ORIGINAL ARTICLE

Intisari Sains Medis 2021, Volume 12, Number 1: 414-419
P-ISSN: 2503-3638, E-ISSN: 2089-9084

Intisari Sains Medis

Published by Intisari Sains Medis

In vitro study of red beetroot ethanol extract (*Beta vulgaris L.*) as xanthine oxidase inhibitor

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Received: 2021-01-16
Accepted: 2021-04-23
Published: 2021-04-30

ABSTRACT

Background: Xanthine oxidase (XO) is an enzyme that catalyzes the oxidation of xanthine to uric acid. Hyperuricemia is a condition in which uric acid levels in the blood are > 6.8 mg/dL. Hyperuricemia is managed by administering uric acid-lowering drugs that work by inhibiting the activity of XO. Allopurinol, as the first-line drug, has several side effects. Flavonoids and betalains have been shown in numerous studies to have the ability to inhibit XO. The purpose of this study is to examine the ethanol extract of red beetroot (*Beta vulgaris L.*) with its flavonoids and betalains content in inhibiting the activity of XO.

Methods: This study was an in-vitro experimental study using UV-Vis spectrophotometry (ë 285 nm). Red beetroot was extracted with 96% ethanol solvent using the maceration method. Allopurinol was used in this study as a positive control. This study was carried out in triplicates, with a test sample consisting of 5 different concentrations. The results of this study were analyzed using the Kruskal Wallis.

Results: The ethanol extract of red beetroot inhibits XO enzymatic activity (55.11%) at 1000 µg/ml. There was a statistical difference between the control group, the ethanol extract of red beetroot group, and the allopurinol group in the inhibition of XO enzyme activity (p= 0.001).

Conclusion: The ethanol extract of red beetroot (*Beta vulgaris L.*) can inhibit *xanthine oxidase* (XO).

Keywords: Betalains, Flavonoids, Red beetroot (*Beta vulgaris L.*), Uric acid, Xanthine oxidase.
Cite This Article: Putri, G.A., Mahendra, A.N., Jawi, I.M. 2021. *In vitro* study of red beetroot ethanol extract (*Beta vulgaris L.)* as xanthine oxidase inhibitor. *Intisari Sains Medis* 12(1): 414-419. DOI: 10.15562/ism.v12i1.934

INTRODUCTION

XO is one of the enzymes that play a role in the uric acid metabolic process by catalyzing the oxidation of hypoxanthine to xanthine and subsequently converts xanthine into uric acid.[1] Increased production and decreased excretion of uric acid contribute to hyperuricemia. Hyperuricemia is a condition in which the serum uric acid level in the body is > 6.8 mg/dL. Age, socioeconomic factors, diet, and drugs are also said to affect uric acid levels. A chronic increase in uric acid levels can lead to gout by forming monosodium urate (MSU) crystals or tophus in the joints.[1,2] The prevalence of gout in Indonesia is estimated to be much higher than that of other Asian countries. Hyperuricemia is a risk factor in other diseases, namely cardiovascular disease, hypertension, coronary heart disease, stroke, kidney disease, metabolic syndrome, and diabetes.[2]

The management of hyperuricemia and gout depends on uric acid-lowering drugs, but the use of medicines in asymptomatic hyperuricemia is still controversial.[2] Xanthine oxidase inhibitors (XOI), namely allopurinol and febuxostat, are the antihyperuricemic drug of choice. The obstacle of administering allopurinol as the first-line drug is the high risk of allopurinol hypersensitivity syndrome (AHS), strongly associated with the HLA-B*5801 gene that is common in Asian populations.[3] An increased risk of cardiovascular death has also been reported in gout patients treated with febuxostat.[4] A good lifestyle, especially diet, is the main principle of prevention and management of hyperuricemic patients due to the limitations of uric acid-lowering drugs that have been described.[3] Compounds with the same effectiveness as XOI and lower side effects need to be developed.

Red beetroot, commonly found on the market, is usually consumed directly or used as a food coloring. Beetroot has also been recommended as a diet for hyperuricemia patients due to its contents.[5] Red beetroot *(Beta vulgaris L.)* contains various bioactive compounds, especially flavonoids.[6,7] Several groups of flavonoids are found in the ethanol extract of beetroot.[8,9] Flavonoids, ubiquitously found in many fruits and vegetables, have been reported to have the ability to inhibit XO by several studies and are said to have a higher effect of inhibiting XO activity than allopurinol.[10,11]

Red beetroot also has antioxidant content.[9] The antioxidant activity of red beetroot is caused by the betalains pigment (betacyanin and betaxanthin). The betalain pigment content is a distinctive characteristic of red beetroot compared to other plants containing anthocyanin pigments. Betalain – with its antioxidant activity that is stronger and more stable than anthocyanins – plays a role in inhibiting XO.[6,12-14] *In vitro* research

on an ethanol extract of red beetroot as XOI has never been carried out. Therefore, this experimental study was conducted as an initial process of discovering and developing novel natural product-based uric acid-lowering drugs.

METHOD

This research was an in vitro experimental study conducted in PT. Sky Pacific Indonesia, Bogor, West Java, Indonesia. Red beetroots *(Beta vulgaris L.)* that fulfilled specific criteria (fresh, free of pests and diseases) were used in the current study.

Materials

The tools needed were a knife, food slicer, oven, 60 mesh sieve, scale, rotary vacuum evaporator, aluminum foil, Erlenmeyer flask, beaker glass, test tube, stirrer, blender, spatula, label, Sartorius No. 393 filter paper, pH meter, tissue, vortex, incubator, pounding mortar, Cary 60 UV-Vis Spectrophotometer, and cuvette. Materials needed in this study are red beetroot *(Beta vulgaris L.)*, ethanol 96%, Dimethyl sulfoxide (DMSO) 99.9%, HCl, NaOH, distilled water, Potassium dihydrogen phosphate (KH_2PO_4), allopurinol (100 mg), xanthine substrate X06626-5G (Sigma Aldrich ≥99.5%), and xanthine oxidase enzyme from cow milk (Roche).

Red Beetroot *(Beta vulgaris L.)* Extraction Process

Red beetroot samples were collected from a supermarket in Bogor, West Java. Beetroots were weighed using a scale. The fresh weight of the beetroot obtained was ± 1.9 kg. The beetroots were washed and sliced using a food slicer to form chips with a thickness of about 1 mm. Beetroots were dried for 6 x 24 hours in an oven at ± 40^{0}C until they become simplicia. The simplicia obtained was blended and sieved using a 60 mesh sieve to form a powder, then weighed. The dry weight of red beetroot simplicia was 112 g.

The extraction process was begun by soaking 25 g of red beetroot simplicia in 50 ml of 96% ethanol solvent for 3 x 24 hours with periodic stirring, then filtering it using filter paper. The filtrate was subsequently concentrated using a rotary vacuum evaporator, then weighed. The concentrated extract obtained, which is 10 ml, could be left for several days in the refrigerator at 4-10^{o}C by covering it with aluminum foil.

Preparation of 0.15 mM Xanthine Substrate Solution

The solution was prepared by dissolving 0.5733 mg of xanthine substrate with a few drops of 1 M NaOH, then adding sodium phosphate buffer (pH 7.5) until the volume reached 25 ml in a beaker glass.[15]

Preparation of 0.2 U / ml Xanthine Oxidase Enzyme Solution

The solution was prepared by adding 85.5 µl of XO enzyme and sodium phosphate buffer (pH 7.5) until the volume reached 10 ml in beaker glass.[15]

Preparation of Red Beetroot Ethanol Extract Solution

The extract of red beetroot solution was made by dissolving 10 mg of ethanol extract with a few drops of 1% DMSO solution, then adding sodium phosphate buffer solution (pH 7.5) until reaching a volume of 10 ml. A standard solution (1000 µg/ml) was obtained, then diluted into 5 different concentrations (100 µg/ml, 200 µg/ml, 500 µg/ml, 800 µg/ml, dan 1000 µg/ml).[15]

Preparation of Allopurinol Solution

Allopurinol solution is made by dissolving 25 mg of allopurinol with distilled water up to 25 ml. The standard solution of allopurinol (1000 µg/ml) was diluted to obtain test samples into 5 different concentrations (100 µg/ml, 200 µg/ml, 500 µg/ml, 800 µg/ml, dan 1000 µg/ml).[15]

Xanthine Oxidase (XO) Activity Inhibition Study

The inhibition test of XO activity by the control group (without test sample), ethanol extract of red beetroot group, and the allopurinol group in this study were observed using a UV-Vis spectrophotometer. Sodium phosphate buffer solution (1.2 ml) was added with 300 µl xanthine oxidase 0.2 U/ml and 300 µl distilled water. The solution was vortexed and incubated for 15 minutes at 37^{0}C, then 600 µl of 0.15 mM xanthine substrate was added, vortexed, and re-incubated for 30 minutes. HCl 0.5 M (600 µl) was added after incubation. Then the absorbance was observed using a spectrophotometer at ë 260-300 nm. After this process, the maximum wavelength with the maximum absorbance value can be obtained and used in the XO activity inhibition test with the test sample. The absorbance obtained from the spectrophotometer is the blank absorbance of XO.[15]

The inhibition test by the sample can be carried out in the same step by adding 300 µl of test sample solution (5 concentrations of red beetroot ethanol extract and 5 concentrations of allopurinol). The absorbances were observed spectrophotometrically at the maximum ë obtained.[15]

The absorbances obtained were calculated using the inhibition percentage formula, which shows XO inhibition activity by the test sample.[16]

$$\% \text{ Inhibition} = \frac{\textit{blank absorbance} - \textit{sample absorbance}}{\textit{blank absorbance}} \times 100\%$$

a. Blank absorbance: the amount of absorption without the addition of the test sample
b. Sample absorbance: the amount of absorption with the addition of the test sample

Statistical Data Analysis

The inhibition percentage was analyzed using the normality test (Shapiro Wilk test) and the homogeneity test (Levene's test). One-Way ANOVA (Bonferroni as a post-hoc test) or Kruskal Wallis (Mann Whitney as a post-hoc test) was subsequently selected according to the results of the normality and homogeneity tests obtained. The data were considered to exhibit statistically significant differences if $p < 0.05$.

RESULTS

The inhibition percentage of XO activity by the control group (without test sample), the ethanol extract of red beetroot group, and the allopurinol group were analyzed using the Kruskal Wallis test because the data in this study were not normally distributed ($p < 0.05$) and not homogeneous ($p =$

Table 1. ***Mann Whitney*** **test results on XO inhibition percentage data**

Group	n	Median (Minimum - Maximum)	p
Control	3	0 (0 - 0)	
XO + 100 µg/ml allopurinol	3	65.23 (64.59 - 65.48)	
XO + 200 µg/ml allopurinol	3	67.15 (66.37 - 67.54)	
XO + 500 µg/ml allopurinol	3	67.15 (66.37 - 67.54)	
XO + 800 µg/ml allopurinol	3	63.99 (62.93 - 64.23)	
XO + 1000 µg/ml allopurinol	3	61.40 (61.06 - 61.61)	**0.037**
XO + 100 µg/ml ethanol extract of red beetroot	3	28.30 (23.25 – 28.62)	
XO + 200 µg/ml ethanol extract of red beetroot	3	51.98 (50.87 – 52.03)	
XO + 500 µg/ml ethanol extract of red beetroot	3	51.34 (50.17 – 53.47)	
XO + 800 µg/ml ethanol extract of red beetroot	3	49.37 (47.74 – 58.06)	
XO + 1000 µg/ml ethanol extract of red beetroot	3	55.57 (53.80 – 55.95)	

Note: Post hoc analyses with significant differences: Control vs. XO + allopurinol at all concentrations (p = 0.037), control vs. XO + ethanol extract of red beetroot at all concentrations (p=0.037).

0.00). The results showed differences in the inhibition ability of XO between the control group (without the test sample), the ethanol extract of red beetroot group, and the allopurinol group (p = 0.001) so that the ethanol extract of red beetroot can inhibit XO.

The Mann-Whitney test results in Table 1 showed a statistically significant difference between the inhibition of XO activity by the control group compared with the ethanol extract of red beetroot group and the control group compared to the allopurinol group (p = 0.037).

There were no statistically significant differences between the five concentrations in the ethanol extract group and the allopurinol group. Table 1 shows that an increase in the extract concentration will increase in inhibition of the XO activity. Still, this study showed that the inhibitory effect between the extract 100 µg/ml and 1000 µg/ml did not differ significantly (p> 0 05). These results were also shown in the allopurinol group. There was no statistically significant difference between the inhibition of XO activity by allopurinol at 100 µg/ml and allopurinol at 200 µg/ml (p > 0.05). There were also no significant differences in the ethanol extract of the red beetroot group compared to the allopurinol group (p> 0.05). Therefore, the XO inhibitory ability of ethanol extract of red beetroot and allopurinol is not statistically different.

The results regarding the action of red beetroot ethanol extract as XOI are shown

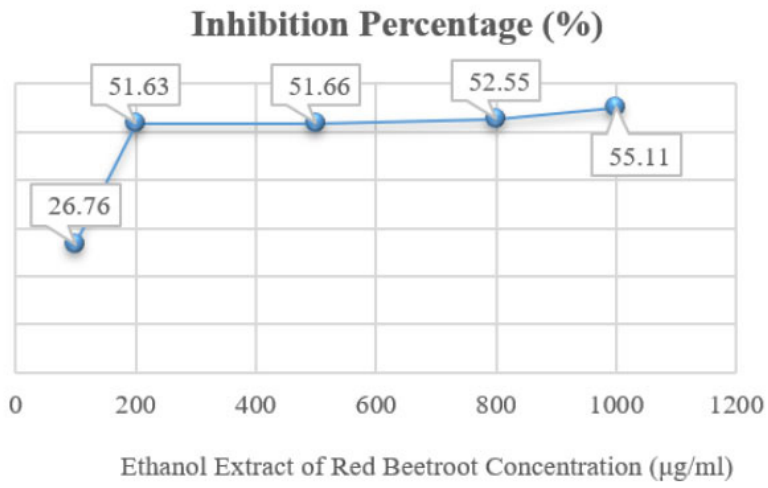

Figure 1. Inhibition of xanthine oxidase (XO) by ethanol extract of red beetroot (*Beta vulgaris L.*)

in Figure 1. There is a slight inhibition percentage difference by ethanol extract of red beetroot at 200 µg/ml up to 1000 µg/ml (in the range of 50%). The inhibition percentage difference in 200 µg/ml extract is approximately 2-folds higher than that of 100 µg/ml extract.

The results among allopurinol groups obtained (there were no statistically significant differences between the five levels of allopurinol concentrations) are shown in Figure 2. It shows that the inhibition percentage of XO enzyme activity from a concentration of 100 µg/ml to 1000 µg/ml has a slight difference in value (around 60%). It also shows a decrease in the inhibition ability by allopurinol and the increase in concentration, but there is an increase from 100 to 200 µg/ ml.

Allopurinol and the ethanol extract of red beetroot can inhibit XO and there were no statistically significant differences between these two groups in this study. Figure 3 shows that the inhibition percentage difference by red beetroot extract and allopurinol is not much different. The greatest inhibition of XO by allopurinol was 67.02% at 200 µg/ml. In comparison, the most extensive inhibition of XO enzyme activity by red beetroot extract was 55.11% at a concentration of 1000 µg / ml. Figure 3 also shows that a higher concentration of red beetroot extract is needed to achieve inhibition XO

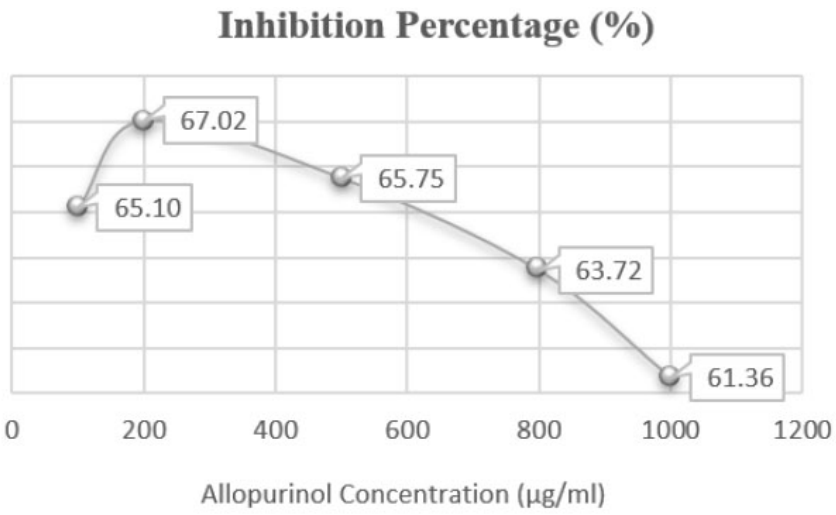

Figure 2. Inhibition of xanthine oxidase (XO) by allopurinol.

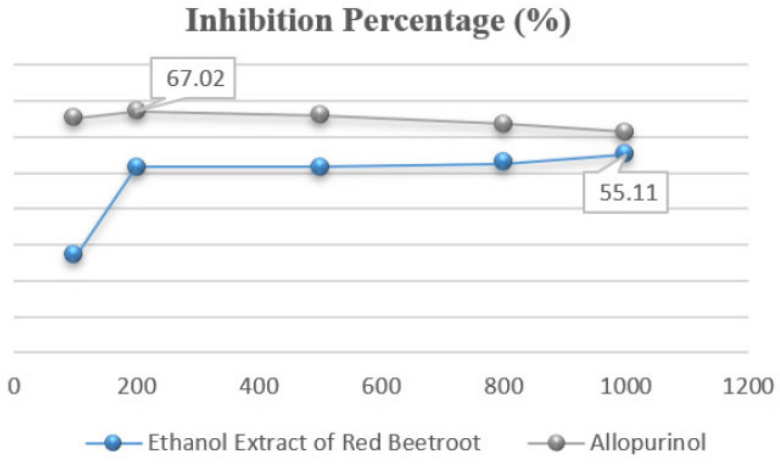

Figure 3. Comparison of inhibition XO by ethanol extract of red beetroot and allopurinol.

ability equivalent to allopurinol.

DISCUSSION

Xanthine Oxidase (XO) Activity

The activity of the XO in this study was observed spectrophotometrically at ë of 260-300 nm range. The UV-Vis spectrophotometer used to measure the amount of the final product (absorbance), namely uric acid, resulting from the reaction between XO with xanthine as a substrate, which is a compound with a strong chromophore group and can absorb UV and visible light.[17]

The maximum absorbance wavelength that shows the most optimum activity of XO was 285 nm in this study. This wavelength belongs to a ë range of 262-295 nm, as found in some studies. The average absorbance value, which describes the amount of uric acid product without the test sample or inhibitor, in this study was 0.7604.

The differences in wavelength and absorbance values obtained between various studies are caused by several factors, such as the number and concentration of analytes in the cuvette, the type of solvent used, the thickness of the cuvette, the distance of light passing through the cuvette, temperature, pH, and technical errors at the time of measurement or instrument errors, such as light leaks.[18]

Inhibition of Xanthine Oxidase (XO) by Ethanol Extract of Red Beetroot (*Beta vulgaris L.*)

The inhibition study of XO by ethanol extract of red beetroot using five different concentrations and three replications was conducted in a spectrophotometer ë 285 nm so that the optimal extract concentration that can inhibit XO and the effect of increasing extract concentration on XO activity can be known. The inhibition percentage of XO by ethanol extract of red beetroot can be seen in Figure 1. Red beetroot ethanol extract can inhibit XO activity from 100 µg/ml (26.76%) and exhibit the greatest inhibition at 1000 µg/ml (55.11%).

The results in this study (i.e., XO activity inhibition by red beetroot ethanol extract) are in line with studies conducted *in vivo*.[13,14] The ethanol extract of red beetroot in inhibiting XO activity is probably due to the flavonoids and betalain content in red beetroot. Flavonoids have a benzopyran ring structure similar to xanthine so that flavonoids can occupy the active site of XO and render the substrate unable to settle the area. This mechanism ultimately leads to the inhibition of uric acid synthesis.[11,19] Betalain as an antioxidant may also inhibit XO activity by transferring hydrogen or electrons to oxygen so that the xanthine oxidation reaction cannot occur.[6,13,20] Several factors that can cause the results of this study and the difference in the inhibition percentage are the number and concentration of different analytes, temperature and incubation time, pH, as well as technical errors during testing, such as the use of pipettes and dilutions.[18]

Temperature, incubation time, and pH can affect enzyme activity and inhibition of XO enzyme activity by the test sample. The increase in temperature and pH can cause the absorbance to decrease, probably due to the enzyme denaturation process resulting in the conformational changes of the enzyme and accompanied by loss of enzyme catalytic ability.[21,22]

The study on the effect of various incubation times on the XO activity is currently lacking. In a previous study, absorbance measurements were carried out every 10 minutes for 40 minutes, and obtained absorbance results showed an increase with an extension of time.[3]

These results can be connected to this study where the addition of reagents, such as the XO and xanthine in the test solution, the vortex process, and sample measurements on the spectrophotometer between each test samples were not carried out simultaneously but gradually starting from low to a high concentration. Therefore, the incubation start time and the reaction for each sample may be different in this study. Some of the beetroot samples in this study may start the reaction first with the measurement time on the spectrophotometer more delayed than the other concentrations sample and result in a more considerable absorbance value, therefore affecting the percentage of inhibition of XO activity. Moreover, the incubation of samples in this study was carried out directly at 37^0C for 15 minutes and 30 minutes in an incubator and pH 7.5, therefore there is a possibility that the temperature and pH are not optimum for the enzyme.

Inhibition of Xanthine Oxidase (XO) by Allopurinol

As a positive control and comparator, allopurinol consists of five different concentrations, examined in triplicates using a spectrophotometer at ë 285 nm. The inhibition percentage of XO with allopurinol shown in Figure 2. Allopurinol can inhibit XO starting from 100 µg / ml (65.10%) and has the greatest XO inhibition ability at 200 µg/ml (67.02%). This result is different from other similar studies. A study showed that allopurinol could inhibit the XO enzyme by 50% at a concentration of 287.82 ppm.[23] Inhibition of XO by allopurinol occurs due to the structural similarity between allopurinol and xanthine substrate. Therefore, allopurinol can occupy the active site of XO, inhibits XO activity and uric acid synthesis.[24,25] These results differ from other studies that an increase in the concentration of allopurinol will lead to an increase in the inhibition ability. However, other studies were conducted with different variations in concentration of allopurinol compared to this study. There was no study with the same concentration variation as this study.

Several factors that can cause differences between each study are the concentration of the enzyme and substrate used, the measurement wavelength, the difference in dilution, temperature, pH, and the type of allopurinol used. Some of the factors that may cause the results of this study and the decrease in the inhibition ability by allopurinol are temperature, incubation time, pH, and technical errors during testing, such as pipette use and dilution.[18]

Enzyme and substrate concentrations can affect enzyme activity. It can be explained by the Michaelis Menten kinetics, where an increase in substrate concentration will cause an increase in enzyme activity and reaction speed so that an increase in the number of enzyme-substrates will also occur. Increasing substrate concentration will not increase the enzyme activity if the maximum speed (Vmax) has been reached. The enzyme is already saturated with the substrate and the free enzymes to form enzyme-substrate complexes are no longer available. The kinetics can be linked to this study where the enzyme may be in a saturated condition, then cause the inhibition ability of XO by allopurinol to remain around 60%.[21]

The percentage of maximum XO inhibition by allopurinol dose of 100 mg and the concentration of uric acid formed by the reaction of 0.15 mM xanthine substrate and 0.2 U/ml XO in this study need to be identified. Calculation of uric acid concentration can be conducted using the Lambert-Beer law with a wavelength of 290 nm, a pH of 7.5, and a cuvette width of 1 cm. The results of the calculation can predict whether the inhibition ability by allopurinol in this study is optimal. The uric acid concentration could not be measured in this study due to the different wavelengths used.[2,18]

Comparison of Inhibition of Xanthine Oxidase (XO) by Ethanol Extract of Red Beetroot (*Beta vulgaris L.*) and Allopurinol

Allopurinol and the ethanol extract of red beetroot can inhibit XO and there were no statistically significant differences between these two groups in this study. This result might be due to the similar red beetroot and allopurinol mechanism in inhibiting XO, i.e., occupying the enzyme's active site. Figure 3 shows that a higher concentration of red beetroot extract is needed to achieve inhibition XO ability equivalent to allopurinol.[11,19]

CONCLUSION

The ethanol extract of red beetroot (*Beta vulgaris L.)* can inhibit xanthine oxidase (XO) activity *in vitro*. Although there are many XO inhibitor drugs, continuous studies should optimize the natural ingredient of red beetroot (*Beta vulgaris L.*). Researchers expect further research to perfect this extract study to benefit patients who suffer from persistent gout.

ACKNOWLEDGEMENT

The authors would like to thank Dr. dr. Bagus Komang Satriyasa, M. Repro and PT. Sky Pacific Indonesia for their helpful guidance and technical supports during the conduct of the current study.

CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

FUNDING

The authors receive no grants during this study conducted.

AUTHOR CONTRIBUTIONS

Gita Almira Putri conducts the data collections/acquisitions and analyze them; Agung Nova Mahendra is the supervisor and a guarantor, provides detail concepts and designs; I Made Jawi is the second supervisor, provides detail concepts, and actively reviews the manuscripts.

ETHICAL CLEARANCE

Number: 124/UN14.2.2.VII.14/LP/2020

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