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*Original Research Article*

# Sub-acute Toxicity Study of Bitter Leaf (*Vernonia amygdalina* Del.) Aqueous Fraction on Haematological Parameters

Fitri Rachmaini, Rahmad Abdillah, Hamimi Anshari, Dian Ayu Juwita*

*Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Andalas, Padang, West Sumatera. 25163, Indonesia.*

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ABSTRACT

*Vernonia amygdalina's* medicinal properties are well established. Furthermore, the safety of the *V. amygdalina* leaf aqueous fraction has yet to be proven. This study aims to determine the sub-acute toxicity of *Vernonia amygdalina* leaf aqueous fraction (VALAF) on haematological parameters in male BALB/c mice. The sub-acute oral toxicity test consisted of three treatment groups of nine animals each and a control group. The BALB/c mice were given repeated oral doses of VALAF at 250, 500, and 1000 mg/kg BW for 21 days. The observed parameters were red blood cells (RBC), haemoglobin (Hb), white blood cells (WBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and hematocrit (HCT). All parameters were measured on the 8$^{th}$, 15$^{th}$ and 22$^{nd}$ days. This study revealed no mortality or morbidity in the subacute toxicity tests. VALAF dose and the duration of administration did not differ significantly from the control group ($p$>0.05). This study showed that VALAF was relatively safe for male BALB/c mice.

***Keywords***: Subacute toxicity; *Vernonia amygdalina*; Haematology, BALB/c mice

## Introduction

The World Health Organization reports that 66% of the population in developing countries, such as India and Indonesia, utilise traditional natural medications.[1] Traditional medications are becoming increasingly popular since they are simple to obtain, reliable, and well-accepted by society. People presume that all-natural products are safe and require no safety assessments. There has been no substantial research on the toxicity of certain herbal extracts. Various plant components, such as fruits, leaves, and bark, expressed phytochemicals with toxicity potentials. Toxicity assessment is essential for conventional drugs, traditional medicine and natural substances to identify any adverse effects that are not obvious before signs and indications arise after prolonged intake.[2] As a result, information from toxicological studies is critical for the risk management of medications. Hence, toxicological investigations of herbal medicines give scientific reasons and evidence of their safety and effectiveness.[3]

*Vernonia amygdalina* (*Asteraceae*), known as "bitter leaf" in the African region, has been reported to offer several health and nutritional advantages. Tea with bitter leaves is frequently used in West Africa to treat diabetes and other metabolic disorders associated with the liver.[4,5] *Vernonia amygdalina* has long been used in Africa to cure various infectious diseases, including ascariasis, malaria, tonsillitis, and pneumonia.[6] *Vernonia amygdalina* is currently found in its natural environment in Indonesia and Malaysia, where it is used to treat diabetes and hypertension.[7]

Several researchers have shown that *V. amygdalina* contains antioxidant,[8] anti-obesogenic,[9] hepatoprotective[10] anti-diabetic, and antihypertensive activities.[5] These activities are attributed to the established phytochemical components of the leaves, such as flavonoids, terpenes, polyphenols, glucosides, and steroidal saponins.[8] Several researchers have isolated and analysed some phytochemical substances with significant pharmacological effects from the leaves of *V. amygdalina*. Vernoniosides A, B, A1, A2, A3, B2, B3, A4 [11], sesquiterpene lactones, flavonoids such as luteolin 7-O-glucosides, luteolin 7-O-glucuronide, luteolin (12) and steroid glycosides are all examples of these compounds.[13] Understanding the safety of *V. amygdalina in vivo* is very important. This plant aqueous fraction toxicity has not yet been recorded. As a result, this study aimed to determine the sub-acute toxicity levels of the aqueous fraction of *V. amygdalina* leaves.

*Corresponding author. E mail: dianayujuwita@phar.unand.ac.id
Tel: +62 813 6300 1618

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Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

## Materials and Methods

*Plant materials*

*Vernonia amygdalina* Del was collected on March 2023 in Pauh, Padang, West Sumatera, Indonesia -0.913548, 100.439067 and authenticated by Assoc Prof. Dr Nurainas at Herbarium ANDA, Department of Biology, Universitas Andalas.

*Chemicals*

Sigma-Aldrich Co. provided gallic acid (USA). The other materials were of analytical quality and obtained from reliable sources.

*Extraction and fractionation procedure*

*V. amygdalina* fresh leaves were washed and dried at room temperature for five days. The leaves were then powdered to a mesh size of 60 and extracted for 18 hours with 70% ethanol. The maceration solution was filtered, and the filtrate was evaporated using a vacuum rotary evaporator (Buchi) at 60°C to produce a thick extract. The crude extract (20 g) was dissolved in 100 mL of distilled water in a separating funnel, to which 100 mL of ethyl acetate was added and capped. The content of the funnel was shaken gently with the release of pressure. The mixed solution was allowed to stand for a few minutes until there was a separation between the ethyl acetate and water layers. The process was repeated three times to achieve exhaustive extraction. Then, the water fraction was concentrated using a vacuum rotary evaporator (Buchi) at 60°C to obtain a dense fraction (VALAF).[14]

*Qualitative phytochemical screening*
Using the Indonesian Herbal Pharmacopea's standard protocols, the plant was evaluated for phytochemicals such as alkaloids, phenols, flavonoids, and tannins.[15]

*Experiment animals*
Male BALB/c mice rats weighing 27-35 g were acquired from the Faculty of Pharmacy, Universitas Andalas. All animals were placed in cages with 12 hours of daylight and night cycles (24 -26°C and 40-60% relative humidity). Food and water were freely accessible. All protocols on animal handling were based on Guidelines by the European Council Directive on the Care and Use of Laboratory Animals (86/609/EEC) issued on November 24, 1986. The protocol was approved by the Andalas University's Committee of Research and Ethics with ethical approval certificate number No /UN.16.2/KEP-FK/2022

*Sub-acute toxicity study*
Thirty-six male BALB/c mice divided into four groups of nine animals each were used for this study. The doses were determined based on previous acute toxicity studies and ethnopharmacological reports. Distilled water was used for standard control. The treatment groups received oral VALAF at 250, 500, and 1000 mg/kg BW doses once daily for 21 days.

*Animal observation, body weight, food intake and water consumption*
All experiment animals were monitored daily for signs and symptoms of death and toxicity, such as changes in eyes and mucous membranes, alterations in skin and hair, signs of tremors, convulsion, salivation, diarrhoea, sleep, and coma, abnormality in respiratory circulation, somatomotor activity and behavioural pattern. Individual animals were watched following treatment, and for 14 days, much attention was paid during the first 24 hours. These observations were done when the animals were being handled and under good lighting conditions. The amounts of food and water consumed were recorded.

*Hematological analysis*
After an overnight fast, animal blood samples were collected through inferior vena cava punctures. Haematological parameters such as hematocrit (HCT), red blood cells (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were assessed using an automated haematology analyser Sysmex XS-800i (Japan). All samples were collected in tubes containing EDTA. Throughout the experiment, each animal's body weight was recorded.
*Statistical Analysis*
Mortality (the number of dead animals) was counted in each group and recorded. All data were analysed by two-way ANOVA followed by Duncan's Multiple Ranking Test (DMRT) as post hoc analysis.

## Result and Discusion

*Physicochemical and Phytochemical analysis*
The physical and physicochemical characteristics are shown in Table 1. Table 2 shows the presence of alkaloids, flavonoids, phenolics, terpenoids and tannins in *V. amygdalina* phytochemical analysis. Several other individuals have mistakenly believed that phytoconstituents found in herbal plants are safe simply because they are derived from nature or obtained from a natural source and hence are deemed safe with no adverse health implications. Self-medication is common for these bioactive phytopharmaceutical formulations developed from herbal medicinal plants.[16,17]
The qualitative phytochemical analysis of the plants showed a diverse variety of bioactive compounds. Tannins, terpenoids, flavonoids, phenolics, and alkaloids are all present. Alkaloids and tannins are among plants' most substantial presence of bioactive chemicals. Similar findings have been reported in the literature.[18–23] The presence of alkaloids and other bioactive compounds invokes the medicinal characteristics of the plants and their therapeutic use in various pharmacopoeias.[24,25]

*Sub-acute toxicity, animal observation, body weight, food intake and water consumption measurement*
A sub-acute oral toxicity study in BALB/c mice indicated zero mortality after repeated VALAF administration for 21 days. There was, however, a change in behaviour, such as grooming and sleeping. There were no alterations in the skin and fur, eyes, or mucous membrane, as shown in Table 3. Body weight differences were statistically significant in all groups. Body weight gain was significantly inhibited in the VALAF 1000 mg/kg BW group, but there was a significant increase in the VALAF 250 mg/kg BW group (Figure 1 A). In all groups, there was no significant change in the mice's food intake or water consumption (Figure 1 B and 1 C). In general, changes in body weight accurately predict overall health and natural compounds may be changed into harmful substances, disrupting food intake. This suggests that VALAF did not affect the animals' normal metabolism. The findings reveal no deaths, physical abnormalities, or abnormal conduct occurred over 21 days of the experiment. Similarly, no apparent toxicity was discovered. *V. amygdalina* aqueous extract showed no influence on treated mice's biochemical and haematological parameters compared to controls. The $LD_{50}$ of *V. amygdalina* aqueous extract was reported to greater than 2000 mg/kg BW, indicating that the extracts are not harmful at the level tested.[27] The $LD_{50}$ of the VALAF studied was greater than 2000 mg/kg BW. Plants with an $LD_{50}$ greater than 1000 mg/kg orally are reported as safe in the literature. [28]
Furthermore, the change in body weight of the animals was recorded throughout the study, regardless of some significant rises in feed intake in the lowest and intermediate-dose groups. The results of this measure show that all animals gained weight, indicating they were in good physiological condition. This evidence demonstrates that VALAF is not hazardous at 250-500 mg/kg. Several studies have reported the safety of *V. Amygdalina*. Legba et al. verify the safety of ethanolic and aqueous extracts of *V. amygdalina* leaf that are utilised as traditional medicine in Southern Benin. [28]
Weight loss is a sign of a drug's or plant's side effects. The analysis of body weight revealed that both the treatment and control groups of rats gained weight at the end of the sub-acute oral toxicity test. Nevertheless, the weight gain of the treated groups was greater than that of the control group. (Figure 1), except for the VALAF at 1000 mg/kg BW. The weight gain was statistically different from the control ($p$<0.05). The increased weight gain of the treatment group animals might be attributed to physiological changes such as food intake and metabolism. [28,29] However, it could also be due to the active chemicals in the *V. amygdalina* aqueous fraction.

**Table 1:** Physical and physicochemical parameters of *V. Amygdala* leaves

| No | Parameters | Values (in percentage) |
|---|---|---|
| 1 | Moisture content | 9.92 ± 0.86 |
| 2 | Volatile oil | Nil |
| 3 | Total ash | 10.58 ± 1.22 |
| 4 | Acid insoluble ash | 1.36 ± 1.30 |
| 5 | Water soluble extractive | 7.88 ± 0.82 |
| 6 | Alcohol soluble extractive | 6.84 ± 1.42 |

**Table 2:** Qualitative analysis of *V. Amygdala* leaves

| No | Phytoconstituents | Result |
|---|---|---|
| 1 | Flavonoids | + |
| 2 | Phenolic | + |
| 3 | Alkaloids | ++ |
| 4 | Tannin | ++ |
| 5 | Sterol | - |
| 6 | Terpenoid | + |

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**Table 3:** Mortality and toxic signs of male BALB/c mice in the sub-acute toxicity test VALAF

| Responses | 24 h after administration | | | | 14 days after administration | | | |
|---|---|---|---|---|---|---|---|---|
| | Normal | VALAF 250 | VALAF 500 | VALAF 1000 | Normal | VALAF 250 | VALAF 500 | VALAF 1000 |
| Tremors | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Convulsion | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Writhing | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Pain response | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Grooming | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Exist |
| Urination | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Diarrhea | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Salivation | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Lacrimation | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Sleep | Absent | Absent | Absent | Absent | Absent | Absent | Exist | Exist |
| Food Intake | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Water Intake | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Coma | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Death | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |

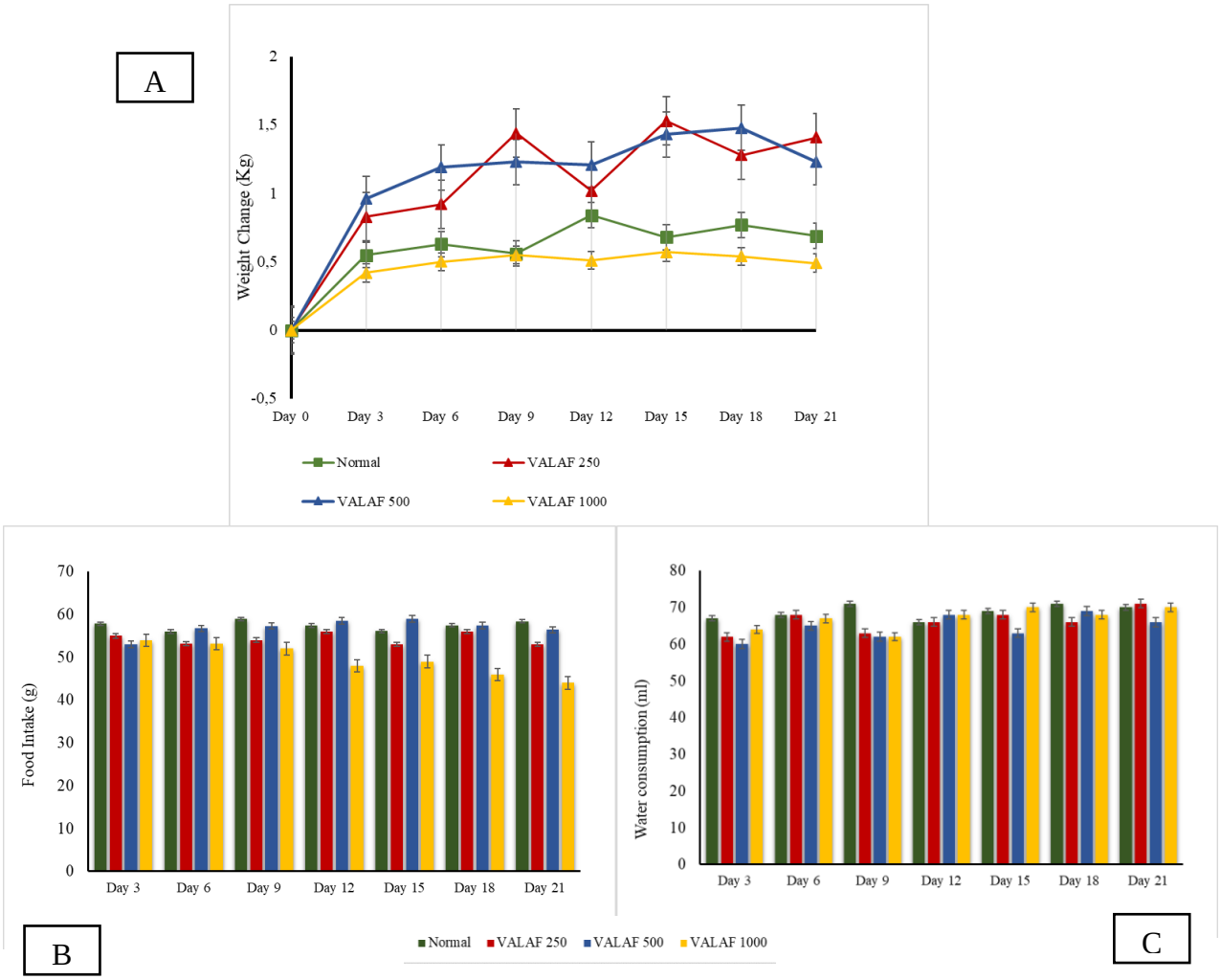


**Figure 1:** Body weight change as a function of time (days) in experimental groups (A); food intake and water consumption pattern as a function of time (days) in various experimental groups (B) and (C)

*Effect on clinical haematology*

Figure 2-8 illustrates the effect of a daily VALAF dosage on animals' haematological parameters. Compared to the control group, the measured parameters display statistically significant alterations. However, all parameters were still within the normal range. Haematology markers may be utilised as accurate indicators to detect the invasion of hazardous toxins or plant extracts. These findings showed that VALAF did not disrupt the haematopoietic system. Haematological and clinical chemistry markers can determine toxicity.[31] It is a crucial indicator of human and animal physiological and pathological states. Every change in haematological parameters is interpreted as a possible risk of anaemia. Since the haematological system has a better predictive ability for toxicity, blood parameter analysis is helpful for risk assessment.[32, 33] In the current investigation, practically all haematological indicators showed a significant difference compared to the control groups. However, all parameters were still in the normal range. This finding demonstrates the lack of haematological toxicity of *V. amygdalina* aqueous extract.

Changes in animal haematological parameters offer more reliable information for determining toxicity consequences.[34] Except for a substantial rise in MCV and a decrease in MCHC, neutrophils, and lymphocytes, repeated administration of VALAF had no significant effect on most indices compared to the control groups. RBC's morphology and osmotic fragility influence the alterations in MCHC and MCV.[27] RBC enlargement, in particular, induces a decrease in MCHC. Furthermore, the whole immune response is managed by lymphocytes and neutrophils as well as antimicrobial function. As a result, further oral chronic toxicity study is required to determine future dosages and identify potential clinical symptoms caused by the substances in concern. It might also be used to determine a drug's therapeutic potential. Although this study offers exciting data on the toxicity of plants, the average lethal dosage was not calculated, and the toxicity was not assessed over an extended time frame.

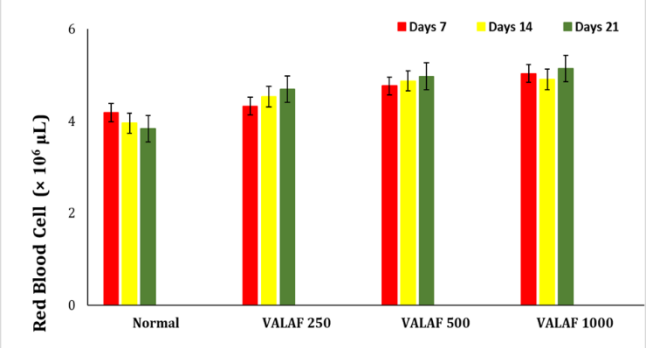


**Figure 2:** Red blood cell parameter of mice after three weeks of administration of VALAF. Each value is a mean of nine determinations ± SEM.

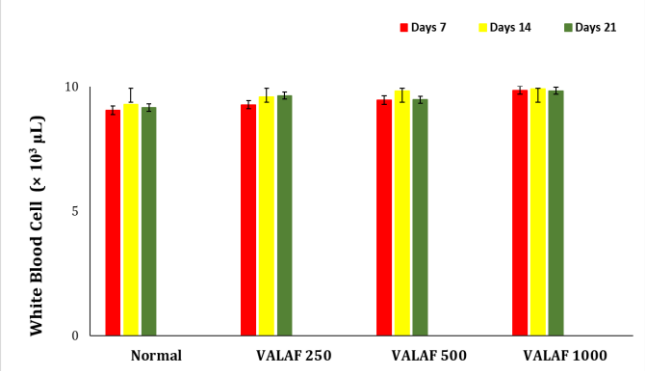


**Figure 3:** White blood cell parameter of mice after three weeks of administration of VALAF. Each value is a mean of nine determinations ± SEM.

## Conclusion

This study revealed no mortality, morbidity or any changes in the general behaviour of mice in sub-acute toxicity trials. VALAF did not induce any severe toxicity that resulted in haematological changes. This study shows that VALAF is tolerable up to 1000 mg/kg BW, implying its safety and promise as a safe pharmaceutical product candidate.

## Conflict of Interest

The authors declare no conflict of interest.

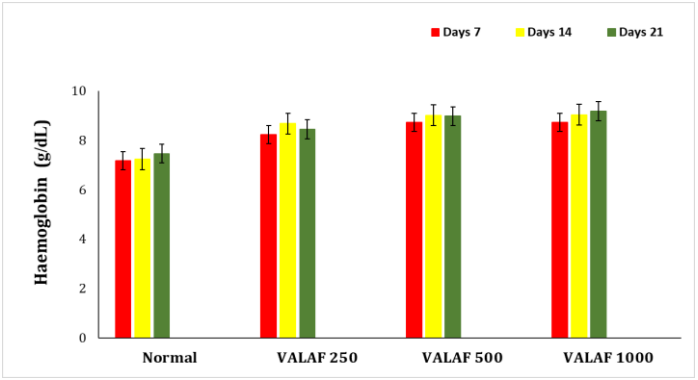


**Figure 4:** Haemoglobin parameter of mice after three weeks of administration of VALAF. Each value is a mean of nine determinations ± SEM.

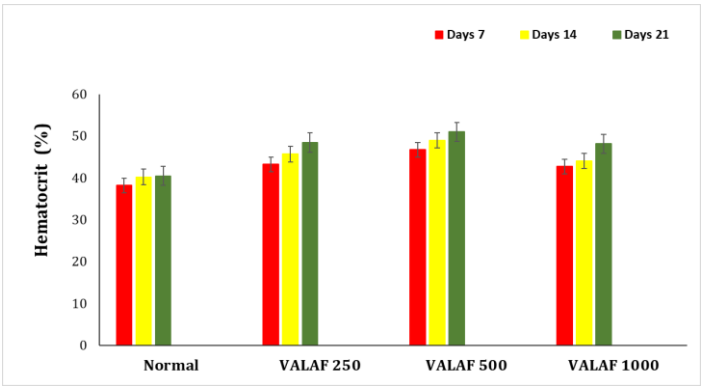


**Figure 5:** Hematocrit parameter of mice after three weeks administration of VALAF. Each value is a mean of nine determinations ± SEM.

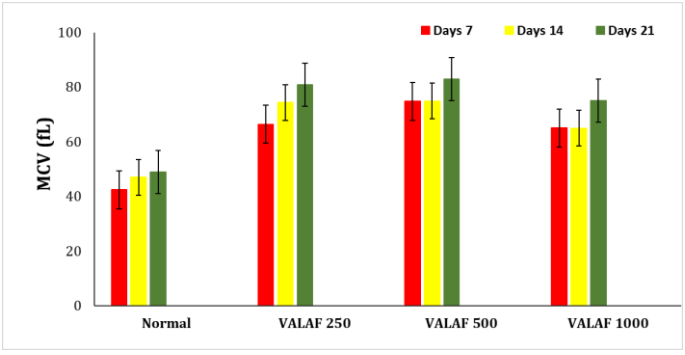


**Figure 6:** MCV parameter of mice after three weeks of administration of VALAF. Each value is a mean of nine determinations ± SEM.

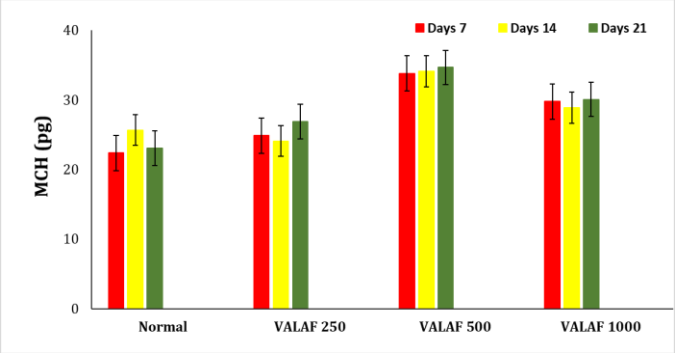


**Figure 7:** MCH parameter of mice after three weeks of administration of VALAF. Each value is a mean of nine determinations ± SEM.

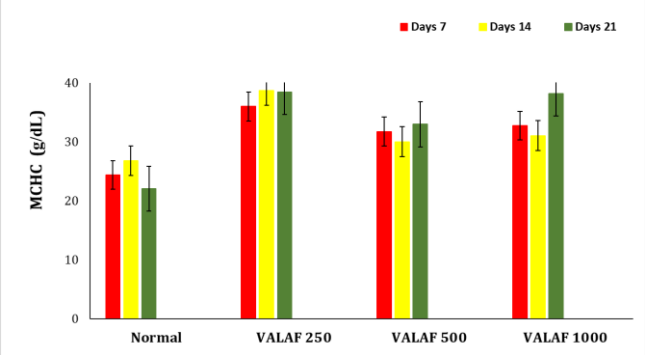


**Figure 8:** MCHC parameter of mice after three weeks of administration of VALAF. Each value is a mean of nine determinations ± SEM.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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