ischemic heart disease, cerebrovascular disease, and diabetes.[2] Degenerative diseases can occur due to damage to cells in the body by free radicals.[3] Free radicals are reactive oxygen atoms because the electrons are unpaired, also called reactive oxygen species (ROS).[4] These free radical compounds are unstable, very reactive, and try to stabilize themselves in attempting to take electrons from other compounds (including proteins, carbohydrates, lipids, and DNA).[5] ROS attacks normal cells in the body and causes cells to become damaged, called oxidative stress.[6] Oxidative stress is a condition of imbalance between prooxidants and antioxidants in our bodies.[7]

Oxidative stress conditions can be overcome by consuming antioxidants to balance prooxidants.[8] Oxidative stress can cause more free radicals; cell damage occurs, cells become susceptible to infection, degenerative diseases, DNA cell division is disturbed, causing cell mutations.[5] This oxidative damage can accompany most or even cancers, diabetes, heart disease, arthritis, neurodegenerative disorders, atherosclerosis, osteoporosis, pancreatitis, special health for women, and pre-eclampsia.[5] We know that free radicals are produced continuously during normal respiration and metabolism, triggered by exposure to air pollution, radiation from X-rays, exposure to sunlight, drugs, viruses, bacteria, parasites, dietary fat, stress, and injury.[5]

Antioxidant compounds can prevent oxidation of other compounds by oxidizing themselves, thus preventing cell damage.[5] Therefore, antioxidants play a role in preventing diseases such as cancer, heart disease, dementia, diabetes, and stroke.[5,9] The most significant source of antioxidants in plants is *Origanum vulgare* (L.), which grows well in Indonesia. This study was conducted to test the antioxidants contained in *O. vulgare* and the ability to neutralize free radicals in vitro (with the DPPH test) and *in vivo* using Sprague Dawley mice. This study hypothesizes that *O. vulgare* has exogenous antioxidant effects on free radicals, both *in vitro* through IC_{50} and *in vivo* values through the MDA level.

This study aimed to provide knowledge to Indonesian people about the potential of *O. vulgare* as one of the herbal antioxidants, which is useful for treating oxidative stress because it is widely used in daily life.

MATERIALS AND METHODS

The activity test of *O. vulgare* extract *in vivo* was carried out with the Faculty of Medicine's ethics committee's approval, the University of Indonesia. The ethical license number is 0071/UN2.F1/ETIK/2018. The making of *O. vulgare* extract and IC_{50} determination were carried out in the Department of Chemical Medicine, Faculty of Medicine, University of Indonesia. The phytochemical analysis and measurement of Malondialdehyde (MDA) levels from the *in vivo* assay were carried out in the Department of Biochemistry, Faculty of Medicine, University of Indonesia. Handling and treating animal models were carried out and followed the Laboratory of Animal Experiments and Toxicology of the Ministry of Health of the Republic of Indonesia.

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Preparation of *O. vulgare* stem bark extract

The stem bark of *O. vulgare* was taken from the Research Institute for Spice and Medicinal Plants in Bogor, West Java. The bark was first cleaned and dried at room temperature for 24 hours, then smoothed using a blender.

Manufacture of *O. vulgare* extract

Making *O. vulgare* extract using bark powder (500 grams) with maceration method and solvent used 70% ethanol (1000 ml). After three days, the solution is filtered, and the filtrate is collected. Maceration is repeated until the filtrate is colorless. The collected filtrate was evaporated using a rotary evaporator (50 rpm, 30-40°C). The concentrated filtrate was then dried using an oven at 40°C. The extract was stored at 4°C.[10]

Phytochemical analysis

Phytochemical analysis was carried out to determine the compounds contained in the extract of *O. vulgare*. Before phytochemical testing, the extract was dissolved with three different solvents, namely 70% ethanol (polar), ethyl acetate (semi-polar), and hexane (non-polar). Phytochemical tests include the saponin test, flavonoid test, terpenoid, steroid test, alkaloid test, tannin test, and glycoside test.[11]

In vitro test

In vitro antioxidant test to find 50% inhibition concentration (IC_{50}) by dissolving the extracted sample in methanol added 0.5 mL of 100 µg/ml 2.2-diphenyl-1picrylhydrazyl (DPPH) solution in a test tube which had been wrapped in aluminum foil. The mixture was vortexed for 30 seconds and incubated inside dark for 30 minutes at 37°C.[12] The absorbance measurements were carried out using a spectrophotometer at a wavelength of 515 nm.[13] DPPH inhibition was calculated using the formula.[12]

$$\%\ Inhibition = \frac{absorbance\ of\ blank - absorbance\ of\ sample}{absorbance\ of\ blank}\ X\ 100\%$$

IC_{50} was obtained using linear regression analysis (Microsoft Excel 2015, version 15.17) from several points between the concentration of the sample (x) to the percentage of inhibition (y) with the equation y = ax + b.

In vivo test

Preparation for sprague dawley rats

This study used 25 healthy male Sprague Dawley rats aged 2 to 3 months with bodyweight between 180-200 grams, which were divided into five groups, namely the positive control group (5 mg vitamin C/200 gr b.w./day, the negative control group, the 5 mg/200 gr b.w./day extract group, the 10 mg/200 gr b.w./day extract group, and the dose group extract of 20 mg/200 gr b.w./day conducted about ten days.[14]

Malondialdehyde (MDA) test

Blood samples (500 µL) were taken at two times, before and after the rats were given extract for ten days, followed by physical exercise (swimming for ten minutes).[15] Blood is taken using a capillary tube in the canthus orbital sinus and stored in a heparin tube.[16] Blood plasma was obtained by centrifuging blood samples and stored at -20°C. A mixture of 250 µL of blood plasma with 100 µL of 8.1% sulfate dodecyl sodium (SDS), 750 µL of 0.5 M HCl, 125 µL aqua bidest, and 750 µL 20 M thiobarbiturate (TBA) vortexed and heated up to 90°C for 15 minutes. After being cooled for 10 minutes, 500 µL aqua bidest and 2.5 mL n-butanol pears were added and divorced. The mixture was then centrifuged at 3000 rpm, and the filtrate was measured for absorbance at a wavelength of 520 nm.[17] To calculate the MDA level, a linear equation was made between the concentration of MDA (Y) and absorbance (X).[18]

Statistical analysis

Analysis of the MDA test data *in vivo* using a normality test followed by a one-way T and ANOVA test with SPSS 24 program.[19] The normality test was used to determine whether the data were normally distributed ($p > 0.05$) or not.[20] The T-test was used to compare MDA levels before and after antioxidant administration and physical exercise.[21] A one-way ANOVA test was used to determine the effectiveness of test samples as antioxidants [22].

RESULTS

Phytochemical analysis

The phytochemical analysis was conducted to observe the antioxidant compound present in the *O. vulgare* stem bark ethanolic extract (Table 1). "+" means those antioxidant substances are present, shown by a reaction in each extract with a different type of solvent, while "–"means the antioxidant substances are absent.

In vitro antioxidant activity of *O. vulgare* extract

In vitro antioxidant activity assay was conducted to know the extract's ability to neutralize free radicals from DPPH, shown by the IC_{50} value (Table 2 for vitamin C, Table 3 for *O. vulgare* stem bark ethanolic extract) and then compared between the IC_{50} value between Vitamin C and *O. vulgare* stem bark ethanolic extract.

In vivo antioxidant activity of *O. vulgare*

The antioxidant activity of *O. vulgare* was also evaluated by comparing the MDA level in the plasma before and after giving the treatment (Table 4).

Statistical analysis

A paired T-Test was used to know if there is a significant difference between two relatable data groups, shown by the value of $p<0.05$ (Table 5).

One-Way ANOVA was used to evaluate if there is a significant difference in the mean of two or more unrelated variables (Table 6). It also determines the most effective dosage of the *O.vulgare* stem bark extract, reducing oxidative stress.

After conducting One-Way ANOVA, the following test is a Post Hoc Test based on the Test homogeneity of variances (Table 7). Post Hoc Test was used to know which data has a significant value.

Table 1: Phytochemical Analysis Result of *O. Vulgare* extract.

Phytochemicals Constituent	Extract		
	Ethanol	Ethyl acetate	n-Hexane
Glycosides	+	-	-
Saponin	+	+	-
Flavonoid	+	+	-
Alkaloid	+	+	-
Terpenoid/ Steroid	+	-	-
Essential oil	+	+	+
Tannin	+	+	-

Table 2: *In Vitro* Antioxidant Activity of Vitamin C.

Concentration (µg/mL)	Absorbance	Inhibition (%)	Equation	IC_{50} (µg/mL)
Blank	2.4718	0	y = 3.1434x + 25.063 R^2 = 0.80956	4.53
1	2.1888	11.45		
5	1.3529	46.05		
10	0.5303	76.28		
25	0.1117	95.35		

Table 3: *In Vitro* Antioxidant Activity of Ethanol Extract of *O. vulgare*.

Concentration (µg/mL)	Absorbance	Inhibition (%)	Equation	IC_{50} (µg/mL)
Blank	2.3238	0	y = 0.3476x + 3.7099 R^2 = 0.84258	133.47
10	2.1472	7.60		
50	2.1080	9.29		
100	1.0366	55.38		
200	0.7506	67.70		

Table 4: Mean and differences in MDA level.

		Mean and Difference of MDA Level		
No.	Groups	Before Treatment (nmol/ mL)	After Treatment (nmol/ mL)	MDA Level Differences (nmol/ mL)
1	Positive Control	0.924	0.670	-0.254
2	Negative Control	0.866	0.986	0.120
3	Extract 5 mg	1.354	0.854	-0.500
4	Extract 10 mg	1.180	0.706	-0.474
5	Extract 20 mg	1.218	0.709	-0.509

Table 5: The result of Paired T-Test of MDA level.

MDA Level	Number of Subjects	Mean (nmol/mL) ± Standard Deviation	Mean Difference (nmol/mL) ± Standard Deviation	95% Confidence Interval of the Difference	P
Before Treatment	25	1.132 (0.168)	0.371 (0.178)	0.298 – 0.445	<0.0001
After Treatment	25	0.761 (0.107)			

Table 6: The result of One-Way ANOVA of MDA level.

Groups	Number of Subjects	Mean (nmol/mL) (Standard Deviation)	P
Positive Control	5	-0.500 (0.135)	0.000
Negative Control	5	-0.474 (0.042)	
Extract 5 mg	5	-0.509 (0.099)	
Extract 10 mg	5	-0.254 (0.057)	
Extract 20 mg	5	-0.119 (0.053)	

Table 7: The result of Post Hoc Test.

		Mean Differences	95% Confidence Interval		p
			Minimum	Maximum	
Positive Control	Dose 10 mg	-0.246	-0.415	-0.077	0.002
	Dose 20 mg	-0.380	-0.549	-0.211	0.000
Negative Control	Dose 10 mg	-0.220	-0.389	-0.051	0.005
	Dose 20 mg	-0.355	-0.524	-0.186	0.000
Dose 5 mg	Dose 10 mg	-0.255	-0.424	-0.086	0.001
	Dose 20 mg	-0.389	-0.558	-0.220	0.000
Dose 10 mg	Positive Control	0.246	0.077	0.415	0.002
	Negative Control	0.220	0.051	0.389	0.005
	Dose 5 mg	0.255	0.086	0.424	0.001
Dose 20 mg	Positive Control	0.380	0.211	0.549	0.000
	Negative Control	0.355	0.186	0.524	0.000
	Dose 5 mg	0.389	0.220	0.558	0.000

DISCUSSION AND CONCLUSION

Phytochemical analysis

The phytochemical tests showed that only glycosides and triterpenoids or steroids were detected in the ethanol fraction. Saponins, flavonoids, alkaloids, and tannins were detected in the ethanol fraction and ethyl acetate fraction. And only essential oils are found in all fractions. Most compounds are detected in the ethanol fraction because they are polar solvents, while ethyl acetate is a semi-polar solvent. Essential oils can be detected in all fractions.

In vitro antioxidant activity of *O. vulgare*

The *in vitro* antioxidant activity of the extract of *O. vulgare* was determined using the DPPH test because of its effectiveness and specificity. The DPPH test also requires only a few extract samples. IC_{50} is an extract concentration that can reduce the number of free radicals by 50%. The DPPH test is based on the reaction between DPPH as a free radical with a hydrogen-donor molecule from the extract of *O. vulgare*. IC_{50} values obtained from the ethanol extract of the bark of *O. vulgare* was 133.47 µg/mL. According to the classification of antioxidant intensity by Jun et al. (2003), includes strong antioxidant (<50 µg/mL), active antioxidant (50-100 µg/mL), moderate antioxidant (101-250 µg/mL), weak antioxidant (251-500 µg/mL), and inactive antioxidant (>500 µg/mL). *O. vulgare* extract has moderate activity [23].

The value of $R^2 = 0.84258$ indicates that with the extract concentration increasing, the antioxidant activity of the extract of *O. vulgare* will also increase.

In vivo antioxidant activity of *O. vulgare* extract

Malondialdehyde (MDA) results from a long chain peroxidation of unsaturated fatty acids found in fat membranes [24]. This peroxidation reaction occurs when the level of oxidative stress increases [25]. The antioxidant activity of *O. vulgare* extract was tested in vivo by comparing the levels of MDA of rats before and after extracts and physical exercise. The comparison was analyzed statistically using the Paired T-Test method. The p-value obtained from the Paired T-Test is 0.000, smaller than 0.05. This showed that there are at least two groups of data that have significant differences between MDA levels before and after treatment. The mean MDA level before treatment was 1.132 ± 0.168 nmol/mL, while the MDA level after treatment was 0.761 ± 0.107 nmol/mL.

One-Way ANOVA was conducted to find out comparisons in the group as a whole. Significance (p) was 0.000 (< 0.05), indicating that there is a minimum of two groups that have significant differences. According to the Post Hoc Bonferroni analysis results, differences in MDA levels between the positive control and some different concentration of *O. vulgare* extract.

O. vulgare extract has IC_{50} value as an antioxidant 133.47 (µg/mL). This shows that antioxidant activity is moderate in reducing DPPH radical levels in *In Vitro*. The ability of the antioxidant extract is not as strong as Vitamin C (positive control). *In vivo* studies showed that *O. vulgare* extract administration at doses of 10 mg and 20 mg can provide antioxidant effects compared to other groups.

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CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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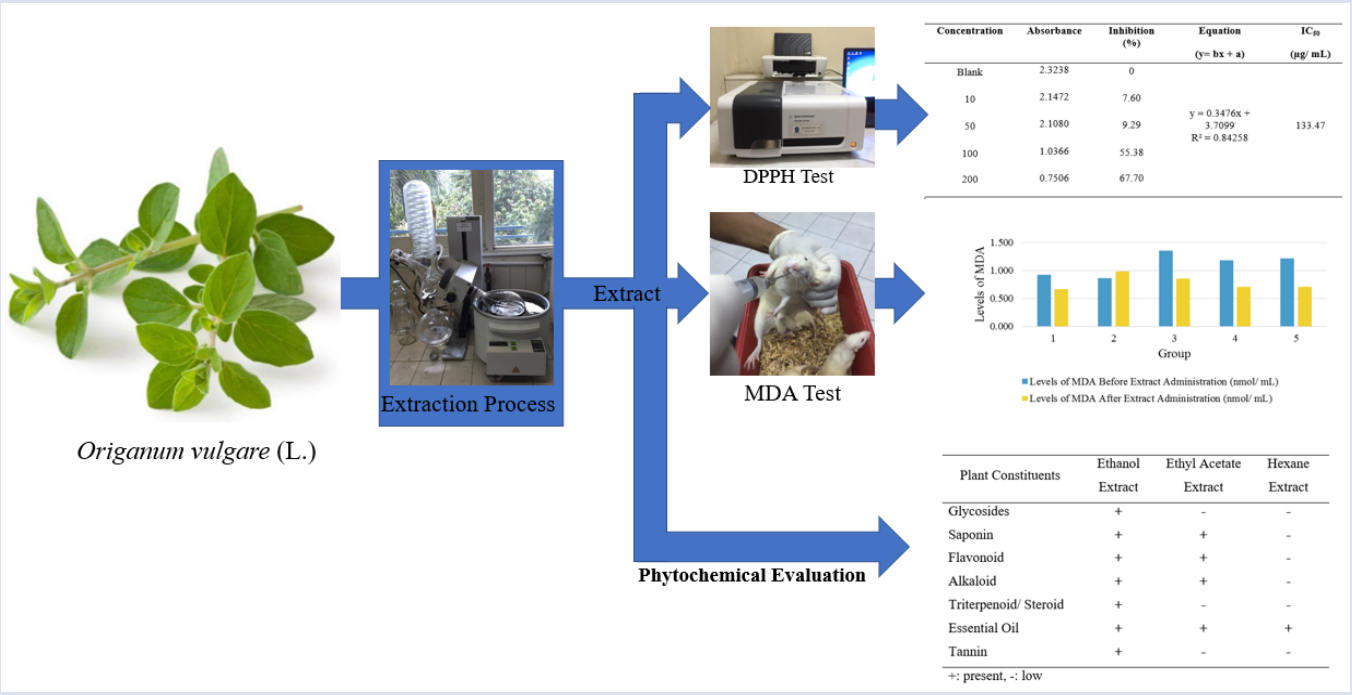
GRAPHICAL ABSTRACT



SUMMARY

- The phytochemical test of *O. vulgare* ethanol extract was showed some compounds, such as a flavonoid, alkaloid, triterpenoid/steroid, essential oil, and tannin, then in ethyl acetate and hexane.
- *In vitro* assay showed that *O. vulgare* extract has strong antioxidant activity with an IC_{50} value of 133.47 µg/mL.
- I*n vivo* test, the most effective dosage is 20 mg/200 gr B.W., represented by a significant decrease of MDA level (0.509 nmol/mL) before and after treatment.

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