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RESEARCH ARTICLE

Antioxidant Properties of Red Betel (*Piper crocatum*) Leaf Extract and its Compounds

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Abstract

Background: *Piper crocatum*, also known as red betel, is a potential herbal medicine. **Aims:** Current study was planned to determine the antioxidant activities of red betel (*Piper crocatum* Ruiz and Pav.) leaves extract (RBLE) as compared to eugenol and hydroxychavicol compounds. **Methods:** DPPH radical scavenging, H_2O_2 scavenging, ABTS reduction, and FRAP reduction assay were carried out. **Results:** In DPPH scavenging, RBLE showed an IC_{50} value of 3.98 µg/mL, eugenol of 2.98 µg/mL, and hydroxychavicol of 18.00 µg/mL. Meanwhile, H_2O_2 scavenging activity showed an IC_{50} value of RBLE, eugenol, and hydroxychavicol as 186.33 µg/mL, 97.36 µg/mL, and 41.06 µg/mL respectively. ABTS reduction assay showed an IC_{50} value of 38.43 µg/mL, 181 µg/mL, and 3.10 µg/mL for RBLE, eugenol, and hydroxychavicol respectively. The highest FRAP reduction activity was shown by Eugenol with a concentration of 50 µg/mL which was equal to 424.67 µM Fe (II)/µg. **Conclusion:** The RBLE and its compounds (eugenol and hydroxychavicol) have antioxidant activity as indicated by the results of the DPPH scavenging, H_2O_2 scavenging, ABTS reduction, and FRAP reduction assays. However, RBLE had the lowest antioxidant activity compared to other compounds.

Keywords: Eugenol, Free Radicals, Hydroxychavicol, *Piper crocatum*

1. Introduction

Molecules or fragment of molecules when they lose an electron in an atomic orbital make them as unstable free radicals. To attain stability, free radicals damage or react with neighboring molecules. External origin of free radicals are X-rays, ozone, cigarette smoke, industrial chemicals, ultraviolet light, and environmental pollutants[1,2]. Imparity between Reactive Oxygen Species (ROS) and the anti-oxidative defense systems cause oxidative stress that could lead to many diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, and atherosclerosis[3].

Piper betle leaves have antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl (OH), anion superoxide (O_2^-) radicals, and lipid peroxidation assay. *P. betle* leaf extract was found to be a powerful trapper of OH radicals[4]. *P. crocatum*, also known as red betel of the family Piperaceae, has a more bitter taste, fragrant aroma, and is a better potential as herbal medicine than the regular betel. However, the volatile oil content and the antimicrobial activity were lowerer[4]. Red betel leaves (*P. crocatum*) extract and its compound namely, eugenol have antioxidant activities including DPPH scavenging activity, Super Oxide Dismutase (SOD) activity and anticancer activity

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against cervical cancer[5]. Red betel has been reported to contain several active compounds such as flavonoids, steroids, tannins, saponins, alkaloids, polyphenolics, quinones, and essential oil[4,6]. Red betel also contains chavicol, chavibetol, carvacrol, caryophyllene, estragole, eugenol, and hydroxychavicol[5].

Hydroxychavicol and eugenol were antifungal compounds[7]. In addition, eugenol at lower concentration has been reported to have antioxidant and anti-inflammatory properties, but at higher concentration exhibits pro-oxidant properties[7]. Hydroxychavicol isolated from the aqueous extract of *P. betle* was the major phenolic component and has been suggested as antimutagenic, anticarcinogenic, antioxidant, anti-inflammatory, and chemopreventive agent[8]. The extract of red betel has been reported to be active against *Colletotrichum gloeosporioides*, *Candida albicans*, and *Botryodiplodia theobromae*[9]. Betel oil nowadays has been used as an antiseptic component in gels, balms, it is anti-inflammatory and also is a treatment for several diseases[10]. The aim of the study was to observe the antioxidant activity of the red betel (*P. crocatum* Ruiz and Pav.) leaf ethanol extract as compared to hydroxychavicol and eugenol.

2. Materials and Methods

2.1 Preparation of Extract

Red betel (*P. crocatum* Ruiz and Pav.) leaves were obtained from Pabuaran Cilendek Timur, Bogor. The plants were identified by Herbarium Bogoriense, Botanical Field Research Center for Biology-Indonesian Institute of Sciences, Bogor, Indonesia. Red betel leaves were dried using food dehydrator (Zhengzhou Well-known) then mashed (160 g) and extracted by 500 mL of distilled ethanol 70% by maceration method. Ethanol filtrate was filtered in every 24 h and wastes were remacerated until there was colourless filtrate. The filtrate was concentrated in evaporator at 50 ºC (Zhengzhou Well-known, RE-201D) to obtain the red betel leaves extract (RBLE)[11-13].

2.2 DPPH Scavenging

A total of 200 µL DPPH (Sigma Aldrich D9132) 0.077 mmol in methanol was added with 50 µL of samples including RBLE, hydroxychavicol (Chengdu Biopurify Phytochemical Ltd, BP3020), eugenol (Chengdu Biopurify Phytochemical Ltd, BP0569) with various concentration added on the 96-well microplate. The mixture was incubated for 30 min at room temperature, then the absorbance value was read at 517 nm wavelength using a micro plate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific). For the sample, 200 µL of DPPH and 50 µL of sample was used, while for negative control 250 µL of DPPH was used, while for blanks, 250 µL of an absolute DMSO was used[11-14]. Calculation of DPPH scavenging activity was done by using the following formula:

DPPH scavenging activity (%) = (A-B)/A × 100

A: control absorbance

B: sample absorbance

2.3 H_2O_2 Scavenging

The scavenging of H_2O_2 was measured based on the method described by Mukhopadhyay *et al*[15,16]. with slight modification. Each sample contained 60 µL of sample, 12 µL of ferrous ammonium sulphate (1mM, Sigma Aldrich 7783859), and 3 µL of H_2O_2 (5mM, Merck 1.08597.1000). For the negative control, 12 µL of ferrous ammonium sulphate and 63 µL of DMSO were used, while for blanks, 60 µL of RBLE and 90 µL of DMSO was used.

After adding H_2O_2, control, sample, and blank solutions was added into the 96-well plate and incubated for 5 min in a dark room at room temperature. Sample and control solutions were added with 75 µL of 1, 10-phenanthrolines and incubated again for 10 min in a dark room at room temperature. Absorbance value was measured at 510 nm wavelength. The percentage of scavenging activities was calculated using the formula:

H_2O_2 scavenging activity (%) = (A-B)/A X 100

A: control absorbance

B: sample absorbance

2.4 ABTS Reduction

Briefly 2 µL of sample in various concentrations was added into the well followed by 198 µL of 2,2'-Azinobis-(3-ethylbenzo thiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) (Sigma Aldrich, A1888) and also in control well with 200 µL ABTS. Blank well was added by 200 µL of

DMSO. Microplate was then incubated at 37˚C for 6 min. Absorbance was measured at a wavelength of 745 nm[11-13]. Calculation of ABTS reduction percentage was carried out by the following formula:

ABTS reduction activity (%) = (A-B)/A X 100

A: control absorbance

B: sample absorbance

2.5 FRAP Reduction

The Ferric Reducing Antioxidant Power Assay (FRAP) reagent was prepared by mixing 10 mL of acetate buffer 300 mM, 1 mL ferric chloride hexahydrate (Merck 1.03943.0250), 20 mM dissolved in distilled water, and 1 mL of 2,4,6-Tris-(2-pyridyl-5-Triazine) (TPTZ) (Sigma Aldrich 368235-7) 10 mM and dissolved with HCl 40 mM. Briefly 7.5 µL of samples were added with 142.5 µL FRAP reagent in 96-well microplate reader then mixed and incubated for 30 min at 37°C. The absorbance was measured at 593 nm with a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific). The standard curve was made using $FeSO_4$, between 0.49 and 62.50 µM $FeSO_4$. The results were expressed in µM Fe (II)/µg extract[11-13].

2.6 Statistical Analysis

Statistical analysis was performed using SPSS software (version 20.0). Values are presented as Mean ± Standard Deviation. Significant differences between the groups were determined using the Analysis of variance (One Way ANOVA) followed by Tukey's HSD Post-hoc Test. The results of DPPH scavenging, H_2O_2 scavenging, ABTS reduction activity were continued by linear regression analysis. Then the value of Median Inhibitory Concentration 50 (IC_{50}) was determined for DPPH scavenging, H_2O_2 scavenging, ABTS reduction.

3. Results and Discussions

Red betel was extracted using 70% ethanol to extract active compounds that have antioxidant activity, such as flavonoids, polyphenols, alkaloids, and tannins. Ethanol is a polar solvent but less polar than water so it is more efficient in degrading cell walls in red betel leaf[17].

DPPH is a free radical that belongs to the hydrogen radical group. DPPH is sensitive to light, oxygen, and pH. However, it is stable in a radical form so it may be quite an accurate measurement of antioxidant activity. DPPH free radical can capture hydrogen atoms from the component of antioxidant sample which are mixed and then react to their reduced form and are characterized by reducing intensity of purple DPPH solution with maximum uptake at 517 nm[11-13]. When antioxidants interact with DPPH, they shift an electron or hydrogen atom (H^+) to DPPH to counteract its free radical character[18]. This process changes the color of the solution from purple to yellow. DPPH assay in this study shows that the radical-scavenging activities of the samples were in the order of hydroxychavicol < RBLE < eugenol. The differences between each concentration were significant (Figure 1) and the IC_{50} value of samples against DPPH free radical scavenging activity is shown in Table 1. IC_{50} value of RBLE, hydroxychavicol and eugenol was 3.98 µg/mL, 18.00 µg/mL and 2.98 µg/mL respectively. IC_{50} of hydroxychavicol and eugenol was equivalent to 119.86 µM and 18.15 µM. Based on the result of DPPH scavenging test, each sample had the highest DPPH scavenging activity at 250 µg/mL concentration.

Widowati *et al*[5]. has reported that DPPH scavenging activity of piper extracts based on IC_{50} value. *P. pellucidum* and *P. umbellatum* had the highest scavenging activity of DPPH of 9 and 15.36 µg/mL respectively. Those piper extracts were also compared to eugenol which had an IC_{50} value of 3.8 µg/mL. Risdian *et al*[1]. suggested that DPPH scavenging activity of *P. betle* L. ethanol extract was strong with an IC_{50} value of 3.48 µg/mL. Alfarabi *et al*[17]. reported that *P. crocatum* leaves at 200 ppm concentration could hamper DPPH by 73.41% with an IC_{50} of 85.82 ppm. As a natural antioxidant, *P. crocatum* leaves extract could hinder the oxidation of linoleic acids because of the presence of flavonoids, tannins, and alkaloids as bioactive compounds. Based on another study, if the compound has IC_{50} value < 200 ppm or < 200 µg/mL, it is considered to possess strong antioxidant activity. Considering these previous studies, RBLE exhibited potential antioxidant property.

Hydrogen peroxide is one of the ROS having supportive roles in energy production *in vivo* systems, phagocytosis, intercellular signal transfer, adjustment

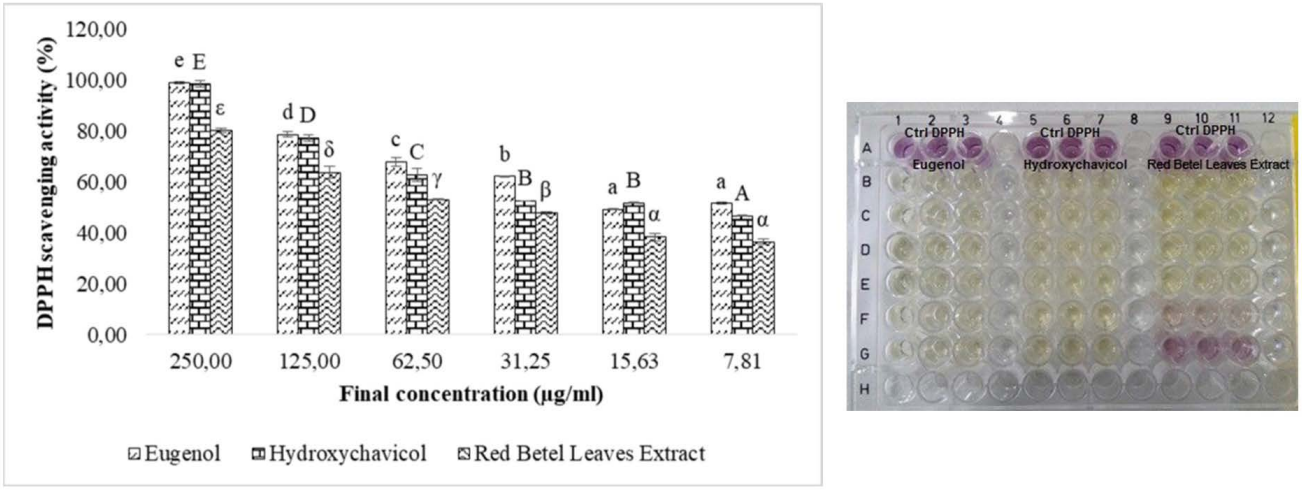

Figure 1. DPPH Scavenging Activity of Eugenol, Hydroxychavicol, and RBLE in Various Concentrations **(A).** DPPH Scavenging Activity **(B).** Eugenol, Hydroxychavicol, and RBLE were diluted in DMSO to reach final concentration of 250, 125, 62.50, 31, 25, 15, 63, 7.81 (µg/mL). Different letter in the same sample of eugenol (a, b, c, d, e), hydroxychavivol (A, B, C, D, E), RBLE (α, β, γ, δ, ε) indicate significant differences among sample concentrations based on Tukey's HSD Post hoc comparisons ($P < 0.05$).

Table 1. IC_{50} of DPPH Scavenging Activity of Eugenol, Hydroxychavicol, and RBLE

Sample	Equation	R^2	IC_{50} (µg/mL)	IC_{50} (µM)
Eugenol	y = 0.1788x + 49.468	0.97	2.98	18.15
Hydroxychavicol	y = 0.2195x + 46.05	0.97	18.00	119.86
RBLE	y = 0.1375x + 49.453	0.96	3.98	-

of cell growth and the synthesis of prominent biological compounds[16]. At a concentration of 250 µg/mL, all the samples showed the highest activity of H_2O_2 scavenging (Figure 2). Almost all of the samples had H_2O_2 scavenging activity which was significantly different at each of the concentrations. Based on IC_{50} value, the radical-scavenging activities of the sample were in the order RBLE < eugenol < hydroxychavicol hydroxychavicol had the strongest activity (41.06 µg/mL) compared to eugenol (97.36 µg/mL) and RBLE (186.33 µg/mL). The result can be seen in Table 2.

H_2O_2 scavenging assay showed that the scavenging activity of hydroxychavicol at concentration of 250 µg/mL was about 1.15 times stronger than RBLE. Tamuly *et al*[19]. has reported that H_2O_2 scavenging activity of *P. wallichii* Miq. Hand. -Mazz. methanol extract which was 49.30 µg/mL. In current study IC_{50} of RBLE, eugenol, and hydroxychavicol was 186.33, 97.36, and 41.06 µg/mL respectively in which, IC_{50} of eugenol and hydroxychavicol was equivalent to 592.92 µM and 250.06 µM respectively. It shows that the samples were not too strong for being potent antioxidant in scavenging H_2O_2.

ABTS-reducing activity assay is for measuring the comparative potential of antioxidant to capture ABTS induced by reacting a strong oxidizing agent (potassium permanganate/potassium persulfate) with the ABTS salt. The long wave absorption spectrum was

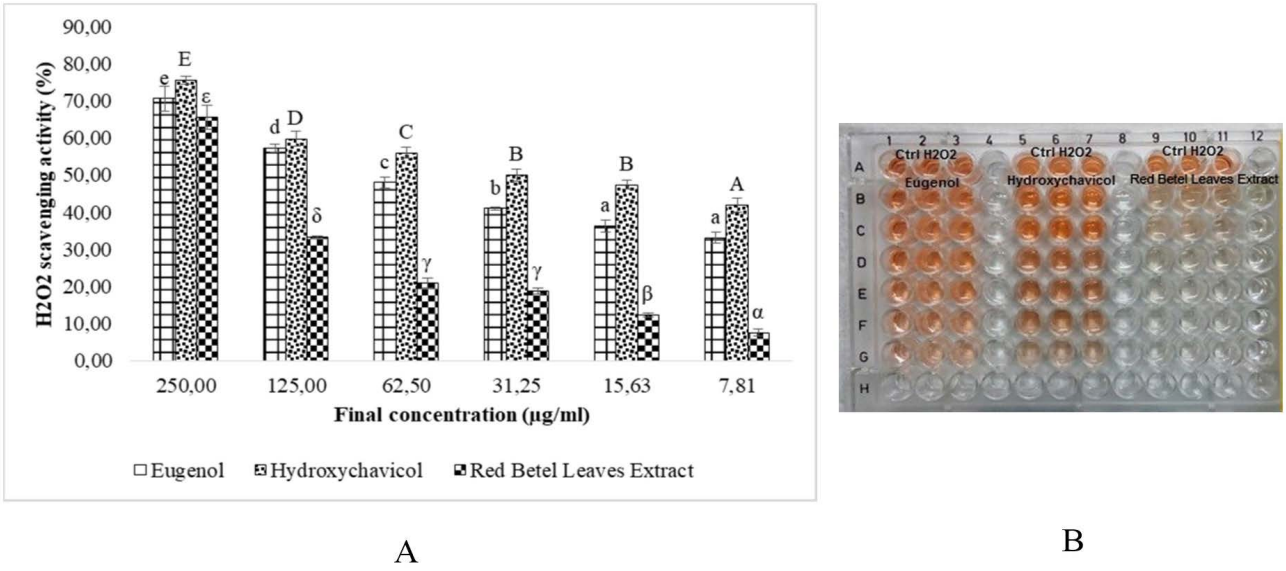

Figure 2. H_2O_2 Scavenging Activity of Eugenol, Hydroxychavicol, and RBLE in Various Concentratios **(A).** H_2O_2 Plate Scavenging Activity **(B).** Eugenol, Hydroxychavicol, and RBLE were diluted in DMSO to reach final concentration of 250, 125, 62.50, 31, 25, 15, 63, 7.81 (µg/mL). Different letter in the same sample of eugenol (a, b, c, d, e), hydroxychavivol (A, B, C, D, E), RBLE (α, β, γ, δ, ε) indicate significant differences among sample concentrations based on Tukey's HSD Post hoc comparisons ($P < 0.05$).

Table 2. IC_{50} of H_2O_2 Scavenging Activity of Eugenol, Hydroxychavicol, and RBLE

Sample	Equation	R^2	IC_{50} (µg/mL)	IC_{50} (µM)
Eugenol	y = 0.1498x + 35.445	0.96	97.36	592.92
Hydroxychavicol	y = 0.1251x + 44.863	0.96	41.06	250.06
RBLE	y = 0.2261x + 7.8717	0.99	186.33	-

used to measure decrease of blue-green colored ABTS radical solution by hydrogen-donating antioxidant.[13] The result of ABTS-reducing activity has been shown at Figure 3. All samples showed high activity at a concentration of 50 µg/mL. RBLE has the lowest ABTS-reducing activity, indicated by the highest IC_{50} value (38.43 µg/mL) compared to hydroxychavicol (3.10 µg/mL) and eugenol compounds (1.81 µg/mL). Based on the result, it can be indicated that RBLE had lower antioxidant activity compared to hydroxychavicol and eugenol (Table 3).

ABTS-reducing assay is based on the ability of the antioxidants to quench the $ABTS^+$ radical cation. ABTS-reducing activities of samples are RBLE < hydroxychavicol < eugenol. IC_{50} of each sample was 38.43 µg/mL (RBLE), 3.10 µg/mL (hydroxychavicol), and 1.18 µg/mL (eugenol). IC_{50} of hydroxychavicol and eugenol was equivalent with 20.64 µM and 7.19 µM. According to Widowati *et al*[11]., eugenol had IC_{50} value of 1.56 µg/mL, equivalent with 9.54 µM.

The Ferric Reducing Antioxidant Power (FRAP) method is based on the decrease of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine ($Fe(TPTZ)^{3+}$) changes to the extremely blue colored Fe^{2+} complex ($Fe(TPTZ)^{2+}$) by antioxidants in acidic medium. Antioxidant reduction of appropriate tripyridyltriazine Fe(III) complex produces absorbance of Fe(II) complex at 593nm [13]. Based on Figure 4, at the highest concentration, each sample had high activity

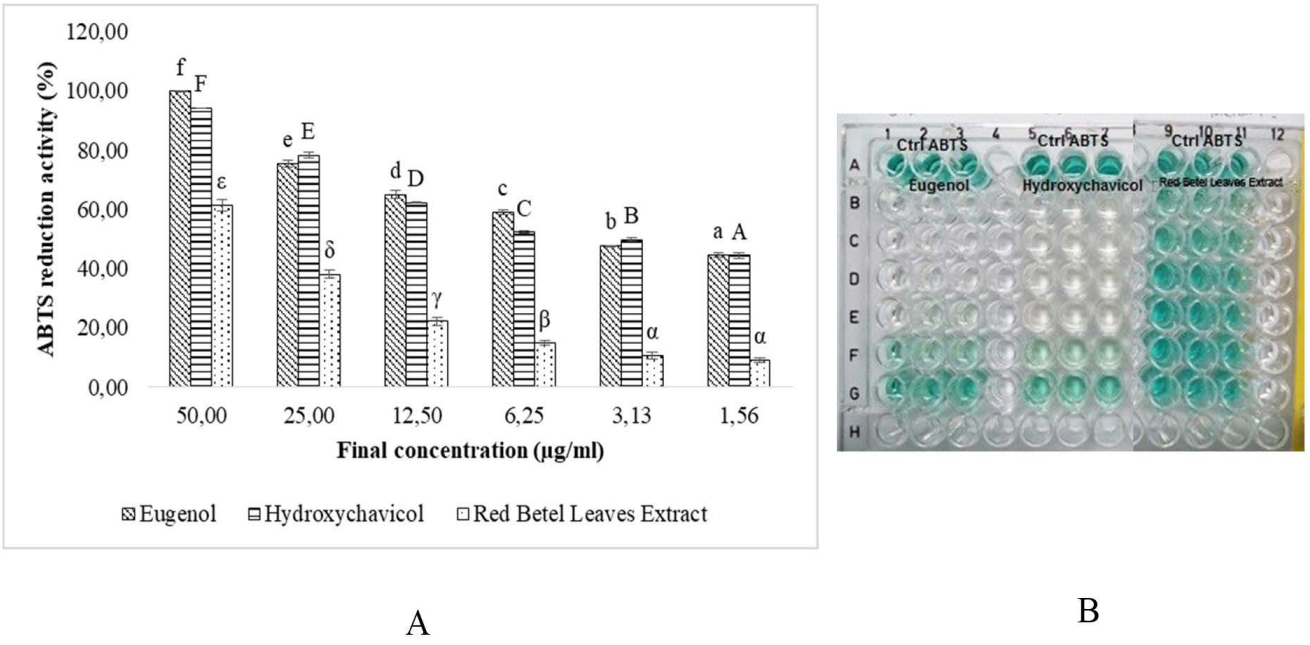

Figure 3. ABTS Reduction Activity of Eugenol, Hydroxychavicol, and RBLE in Various Concentratios **(A).** ABTS Plate Reduction Activity **(B).** Eugenol, Hydroxychavicol, and RBLE were diluted in DMSO to reach final concentration of 50, 25, 12.50, 6.25, 3.13, and 1.56 (µg/mL). Different letter in the same sample of eugenol (a, b, c, d, e, f), hydroxychavivol (A, B, C, D, E, F), RBLE (α, β, γ, δ, ε, ζ) indicate significant differences among sample concentrations based on Tukey's HSD Post hoc comparisons ($P < 0.05$).

Table 3. IC_{50} of ABTS-Reducing Activity of Eugenol, Hydroxychavicol, and RBLE

Sample	Equation	R^2	IC_{50} (µg/mL)	IC_{50} (µM)
Eugenol	y = 1.1058x + 48.004	0.95	1.81	11.03
Hydroxychavicol	y = 1.0133x + 46.857	0.96	3.10	20.64
RBLE	y = 1.0884x + 8.1753	0.99	38.43	-

of FRAP reduction (eugenol= 424.67 µM Fe(II)/µg, hydroxychavicol= 371.17 µM Fe(II)/µg, and RBLE= 227.75 µM Fe(II)/µg) which were significant. On the other hand, at the lowest concentration, each sample had almost the same activity values (eugenol= 64.17 µM Fe(II)/µg, hydroxychavicol= 44.33 µM Fe(II)/µg, and RBLE= 50.58 µM Fe(II)/µg).

On the other hand, RBLE had the highest FRAP-reducing activity compared to eugenol and hydroxychavicol. The order can be as hydroxychavicol < eugenol < RBLE. The highest percentage of activity was RBLE (227.75 µM Fe(II)/µg) at the concentration of 50 µg/mL while the lowest was hydroxychavicol (44.33 µM Fe(II)/µg). Srivastava *et al*[20]. reported that *P. betle* had FRAP-reducing activity around 3.44 GAE/g. In addition, Widowati *et al*[11]. suggested that FRAP-reducing activity of eugenol at concentration of 250 µM and 50 µM was 402.42 µM Fe(II)/µg and 155.13 µM Fe(II)/µg respectively.

4. Conclusion

The red betel leaf extract (*P. crocatum* Ruiz and Pav.) and its compounds (eugenol and hydroxychavicol)

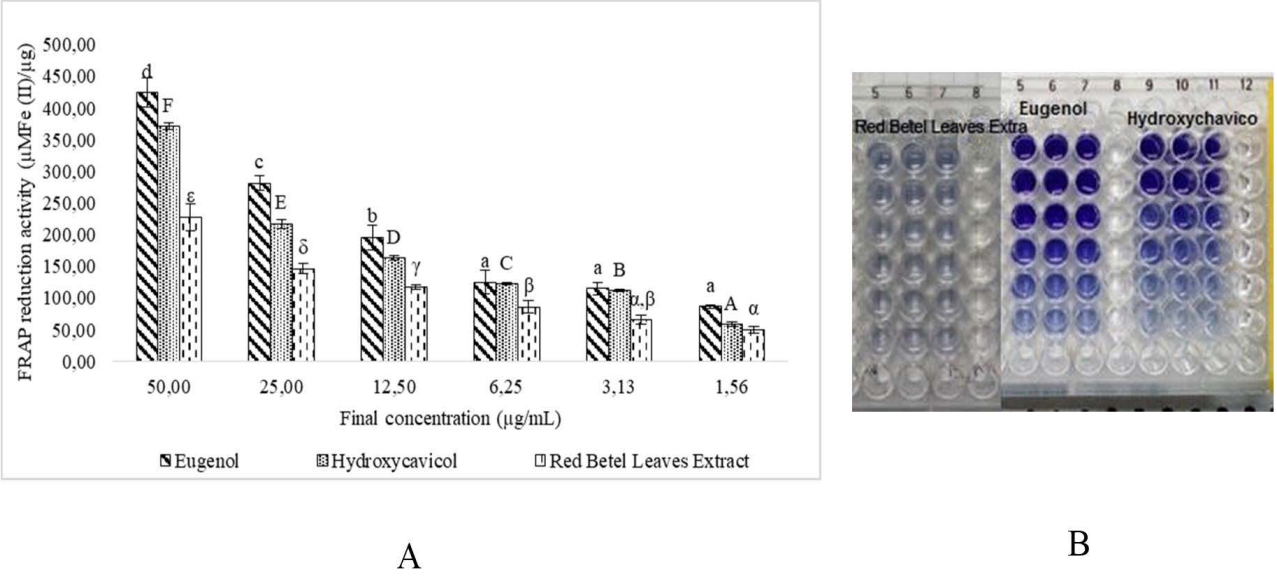

Figure 4. FRAP Reduction Activity of Eugenol, Hydroxychavicol, and RBLE in Various Concentratios **(A).** FRAP Plate Reduction Activity **(B).** Eugenol, Hydroxychavicol, and RBLE were diluted in DMSO to reach final concentration of 50, 25, 12.50, 6.25, 3.13, and 1.56 (µg/mL). Different letter in the same sample of eugenol (a, b, c, d, e), hydroxychavivol (A, B, C, D, E, F), RBLE (α, β, γ, δ) indicate significant differences among sample concentrations based on Tukey's HSD Post hoc comparisons ($P < 0.05$).

have antioxidant activity as indicated by the results of the DPPH scavenging test, H_2O_2 scavenging, ABTS reduction, and FRAP reduction. However, RBLE had the lowest antioxidant activity compared to eugenol, hydroxychavicol.

5. Conflict of Interest

All authors state there is no conflict of interest.

6. Acknowledgement

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