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Research Article

Natural Antioxidants Effect on the Performance of Erythrocytes, Leucocytes, and Hb Levels of Pregnant Mice Exposed to the Plastic

Endah Sri Palupi[1,2*], Eko Setio Wibowo[3], Iga Ayu Ratna Puspita Sari[4], Atang[5]
[1] Animal Structure and Development Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia
[2] Research Center of Biodiversity and Marine, Research and Community Service Institute (LPPM), Universitas Jenderal Soedirman, Purwokerto, Indonesia
[3] Physiology Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia
[4] Animal Taxonomy Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia
[5] Animal Structure and Development Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia
*E-mail:endah.sri.palupi@unsoed.ac.id

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ABSTRACT
Plastic is a widely used material containing several toxic components, such as free radicals. HDPE plastic is considered safer but requires more attention due to its increased usage. Exogenous antioxidants are needed to neutralize free radicals. This research aimed to evaluate the effect of natural antioxidants on the performance of erythrocytes, leucocytes, and hemoglobin levels in pregnant mice exposed to plastic compounds. The study used a completely randomized design with six groups and four replicates each. These groups were aquadest control, plastic chemical compounds (PCC) control, PCC + carrot juice, PCC + orange juice, PCC + purple sweet potato juice, and PCC + mung bean sprouts juice. The PCC was obtained from boiling HDPE plastic, while natural antioxidant juice was derived from pure sources. Pregnant mice were treated with 0.25 mL of each substance orally for 21 days, starting from the first gestational day. Blood was collected from a caudal vein. Erythrocytes and leucocytes were counted using a hemocytometer, and Hb levels were measured by the Sahli method. Blood cell morphology was assessed using Giemsa-stained blood smears. Statistical data were analyzed using one-way ANOVA with GraphPad Prism ver.10.0 software, while morphology data were analyzed descriptively. The results showed that PCC and natural antioxidants did not significantly impact the number of erythrocytes, leucocytes, cell morphology, and Hb levels in pregnant mice. However, natural antioxidants may stabilize erythrocyte counts and Hb levels after treatment.

Keywords: *Erythrocytes;HDPE plastic; Hemoglobin; Leukocytes; Natural antioxidants.*

Introduction

Plastic poses problems due to its widespread use and harmful chemical components, such as bisphenol A (BPA) and phthalates [1]. These substances have proven adverse effects on human health [1], causing issues such as cardiovascular, reproductive, respiratory, and gastrointestinal systems problems [2], as well as birth defects and cancer [1]. Previous research demonstrated that High-density Polyethylene (HDPE) microplastic exposure causes malformation in larval Pacific oysters (*Crassostrea gigas*). Smaller HDPE microplastic sizes result in higher rates of larval defects. Moreover, HDPE microplastic has been shown to induce anomalies in swimming behaviour [3] and inhibit larval development in Eastern oysters (*Crassostrea virginica*) [4].

Nowadays, several types of plastic exist based on their raw materials and use. Polystyrene (PS) and Polyvinyl Chloride (PVC) are harmful and should be avoided for food storage. On the other hand, polyethylene terephthalate (PET) is a disposable material that can only be used once, posing less harm when guidelines are followed. This type is less harmful as long as it complies with the guidelines. Furthermore, some plastic types are safe for human daily use. These are HDPE, Low-density Polyethylene (LDPE), and Polypropylene (PP) [1], [2].

HDPE plastic is considered safer for human health due to the absence of BPA and phthalate [2]. Considering its safety, HDPE plastic has become the main choice for humans; thus, it has high frequencies to use. However, HDPE plastic is composed of ethylene monomers derived from petroleum [5]. Besides that, all plastic types involve chemical reactions during production [6], which may affect human health, including hematology aspects. Studies have shown that polyethylene microplastics have genotoxic effects on human lymphocytes [7]. Moreover, the microplastic of HDPE and PS plastics alters gene expression in the immune system [8].

One of the dangerous substances from plastic is free radicals that may become reactive oxygen species (ROS) that induce several diseases [9]. Therefore, exogenous antioxidants from natural resources may protect cells and tissue from free radicals released by plastics. Several natural resources contain antioxidant properties, such as carrots (*Daucus carota*) with carotenoid [10], oranges (*Citrus* sp.) with vitamin C [11], purple sweet potatoes (*Ipomoea batatas*) [12], [13], and mung bean (*Vigna radiata*) sprouts [14], [15]. Those natural antioxidants may play a role against free radicals produced by plastic monomers. Therefore, this research aims to investigate the natural antioxidant effect on erythrocytes, leucocytes, Hb, and its morphology after plastic chemical compound treatments in pregnant mice.

Materials and Methods

Animals

The procedures were carried out at the animal house of the Faculty of Biology, Universitas Jenderal Soedirman, with ethical approval (certificate number: 8080/KEPK/XI/2019). Twenty-four pregnant mice (*Mus musculus*), aged eight weeks and weighing 20-30 g, were used. Mice were confirmed pregnant by the presence of a vaginal plug.

Pregnant mice were obtained by mixing male and female mice in a ratio of 1:4. Female mice have been confirmed in the estrus phase by vaginal smear method using methylene blue staining. The subsequent step was observing the presence of a vaginal plug the following morning, a day after mating, to evaluate the presence of gestation. This time was the first day of the treatment.

The study used a Completely Randomized Design with six groups: aquadest control (C-1), PCC control (C-2), PCC + carrot juice (T-1), PCC + orange juice (T-2), PCC + purple sweet potato juice (T-3), and PCC + mung bean sprouts juice (T-4). PCC was prepared by boiling 100 g HDPE plastic in 1000 mL aquadest until 100 mL remained. Natural antioxidant juices were prepared from pure sources. Treatments (PCC and juices) were given orally (0.25 mL) daily (09.00-08.00 a.m.) for 21 days. The mice were fed a standard diet *ad libitum*. Blood samples were collected from the caudal vein on day 22.

Sample preparation and cell counting

The procedure of counting the number of erythrocytes [16] and leucocytes [17] follows these procedures. Both methods have similar steps, excluding the pipetting scale and dilution solution. The red blood cells were diluted using Hayem solution, while leucocytes were used in Turk solution. By using a Thoma pipette, blood from the caudal vein was sucked up to 0.5 mark, then Hayem solution up to 101 mark for erythrocytes counting. Whereas it was sucked up to 0.5 mark and Turk solution up to 11 mark for leucocyte counting. Afterwards, the fluid in the Thoma pipette was homogenized by gently mixing. Three drops first were discarded before loading to the hemocytometer. The erythrocytes on the five central squares (consisting of 25 central squares) were counted. Meanwhile, leucocytes were counted from four large squares at the corner. Subsequently, by using these formulas, the number of erythrocytes (Eq.1) and leucocytes (Eq.2) were counted. The number of erythrocytes and leucocytes was counted every week, starting from week 0, week 1, week 2, and week 3.

$$\text{Total erythrocytes count} = \mathit{The\ number\ of\ cells\ in\ 5\ squares} \times 10^4 \mathit{cells/mm^3} \quad \text{........ (1)}$$

$$\text{Total leucocytes count} = \mathit{The\ number\ of\ cells\ in\ 4\ squares} \times 50\ \mathit{cells/mm^3} \quad \text{......... (2)}$$

Hemoglobin level measurement

The Hb level measurement using the Sahli method is based on the conversion principle from hemoglobin to acid hematin. HCl 0.1 N was poured into a Hb tube up to the 20 mark. Blood from the caudal vein in the amount of 20 µL was pipetted using a Hb pipette, then poured into a Hb tube containing HCl 0.1 N. Subsequently, the Hb tube was placed in the column of Sahli's Comparator box for dilution using HCl 0.1 N up to colour matching between test and standard. The scale of the endpoint blood test was the Hb level. Hb level was measured every week, starting from week 0, week 1, week 2, and week 3.

Blood smear procedure

The blood was collected from the caudal vein using a syringe 23G the following day after the treatment (day 22). The blood smear procedures followed Bancroft's histological method [18]. Then, blood was dropped on the edge of the object glass; by using another object glass, blood was smeared in the opposite direction. After 10 – 15 minutes, the blood smear was fixed using methanol for 10 minutes. Giemsa staining was for blood smear dying. Subsequently, it was rinsed with running water and then air-dried. A blood smear slide was mounted with entelan (Merck; cat. no. 1079600500) and cover glass (Menzel coverslip 22 x 22). The morphology of erythrocytes and leucocytes was observed using a binocular microscope (Olympus). A blood smear was made at the end of the treatments.

Statistical analysis

The quantitative data were analyzed using one-way ANOVA (α=0.05), continued Duncan post-hoc test, while the morphological data was analyzed using descriptive statistics. Pearson Correlation determined the correlation between erythrocytes and Hb level. All the analyses and graphs were processed by using GraphPad Prism ver.10.0 software.

Results and Discussion

The mice used in this study were confirmed to be pregnant conditions by the presence of a vaginal plug (copulation plug). However, after 21 days of treatments, None of the mice delivered their babies. The mouse strain, age, and previous pregnancy may cause this condition [19]. For the information, the mice used in this study had not experienced any previous pregnancies. Another reason could be that no embryos developed due to the treatments. Additionally, the vaginal plug is not a definitive parameter for determining pregnancy in mice, as false pregnancies can exceed 50% [20].

The study of the effect of HDPE plastic on reproduction is limited, particularly its impact on Mammals. However, nano plastic from polystyrene might induce reproduction alterations and neonate malformation of *Daphnia magna*, a water organism [21]. In addition, another plastic-type, PET plastic, induced embryo abnormalities and oxidative damage in Zebrafish embryos [22]. While HDPE plastic induced Pacific oyster larva defect (*Crassostrea gigas*) [3]. Therefore, the use of HDPE plastic might be dangerous in long-term exposure. Despite this, it is believed to be a less harmful plastic than previously described types.

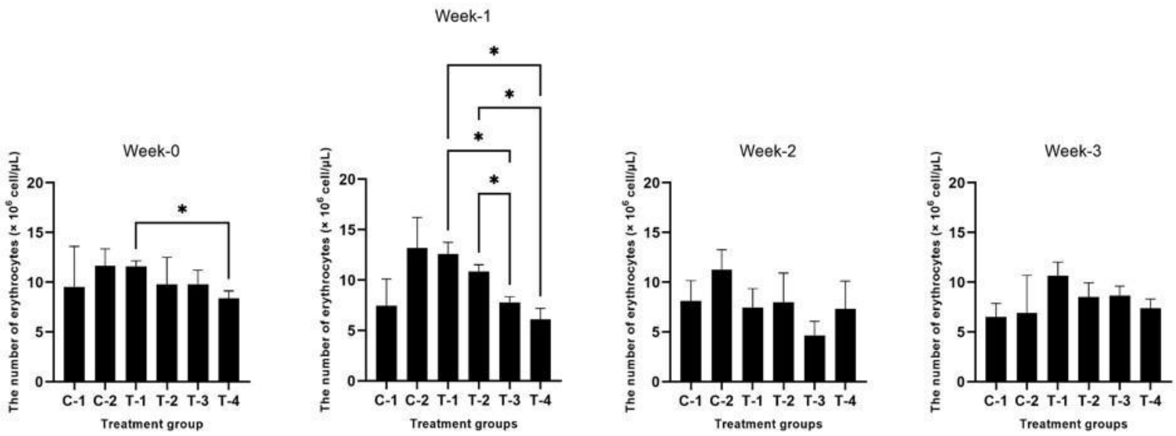

Figure 1. The number of erythrocytes each week among groups after plastic chemical compound and natural antioxidant juice treatment (ANOVA, *P<0.05).

The effect of natural antioxidants on the erythrocytes performance of pregnant mice exposed to the plastic chemical compound

The effect of natural antioxidants on the number of erythrocytes after plastic chemical compound treatment is shown in Figure 1. There were no significant differences among groups from the third week (Week 3) of treatment (P>0.05). A significant difference occurred in the second week among groups using natural antioxidants. Based on Figure 1, it is clear that the treatment group with carrot juice (T-1) tends to have a high number of erythrocytes, while other natural antioxidants tend to show similar results.

Principally, based on the research results, the number of erythrocytes was similar among groups. The natural antioxidants did not affect the number of erythrocytes after three weeks of treatment. This study used HDPE plastic, which is known to be a less harmful plastic-type [1], [2]. There is no reported information on its health effects [2]. This characteristic may influence the results. The limited data on the effect of HDPE compound from plastic on hematological performance is assumed due to its safety. The toxic compounds in plastic are usually caused by BPA and phthalates, which HDPE plastic does not contain [2]. The possible toxic substance is free radicals [9]. The natural antioxidants consumed may normalize the free radicals produced. Therefore, there are no differences in the total erythrocytes and morphology from treatment groups after three weeks of exposure compared to the previous data collected. The number of erythrocytes tends to be stable with the addition of natural antioxidants.

However, considering the high frequency of using HDPE plastic needs more concern. Previous research to prove HDPE safety has shown no impact on the organism. HDPE plastic did not affect earthworm gut microbiota [23] nor the growth and survival of yellow perch [24]. However, it may have side effects in fish after a long period of exposure [24].

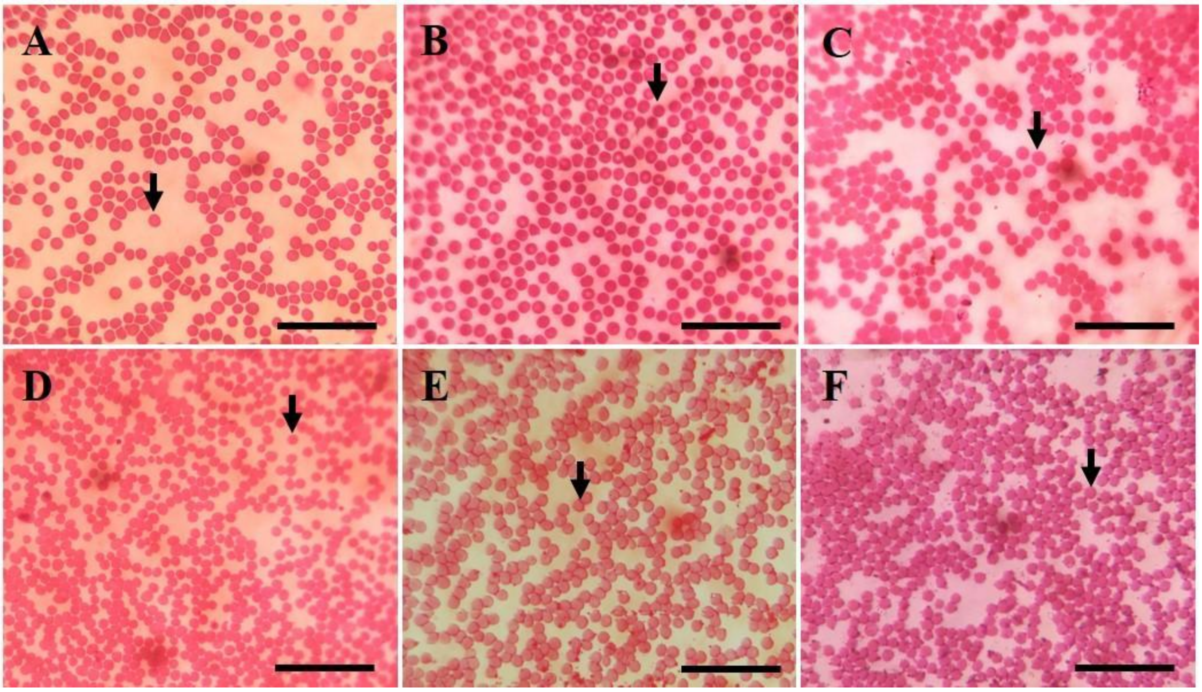

Figure 2. Morphology of erythrocytes among groups after three weeks of treatment. A. Control-1; B. Control-2; C. Treatment-1; D. Treatment-2; E. Treatment-3; F. Treatment-4. Giemsa staining; Black arrow: erythrocytes; Bar Scala = 50 µm.

The PCC may cause oxidative stress in erythrocytes. This condition results from excessive free radicals produced by the PCC treatment. The increased free radicals will elevate reactive oxygen species (ROS), impacting lipid peroxidation, nucleic acid, protein, and membrane structure alteration [25]. Oxidative stress in erythrocytes induces membrane permeability disruption [26]. Therefore, natural antioxidants may protect the erythrocytes from oxidative stress by neutralizing free radicals.

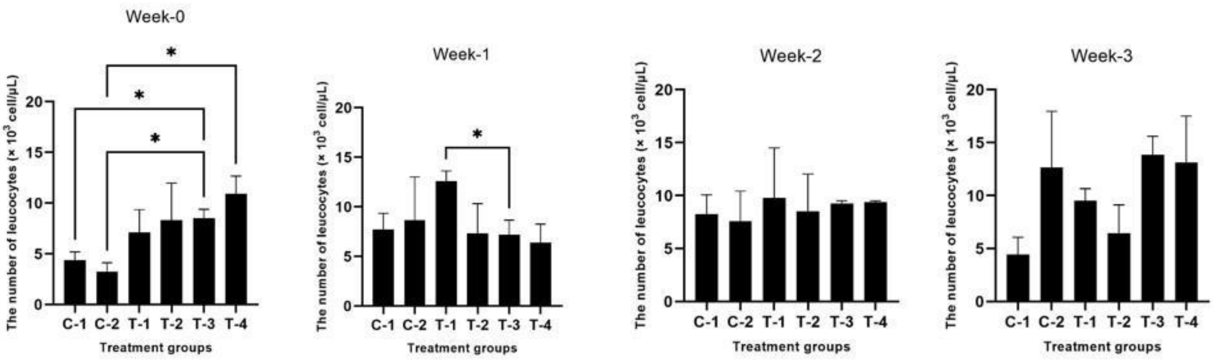

Figure 3. The number of leucocytes each week among groups after plastic chemical compound and natural antioxidant juice treatment (ANOVA, *P<0.05).

Antioxidant mechanisms to neutralize free radicals are mediated by several pathways, depending on the type of antioxidants [27]. Natural antioxidants are low molecular weight antioxidants, including Vitamin C, Vitamin E, carotenoids, and flavonoids [27]. Furthermore, carotenoids have radical reactions by binding lipid peroxyl radicals. On the other hand, flavonoids might mediate cell signalling pathways. Subsequently, it interferes with the regulation of gene expression in cellular processes, such as mitosis, inflammation, apoptosis, and survival [27]. Flavonoid contents are also known to be membrane lipid protectors from lipid peroxidation in erythrocytes [28].

The effect of natural antioxidants on the leucocyte performance of pregnant mice exposed to the plastic chemical compound

The number of leucocytes fluctuated among the data collected periods, as shown in Figure 3. Besides, there were no significant differences in total leucocytes among groups after three weeks of treatment. However, the PCC-control group (C-2) had more leucocytes than the aquadest control (C-1), purple sweet potatoes, and mung bean groups. At the same time, the orange juice group had the most similar leucocytes in total with aquadest control (C-1).

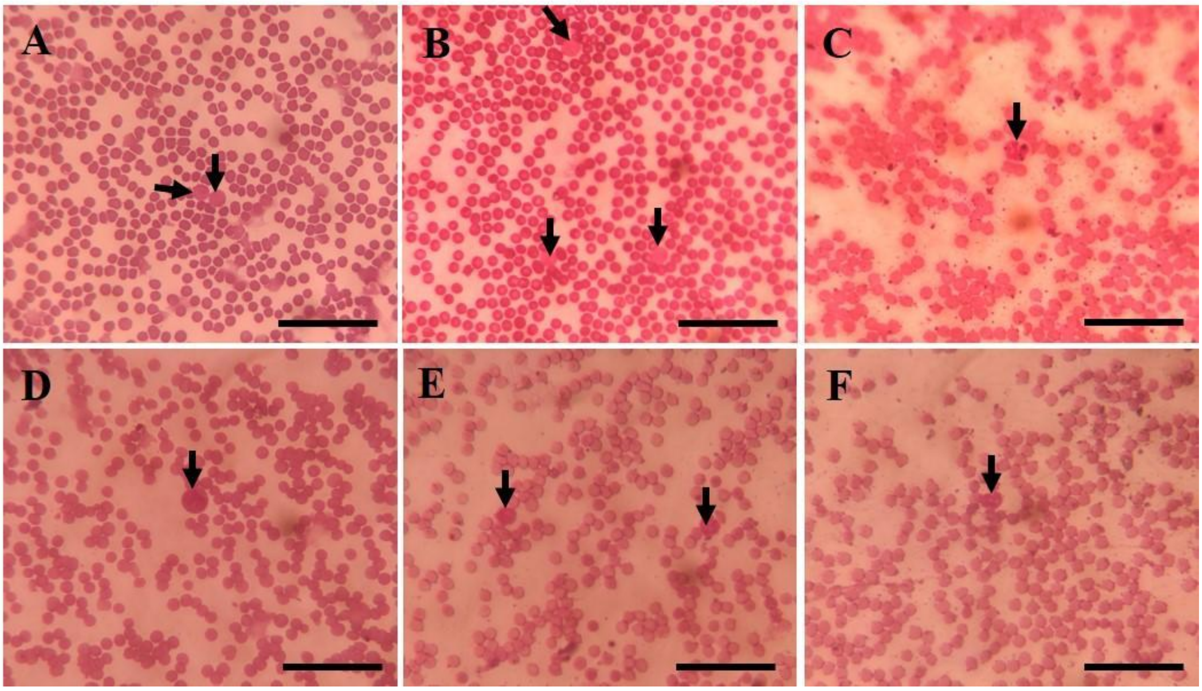

Figure 4. Morphology of Leucocytes among groups after three weeks of treatment. A. Control-1; B. Control-2; C. Treatment-1; D. Treatment-2; E. Treatment-3; F. Treatment-4. Giemsa staining; Black arrow: Leucocytes; Bar scale = 50 µm.

The leucocyte morphology is shown in Figure 4. The leucocyte morphology found on the blood smear slides is clearly varied. Leucocytes are larger than erythrocytes and have various nuclei shapes depending on the leucocyte type. These size and nucleus differences make leucocytes distinctly different compared to the erythrocyte.

The number of leucocytes varied among groups after three weeks of treatment, as did their morphology. Leucocytes play a role in the immune system against infectious agents, pathogens, and foreign invaders [29], [30]. PCC can be categorized as a triggered agent in the increase of leucocytes. Thus, the groups with PCC had a higher number of leucocytes than the aquadest control group. After three weeks of treatment, the total leucocytes in the group with PCC exposure increased. The longer the PCC exposure, the higher the leucocytes total.

Based on Figure 3, it can be seen that the orange juice group (T-2 group) had a similar amount compared to the aquadest control. Orange juice may normalize the number of leucocytes. It may be caused by the antioxidant content of orange [11], which may neutralize free radicals produced from PCC. Therefore, no more free radicals or other toxins can induce an increase in leukocytes.

On the other hand, the increase of total leucocytes in the T-3 and T-4 treatment groups may be affected by the administration of natural antioxidants. Vitamin E, such as the α-tocopherol isoform produced by seeds, could regulate the immune system, such as lymphocytes, which belong to leucocytes [31]. Mung bean sprouts in this study may play a role as a vitamin E source.

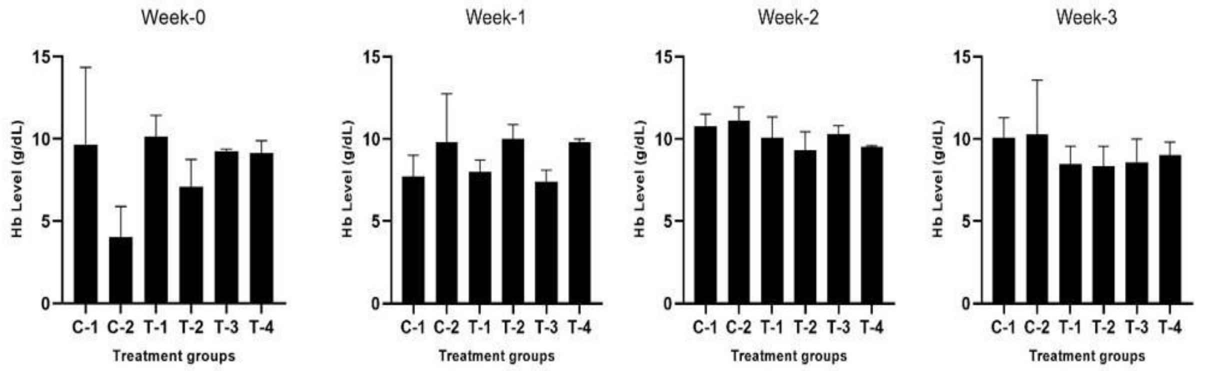

Figure 5. The Hb level of mice each week among groups after plastic chemical compound and natural antioxidant juice treatment (ANOVA, P>0.05).

The effect of natural antioxidants on the Hb level of pregnant mice exposed to the plastic chemical compound

The Hb level among treatment groups after the PCC and natural antioxidant exposure is shown in Figure 5. After three weeks of treatment, the Hb level was almost similar among groups, as well as after the first and second weeks of treatments. There were no differences among groups in all of the data collected periods. However, it can be seen that the Hb level after three weeks of treatment is more stable than in previous weeks.

Hemoglobin is a protein with a quarternary structure. Its function is to bring respiratory gases, both oxygen and carbon dioxide [32], [33]. It is located inside the erythrocyte. Thus, there is a correlation between Hb and erythrocytes [34]. Based on Figure 1 and Figure 5, it is clear that after three weeks of treatment, the number of erythrocytes and the Hb level showed opposite trend. However, both data show stable values among groups. The following section will provide more details about the correlation between both.

The correlation between the number of erythrocytes and the Hb level of pregnant mice exposed to the plastic chemical compound

The correlation between erythrocytes total and Hb level is shown in Figure 6. Based on that figure, the correlation can be seen after three weeks of treatment, while the previously collected data did not show any correlation.

Based on Figure 6, it is clear that there was a correlation between the number of erythrocytes and Hb level after three weeks of treatment. The negative correlation shows that mice with many erythrocytes have low Hb levels and vice versa. This condition is possible as an adaptive mechanism of mice to PCC exposure and natural antioxidant treatments.

Hemoglobin and erythrocytes work together to transport gasses in the circulatory system. Furthermore, hemoglobin, located within erythrocytes, binds oxygen and carbon dioxide [32]. This relationship suggests that the number of erythrocytes and hemoglobin levels should be positively correlated, with a high erythrocyte count accompanied by elevated Hb levels. However, the correlation between erythrocytes and Hb observed in this research was negative after three weeks of treatment. This correlation was evident in the treatment groups receiving natural antioxidants (T-1, T-2. T-3, and T-4). Natural antioxidants may protect erythrocytes while triggering adaptive mechanisms to increase erythrocyte numbers when Hb levels decrease during the treatments.

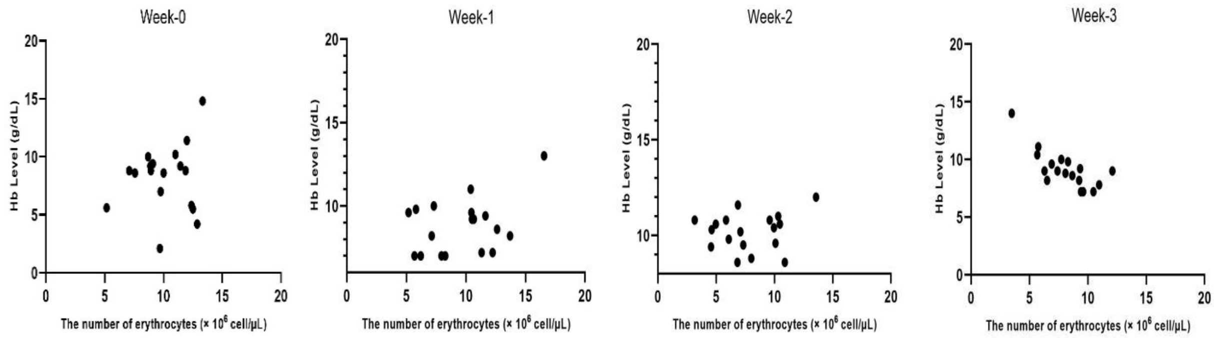

Figure 6. The correlation between the number of erythrocytes and Hb levels after plastic chemical compound and natural antioxidant juice treatment (Pearson Correlation, $P<0.05$, $r = -7518$).

The absence of a natural antioxidant effect significantly on the observed parameters is argued by PCC's lack of free radicals since HDPE plastic is safer than other plastic types. However, providing the natural antioxidant in the long term may trigger the stabilization and adaptation of mice blood cell parameters exposed to PCC. Furthermore, HDPE plastic appears to be safer for blood cells, as it did not have a significant impact on these parameters compared to the control.

Overall, PCC derived from HDPE plastic in this study had varying impacts on the observed blood parameters. Short-term exposure did not affect white blood cells, while red blood cells were influenced, with or without natural antioxidants. In contrast, long-term exposure revealed that natural antioxidants provided greater protection for erythrocytes than leucocytes. Natural antioxidants may help stabilize erythrocyte numbers after three weeks of treatment. Meanwhile, leucocyte numbers tended to increase over the same period, likely due to an immune response to the foreign agent [29], [30]. Besides, several natural antioxidants may modulate the immune response [31].

Natural antioxidants might stabilize erythrocyte numbers through their free radical scavenging activity. These mechanisms align with the characteristics of the antioxidant types explained previously. Overall, the administration of natural antioxidants may protect observed blood cell parameters. The insignificant differences among treatment groups provide flexibility in choosing consumable natural antioxidants.

Conclusion

Natural antioxidants did not significantly affect erythrocytes, leucocytes, and Hb levels, as well as the blood cell morphology in mice exposed to plastic chemical compounds. However, the negative correlation between erythrocyte counts and Hb levels suggests that natural antioxidants may stabilize and trigger adaptive mechanisms in the blood cells of PCC-exposed mice.

Acknowledgments

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Perkembangan Embrionya Akibat Paparan Senyawa Kimia Plastik".

Conflict of interest

We declare that there is no conflict of interest.

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