

Anti-anemia Effect of Chlorophyll from *Katuk (Sauropus androgynus)* Leaves on Female Mice Induced Sodium Nitrite

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ABSTRACT

Context: Sodium nitrite (NaNO₂) in blood is highly reactive with haemoglobin (Hb), thus affecting hematopoiesis and induction of methemoglobinemia.

Aim: This study was conducted to determine the effect of chlorophyll from *katuk (Sauropus androgynus)* leaves on the level of Hb, Malondialdehyde (MDA), ferritin, and schistocytes percentage in female mice induced NaNO₂. **Settings and Design:** Experimental research was conducted using 24 female mice strain Balb-c. **Methods and Material:** NaNO₂ 0.3 ml/head/day given during 18 days, while the chlorophyll or Cu-chlorophyllin as much as 0.7 ml/head/day given the following day for 14 days. **Statistical analysis used:** Results are reported as mean values ± SD and statistically analyzed by One Way Anova test with 95% significance level. **Results:** The Hb levels of blood plasma in the control group, NaNO₂ induction, induction NaNO₂ and chlorophyll of *katuk* leaves (NaNO₂+*katuk*), induction of NaNO₂ and Cu-chlorophyllin from K-Liquid™ (NaNO₂+Cu-chlorophyllin) in sequence is 13.29 g/dl; 11.83 g/dl; 14.54 g/dl; 13.99 g/dl, whilst the MDA levels in each group is 2.10 ± 0.11 mol/L, 3.44 ± 0.38 mol/L, 2.31 ± 0.18 mol/L, 2.31 ± 0.13 mol/L, and the ferritin levels is 62.71 ± 6.42 ng/ml; 63.22 ± 7.59 ng/ml; 67.45 ± 8.03 ng/ml, and 64.74 ± 7.80 ng/ml, respectively. The fragment schistocytes percentage's in each group is 0%, 0.11%, 0.01%, 0.03%.

The ferritin levels tend to increase in NaNO₂+*katuk*. Mann Whitney test results obtained no significant difference in Hb, MDA level and schistocytes percentage between the groups of mice that received NaNO₂+*katuk* NaNO₂+Cu-chlorophyllin (*p*>0.05). This indicates that chlorophyll from *S. androgynus* leaves as effective as Cu-chlorophyllin in decrease the MDA levels after NaNO₂ treatment, and although not significant, it can increase ferritin levels. **Conclusion:** The antioxidant activity of chlorophyll from *katuk* leaves are able to decrease schistocytes percentage's and MDA level. The increasing of Hb and ferritin level indicates its potential in the treatment of haemolytic anaemia. Further studies aimed at the mechanisms of action of this chlorophyll are needed.

Key words: Anemia, Chlorophyll, Ferritin, Sodium nitrite, Schistocytes.

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INTRODUCTION

Sodium nitrite (NaNO₂) is an inorganic salt used as a common preservative and color fixative in cured meat products, fish and some types of cheese and occurs naturally in many foods, particularly vegetables. Recent studies about toxicology of NaNO₂ show that sodium nitrite in blood is highly reactive with hemoglobin, thus affecting hematopoiesis and induction of methemoglobinemia—a condition in which there is a reduction in hemoglobin's ability to transport oxygen.¹ The adduction of NaNO₂ in acute doses significantly decrease the number of erythrocyte causing a state of anaemia in rat.² Therefore, it important to prevent the anemia due to the negative effects of NaNO₂.

The rate of anaemia occurrence in Indonesia is considerably high. According to annual report of Dinkes Prov. Jateng (2013), the rate was 40.1% in pregnant mother in 2013. The percentage of anaemia prevalence in women is higher than that of men, that was 23.9%.³ The condition of anaemia that is not treated immediately will lead to complications such as cardiomegaly since erythrocytes and blood viscosity decreased causes an increase in cardiac output and blood flow as a result of tissue hypoxia.⁴ Prevention of anaemia has been undertaken by the government through the provision of iron supplementation.⁵ However, the use of the supplementation emerge side effects such as less comfortable circumstances in the pit of the stomach, nausea, vomiting, and constipation. Therefore, it is needed an anaemia therapy with natural ingredients, one of which is using chlorophyll of plants.

In Indonesia, *Katuk (Sauropus androgynus)* leaves are often consumed as a breastmilk booster. The leaves contain plenteous chlorophyll. Nurdin

et al. (2009) notified that 8% of the leaves dry weight contain chlorophyll-a and chlorophyll-b as much as 1136.6 mg/kg and 372.5 mg/kg of the substances, respectively.⁶ Porphirin structure of the chlorophyll can reduce *diphenilpicrylhydrazil* (DPPH) free radical form *diphenilpicrylhydrazine* that is non radical.⁷ Heme structure of the chlorophyll can bind a Magnesium (Mg) ion acting as an iron ion in human and animals.⁸ However, a role of chlorophyll as antioxidant with parameter hemoglobin (Hb), *Malondialdehyde* (MDA) and ferritin level, also schistocytes percentages in experimental animals induced by NaNO₂ as pathological treatment for anaemia have not been investigated.

MDA is an end product of lipid peroxidation.⁹ State of increasing oxidative stress in the body induces the increase of MDA level as well. Oxidative stress which is uncompensated in normal erythrocyte will bring in hemoglobin oxidation become methemoglobin and membrane ravage in the form of uplift of membrane permeability to cell lysis.¹⁰ This condition causes the level of iron (Fe) stock in the body decrease progressively, reflected by deflation of ferritin concentration in serum.¹¹ Examination of routine ferritin level is carried out to determine diagnosis of iron deficiency, because it is proven that ferritin level as an earlier indicator declines in the condition where anaemia of iron deficiency occur.¹² Schistocytes are fragments of red blood cells (RBCs) produced by extrinsic mechanical damage within the circulation. The detection of schistocytes is an important morphological clue to the diagnosis of thrombotic microangiopathic anemia (TMA).¹³

This study aimed to examine the effect of chlorophyll in anaemic female mice induced NaNO₂ during 18 days. Antianemia effect of chlorophyll is observed after *Katuk* leaves treatment for 14 days by measuring

hemoglobin, MDA and ferritin levels of blood plasma, also the percentage of schistocytes in blood smear. The result of this research is expected to encourage the use of *Katuk* leaves chlorophyll as anaemia drug.

SUBJECTS AND METHODS

Material

Fresh leaves of *katuk* (*Sauropus androgynus*) (Figure 1) were obtained from the inhabitant park in Penggaron Lor Village, Genuk, Semarang, Indonesia. All chemicals were of analytical grade. Cu-chlorophyllin from K-Liquid™ was obtained from a drug store in Semarang. Ethical clearance was obtained from the Ethics Committee of the Faculty of Medicine Universitas Islam Sultan Agung, Indonesia.

Chlorophyll Extraction

Chlorophyll crude extract was prepared from *S. androgynus* leaves by extraction with acetone: methanol (7:3, v/v).¹³ A given amount of leaves (200 g) was mixed with acetone: methanol (7:3, v/v) and magnetically stirred until the pigment was removed. Ascorbic acid was added to the solution to avoid degradation of the pigment. The extract was filtered to remove insoluble material. The filtrate obtained was then partitioned with diethyl ether three times sequentially. The organic phase material resulted were added with Na₂SO₄ anhydrate as far as water contained was bound maximum. The solvent removal and drying process of chlorophyll used rotary evaporator and N₂ gas.

Experimental design

The experiments were carried out on two-month-old female Balb-C mice. The animals were divided into three sodium nitrite-treated groups (n=6 in each group) and an age-matched control group (n=6). Mice were maintained in the animal house of Faculty of Medicine Universitas Islam Sultan Agung in standard hard bottom polypropylene cages at 23°C ± 2°C, 12:12 h light/dark cycle and free access to laboratory chow and tap water throughout the study.

NaNO₂ was administered orally by gavage at 3.75 mg/kg body weight (dissolved in 1 ml aquades), refers to rate of LD₅₀ on mice.¹⁴ NaNO₂ as much as 0.3 ml/g of body weight were given to the mice for 18 days for treated animals (group II, III and IV).¹⁵ The control rats were injected with the same volume of distilled water. The following days after day 18 until day 32, the mice from group III were given with chlorophyll solution from *S. androgynus* leaves, whereas the mice from group IV were given with Cu-chlorophyllin from K-Liquid.™ The doses of chlorophyll and Cu-chlorophyllin those were given to mice were 0.7 ml/head/day according to the conversion of adult man doses.¹⁶

Blood collection and plasma analysis

Blood samples from the mice were taken at the day 33 to measure Hb, MDA and ferritin level in the blood plasma. Whole blood samples were obtained in heparinized tubes, centrifuged and plasma was stored at -20°C until further analysis. Hb level was measured by cyanmethemoglobin methods using spectrophotometer MDA level was measured using Thio Barbituric Acid Reactive Substance (TBARS) test with 532 nm wavelength spectrophotometer, whilst ferritin level was measured using Enzyme Linked Immunosorbent Assay (ELISA) method. Schistocytes are detected in the peripheral blood smear stained using wedge procedures and observed by microscopy in 100X every 1000 erythrocyte.

Statistical analysis

Results are reported as mean values ± SD and statistically analyzed by One Way Anova test with 95% significance level. If the data characteristics did not allow for the One Way Anova test to be conducted, then the Kruskal Wallis test became the alternative. A post hoc test was conducted where needed.



Figure 1: *Katuk* (*Sauropus androgynus*).

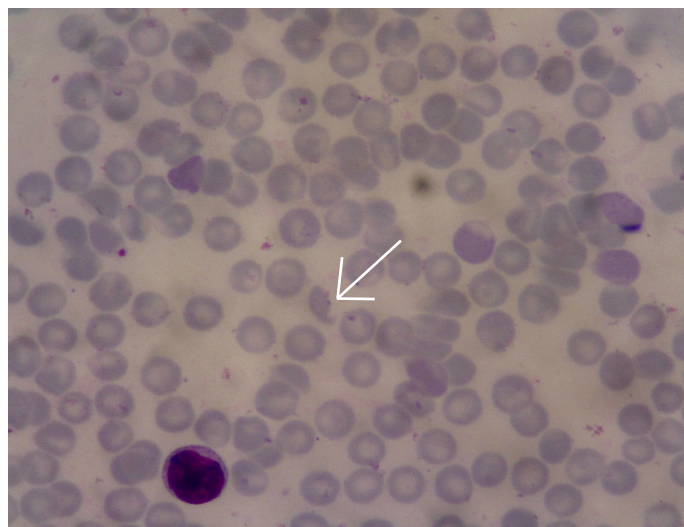


Figure 2: Typical shapes for specific identification of schistocytes showed a polychromatophilic erythrocyte in the white arrow.

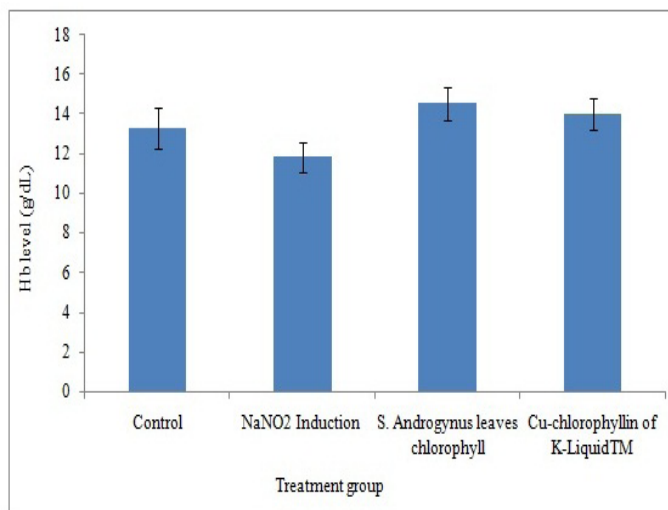


Figure 3: Hb levels in four treatment groups after 18 days NaNO₂ treatment followed by 14 days chlorophyll from treatment. *S. androgynus* leaves or Cu-chlorophyllin of K-Liquid™.

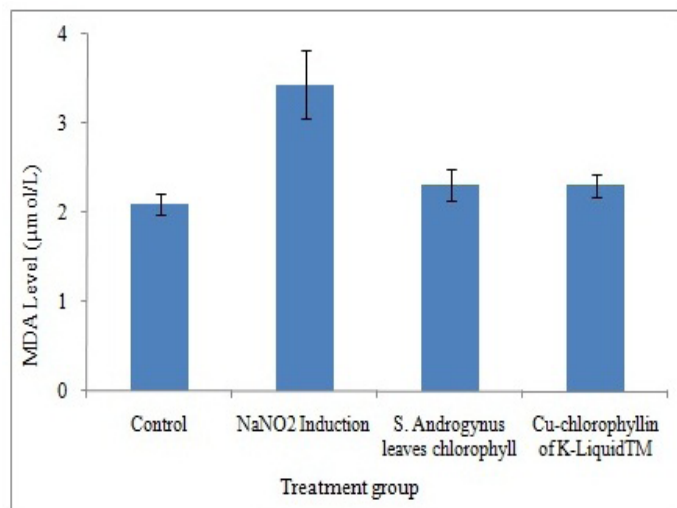


Figure 4: MDA levels in four treatment groups after 18 days NaNO₂ treatment followed by 14 days chlorophyll from treatment. *S. androgynus* leaves or *Cu-chlorophyllin* of K-Liquid TM.

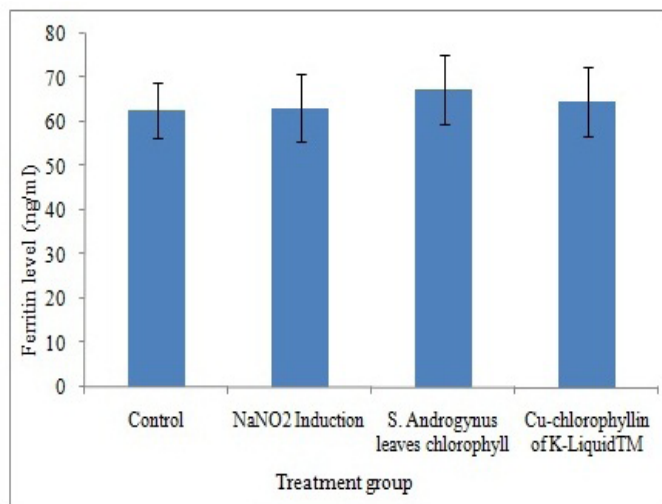


Figure 5: Ferritin levels in four treatment groups.

Table 1: Means of MDA and ferritin levels in four treatment groups

Treatment group	Hb level (g/dL)	MDA level (µmol/L)	Ferritin level (ng/ml)	Schistocytes (%)
Control	13,29 ± 1,06 ^a	2.10 ± 0.11 ^a	62.71 ± 6.42 ^a	0 ± 0 ^a
NaNO ₂ induction	11,83 ± 0,77 ^b	3.44 ± 0.38 ^b	63.22 ± 7.59 ^a	0,11 ± 0,07 ^b
<i>S. androgynus</i> leaves chlorophyll	14,54 ± 0,84 ^a	2.31 ± 0.18 ^a	67.45 ± 8.03 ^a	0,01 ± 0,04 ^a
Cu-chlorophyllin from K-Liquid TM	13,99 ± 0,81 ^a	2.31 ± 0.13 ^a	64.74 ± 7.80 ^a	0,03 ± 0,05 ^a

NB: Superscripts in different letter showed the significantly different at a 5%.

RESULTS

The hemoglobin (Hb) decreased after NaNO₂ induction treatment group for 18 days. In contrast, mean of, MDA, ferritin level, and schistocytes percentages in mice which were induced with NaNO₂ induction increased draw on the control group level (Table 1). Control group showed normal erythrocytes, whereas in the group induced NaNO₂ found schistocytes (Figure 2). *Katuk* chlorophyll and Cu-chlorophyllin group showed the lower schistocytes percentage compared with the group with the induction of NaNO₂.

Adduction of *Katuk* leaves chlorophyll and Cu-chlorophyllin proven to increase Hb level in mice. Although it has been induced by NaNO₂ (Figure 3). Adduction of *Katuk* leaves chlorophyll and Cu-chlorophyllin proven to reduce MDA level in mice. Although it has been induced by NaNO₂, the levels were approaching the level of MDA in the control group after treatment, even Mann Whitney statistical test shown that MDA level of *Katuk* leaves treatment was not different from the level of control group ($p > 0.05$). Moreover, MDA level of *Katuk* leaves was equal to that of Cu-chlorophyllin from K-Liquid, with the statistical test result shown that there was no differences between them ($p > 0.05$, Figure 4), proving that chlorophyll from *Katuk* leaves as effective as chlorophyll from market products. One Way Anova test resulted that there were no differences in ferritin levels between all groups. However, the level in *Katuk* leaves (67.45 ± 8.03) tend to be higher than those of control group and other groups, followed by Cu-chlorophyllin from K-Liquid (64.74 ± 7.80) (Figure 5).

DISCUSSION

The present study demonstrates the effect of acute NaNO₂ treatment caused a significantly reduced decrease in hemoglobin level, as well as

increased MDA, ferritin level and schistocytes percentages. It is suggested that the decrease in hemoglobin level may be attributed to microcytic and/or hypochromic anemia possibly as consequence of the toxic effect of NaNO₂. Sodium nitrite (NaNO₂) can trigger oxidative stress condition which is indirectly caused haemolytic anaemia. It can be seen from the mean of *Malondialdehyde* (MDA) level in control group compare to those of the other groups that were induced by NaNO₂. Adduction of NaNO₂ leads free radical formation from nitrite ion, and then the reaction continues to form lipid peroxidase and produce one of some products in the form of MDA. Sodium nitrite is one of substances that are classified as moderately toxic based on LD₅₀. Toxic effect from NaNO₂ in this research was demonstrated by death of 4 mice which experiencing skins discoloration, feathers loss and the eyes excreting ichor.¹⁷ The decrease of erythrocyte is caused by lysis of membrane structure of the erythrocyte by dint of nitrite ion and nitrite metabolism and as a product of lipid peroxidase which reacted with sulfhydryl group from fat layer and protein components of erythrocyte membrane. Nitrite triggers the formation of free radicals due to its ability to stimulate iron ion oxidation on the oxyhaemoglobin forming methemoglobin as ROS.^{18,19} Therefore, anaemia that comes up is not iron deficiency anaemia, but rather haemolytic anaemia.

In case of haemolytic anaemia, receptor transferrin serum increase when bone marrow erythropoiesis activities increase as well and found no functional iron depletion, it means the level of serum ferritin is normal or increase. Ferritin is an acute-phase protein, so that the value increases in inflammation condition, in pre-latent phase it is familiar known as iron depletion or storage iron deficiency. At this state, iron stock decrease, but the iron in plasma and erythrocyte is still normal.²⁰

Excessive oxidant compounds induce a chain reaction of free radicals or lipid peroxidase which through several processes to form MDA. Oxidant

compounds which successfully suppressed will not cause a continued reaction or lipid peroxidase, so that the MDA level will decline. Oxidative stress circumstances of NaNO₂ as anaemia pathologic treatment in female mice can be mitigated by antioxidant activities of *Katuk* leaves chlorophyll which MDA level of *Katuk* leaves chlorophyll treatment group is identical to that of the control group.

Katuk leaves chlorophyll treatment can increase ferritin level in mice, although statistically there was no significant difference. Among other treatment groups, *Katuk* leaves treatment group gave the highest level of ferritin compare to the control group. The increase equal to the level of Cu-chlorophyllin from K-Liquid, however there was no significant difference between them. *Katuk* leaves chlorophyll potentially can be used as antioxidant, because it is able to prevent the formation of oxidant compound excessively.⁹ In the state of infection and inflammation, it occur disruption of iron release from reticuloendothelial cells, consequently, the level of ferritin intracellular and serum increase.²¹ Copper (Cu) of Cu-chlorophyllin is a micro essential substance which serves as part of the enzymes in the body and plays a role in iron absorption. Copper of seruloplasmin (a glycoprotein synthesized in liver) contribute in Fe oxidase process produce other copper fmetaloproteins i.e. cytochrome oxydase, tironase, monoamin oxydase, superoxide dismutase (SOD) and lysyl oxydase before transported into plasma.²²

This is in accordance with Togatorop (2013) revealed that chlorophyll of *Katuk* leaves can relieve toxic chemicals caused by cigarette smoke including free radicals in the body. Antioxidative effect of chlorophyll coming from porphyrin structure that is tetrapirrol shaped and conjugated polyenes which can capture oxygen singlet²³ along with the existence of Mg ion that makes free radicals tend to give electrons to the Mg, thus neutralize free radicals.²⁴ As a metaloenzim, SOD activities depend on the presence of Cu, Zn, and Mn metals. Copper in erythrocyte have the form of dismutase superoxide metaloenzyme that act as antioxidants.²⁵ Treatment of *Katuk* leaves chlorophyll can decrease *Malondialdehyde* (MDA) level. The decrease of MDA level in *Katuk* leaves treatment was very effective and significant draw on negative control group. The decline is also almost the same as that of the Cu-chlorophyllin treatment group as a positive control group. According to this, chlorophyll of *Katuk* leaves potentially can be used as an alternative for healing anaemia caused by oxidative stress. The point must also be considered for future research is necessary to assess the level of oxidative stress that is caused by NaNO₂ induction. Besides, it is needed to measure quantitatively the level of chlorophyll from *Katuk* leaves extract and Cu-chlorophyllin from K-LiquidTM along with assessment on ferritin level in latent phase of anaemia.

CONCLUSION

The results of this study demonstrate the potential of chlorophyll from *katuk* (*Sauropus androgynus*) leaves as antioxidant caused by oxidative stress due to NaNO₂ induction as indicated by increasing of MDA level. Furthermore, our studies also indicate that the *katuk* chlorophyll may also be useful in the treatment of haemolytic anaemia, indicated by its ability to decrease Schistocytes percentages, also increase Hb and ferritin level. Further studies aimed at the mechanisms of action of this chlorophyll are needed.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ABBREVIATION USED

MDA: Malondialdehyde; SD: Standard deviation; DPPH: Diphenylpicrylhydrazil; RBCs: Red blood cells; TMA: Thrombotic microangiopathic anemia; v/v: Volume per volume; LD₅₀: Lethal dose 50%; TBARS: Thio Barbituric Acid Reactive Substance; ELISA: Enzyme Linked Immunosorbent Assay.

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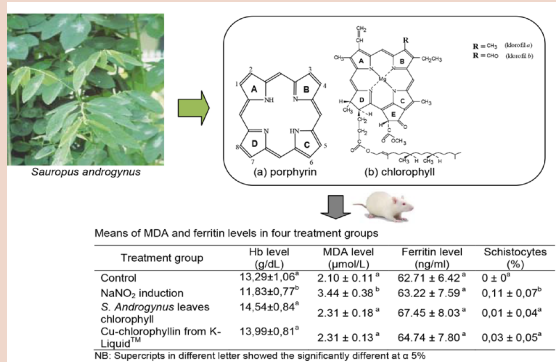
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PICTORIAL ABSTRACT



SUMMARY

- Sodium nitrite (NaNO₂) in blood is highly reactive with haemoglobin (Hb), thus affecting hematopoiesis and induction of methemoglobinemia.
- Chlorophyll from *S. androgynus* leaves was as effective as Cu-chlorophyllin in decrease the MDA levels after NaNO₂ treatment, and although not significant, it can increase ferritin levels.
- Chlorophyll from *S. androgynus* is potential as food supplement in anemic conditions caused by sodium nitrite consumptions.

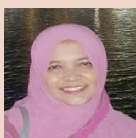
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Suparmi: Is a lecturer and researcher at Department of Biology, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang-Indonesia. She is passionates in research on chlorophyll and natural pigments, especially its application for food, industry and health application. Since 2013, she is a member of Indonesian Pigment Researcher Association and Indonesian Food Technologists. Currently, she is a PhD student studying food toxicology in Wageningen University, The Netherlands.



Sampurna: Is positioned as a member of Department of Clinical Pathology, also a lecturer and researcher at Faculty of Medicine, Universitas Islam Sultan Agung. He is also as a deputy director of Sultan Agung Islamic Hospital. He is working on various aspects of tropical medical pathology specialty that is concerned with the diagnosis of diseases based on the laboratory analysis of bodily fluids, such as blood and urine.



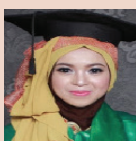
Nur Ana CS: A lecturer and researcher at Department of Internal Medicine, Faculty of Medicine, Universitas Islam Sultan Agung. She is also an internist at Sultan Agung Islamic Hospital. She has experience in the area of tropical internal diseases and frequently become a guest speaker on national radio talkshows.



Alvenia Meilina Ednisari: Was a student at Universitas Islam Sultan Agung, Semarang-Indonesia, where she graduated in Bachelor of Medicine. Her research focused on the effect of *katuk* leaves (*Sauropus androgynus*) on haemoglobin level in sodium nitrite-induced mice (*Mus musculus* Balb/c).



Galuh Dea Urfani: Was a student at Universitas Islam Sultan Agung, Semarang-Indonesia, where she graduated in Bachelor of Medicine. Her research focused on the effect of *katuk* leaves (*S. androgynus*) and Cu-Chlorophyllin on malondialdehyde level as an experimental assessment on sodium nitrite-induced mice.



Iqrommatul Laila: Was a student at Universitas Islam Sultan Agung, Semarang-Indonesia, where she graduated in Bachelor of Medicine. Her research focused on the comparison of *katuk* leaves (*S. androgynus*) and Cu-Chlorophyllin effects on ferritin level on sodium nitrite-induced mice.



Heavin Rakhmat Saintika: Was a student at Universitas Islam Sultan Agung, Semarang-Indonesia, where she graduated in Bachelor of Medicine. His research focused on the effect of *katuk* leaves (*S. androgynus*) and Cu-Chlorophyllin on the fragment schistocyte percentages on sodium nitrite-induced mice.