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Clinical Manifestations and Behavior Changes in Rats Treated with Jamaican Cherries Extract (Muntingia Calabura)

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A B S T R A C T

Iron Deficiency Anemia (IDA) in female adolescents is a health problem that occupies the 8th position as a cause of global disability and is the common cause of anemia in reproductive women. Iron tablet supplementation is the government program for the depletion of IDA prevalence in Indonesia but often results in gastrointestinal side effects. Jamaican cherries contain vitamin C, iron, and flavonoids, which have important roles in inducing iron metabolism. This study aimed to investigate the influence of Jamaican cherries' ethanol extract on clinical manifestations and behavior changes in female Wistar rats. The study is conducted in a randomized controlled trial with pre-posttest control groups design using 24 female Wistar rats, aged between 10 -12 weeks which randomly divided into six groups: N, positive control (PC: AIN-93M low iron food + elemental iron), and treatments (T1-4: AIN-93M low iron food + 0.26, 0.35, 0,5 or 0.75g/ 100g Body Weight (BW) Jamaican cherries extract respectively for 28 days). The clinical manifestations, reflexes, and behavior changes in rats were observed on days 0, 14, and 28 of dose administration. BW data were analyzed using one-way ANOVA and ANOVA Repeated-Measure tests. All rats had normal reflexes and did not show clinical manifestations and behavior changes at the end of this study. The average number of rats' BW in T1-4 groups did not significantly differ before (p=0.896), during (p=0.884), and after the treatment (p=0.775). However, the average of the rats' BW in the T1 group increased significantly after the treatment (p=0.012). In conclusion, the administration of Jamaican cherries' ethanol extract up to 0.75g/100g BW has no toxic effects in female Wistar rats.

Kata kunci:

Anemia defisiensi besi
Berat badan
Ekstrak buah kersen
Manifestasi klinis
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A B S T R A K

Anemia defisiensi besi (ADB) pada remaja putri merupakan salah satu masalah kesehatan yang menempati posisi ke-8 sebagai penyebab disabilitas tingkat global dan penyebab utama anemia pada ibu hamil. Suplementasi tablet besi merupakan program pemerintah untuk mengurangi prevalensi ADB di Indonesia tetapi sering menimbulkan efek samping di saluran percernaan. Buah kersen mengandung vitamin C, zat besi dan flavonoids yang berperan penting dalam metabolisme besi. Penelitian ini bertujuan untuk mengetahui pengaruh ekstrak buah kersen terhadap manifestasi klinis dan perubahan perilaku tikus Wistar betina. Penelitian randomized controlled trial ini dengan pre-posttest control group design dengan menggunakan 24 ekor tikus Wistar betina umur 10 - 12 minggu dan dibagi secara acak menjadi 6 kelompok: normal, kontrol posistif (KP) dan perlakuan (P1-4). Tikus di KP diberi pakan AIN-93M dengan besi rendah + besi elemental sedangkan P1-4 diberi pakan yang sama ditambah 0.26, 0.35g, 0,5g atau 0.75g/ 100g berat badan (BB) secara berturut-turut ekstrak buah kersen selama 28 hari. Manifestasi klinis, reflek dan perubahan perilaku tikus diamati pada hari ke-0, 14 dan 28. Data BB

dianalisis menggunakan uji one way ANOVA dan uji repeated measures ANOVA. Semua tikus memiliki reflek yang normal dan tidak menunjukkan manifestasi klinis dan perubahan perilaku. Rerata BB tikus pada P1-4 tidak berbeda signifikan sebelum (p=0,896), selama (p=0,884), maupun setelah perlakuan (p=0,775) tetapi rerata BB tikus P1 meningkat secara signifikan (p=0,012). Kesimpulan, pemberian ekstrak buah kersen hingga 0,75g/100g BB tidak menyebabkan toksisistas tikus Wistar betina.

INTRODUCTION

Iron Deficiency Anemia (IDA) is a nutritional problem in adolescents that currently occupy the 8th position as the common cause of global disability-adjusted life years (Vos et al., 2020). It is also the most common cause of anemia in reproductive women (World Health Organization, 2021), which is associated with decreased economic productivity and an increased mortality rate among women (Kinyoki et al., 2021). According to the Indonesian basic research of health, the prevalence of anemia in young people (15–24 years old) had increased from 18.4% in 2013 to 32% in 2018. In addition, the prevalence of anemia in young women (27.2 %) is higher than in young men (20.3%) (Indonesian Ministry of Health, 2018; Indonesian Ministry of Health, 2013).

Iron tablet supplementation is one of the most common programs worldwide used for treating anemia including in Indonesia (Indonesia Ministry of Health, 2014). For young women with anemia, the iron supplement is taken once per week for a year and every other day during menstruation (Indonesia Ministry of Health, 2020). However, frequent usage of iron supplements may cause gastrointestinal side effects such as nausea, vomiting, abdominal pain, and constipation. As a result, it may cause low compliance and discontinuation of usage, even though the national purchase of iron supplementation is increased during the last five years (Jimenez et al., 2015). This situation requires innovative solutions to use natural resources to reduce symptoms of anemia (Christian, 2021).

Red guava, moringa leaves, and snake fruit seeds are several natural resources that have been used as alternative iron supplements for IDA treatment (Arini, 2018; Ristanti, 2019; Yusnaini, 2016). However, the usage of those alternatives has its disadvantages. For example, the administration of ethanol extract of snake fruit seeds needs higher doses, which potentially cause hypersensitivity reactions (Ristanti, 2019). The Jamaican cherries have widely been distributed in many countries worldwide and are available for every season (Ningsih, 2016). Furthermore, every 100 g of this fruit may potentially induce iron metabolism because it contains important nutrients such as 178.96 mg vitamin C, and 0.10 mg Fe (Makahity et al., 2019). Both unripe and ripe Jamaican cherries also contain flavonoids such as rutin, myricetin, luteolin, quercetin, apigenin, and kaempferol (Kubola et al., 2011). In general, flavonoids have been used in some studies to treat anemia (Borawska et al., 2014), while flavonoids in Jamaican cherries also have a role in the regulation of iron homeostasis. A combination of Fe and 50 mg/Kg BW pure quercetin improves red blood cells and increases Hb and serum iron levels in the rats' anemia model (Mazhar et al., 2018). High Fe exposure is related to changes in body composition, hence it is critical to consider the effect on body weight (Moreno-Fernandez et al., 2019). Iron is very important in the body biologically, but it also has the potential to be toxic under certain conditions, thus it must be strictly controlled at the cellular and systemic levels to prevent cases of deficiency or overload (Camaschella et al., 2020). Human consumption of medicinal plants must be reviewed for safety in terms of dosage, processing methods, and unwanted side effects (Nurfaat & Indriyati, 2016). However, there are still a limited amount of studies that reported the administration of ethanol extract of Jamaican cherries for anemia treatment. The other acute and chronic toxicities of ethanol extract in Jamaican cherries remain unknown. Therefore, this study aimed to evaluate whether or not the administration of Jamaican cherries extract has sub-chronic toxicity on body organs of female Wistar rats.

METHODS

Extraction of Jamaican cherries

The ripe Jamaican cherries used in this study are collected from the cherries plants alongside Ambarketawang street, Sleman, Yogyakarta, in the period of 2 months. The extraction process of Jamaican cherries in this study was based on the Afdhaliya study (in press). Briefly, Simplicia of Jamaican cherries was extracted using the maceration method with ethanol solvent, according to Melati, *et al* (2019) and Ristanti (2019). Collected filtrates of Jamaican cherries were dried using a rotary vacuum evaporator (Rotavapor R215 Merck KGaA, Darmstadt, Germany) in the Natural Resource Technology Laboratory, Faculty of Pharmacy, University of Setya Budi Surakarta at 60ºC, 60 rpm and 175 bar for 60 minutes. The ethanol extracts was then kept at 4ºC before further analysis.

Animal model and research method

This study was an experimental laboratory with a pre-posttests control group method. Animals used in this study were female Wistar rats (*Rattus Norvegicus*), weighed around 150-170g, aged between 10-12 weeks, and obtained from the Integrated Biomedical Laboratories, Faculty of Medicine, Islamic University of Sultan Agung Semarang. The rats were housed in a standard cage with a 12 hours light/dark cycle, 22°C ± 3°C temperature, and 60-65% humidity. Each group consisting of 4 rats was kept in the same environmental conditions using a 30 x 40 x 10 cm cage and was acclimatized for 6 days.

Sample Size and Study Protocol

A total of 24 female Wistar rats were used in this study which was selected using the resource Equation (E) degrees of freedom by ANOVA (Nadeem et al., 2017). Rats were randomly divided into 6 groups: Normal (N), Positive Control (PC), and Treatment (T1, T2, T3, and T4) groups. Rats in the normal groups were fed with a standard food of the American Institute of Nutrition 1993 Maintenance (AIN-

93M). The PC group was fed with the AIN-93M food with low iron levels and was given 0.05 ml/200 g BW per day iron supplement. The T1 – T4 groups were fed as same as the PC group and were given 0.26g, 0.35g, 0.5g, and 0.75g/100g BW of ethanol extract of Jamaican cherries, per day. All rats could access ad libitum to drinking water.

The experimental protocol and animal handling procedures in this study were approved by the Health Research Ethics Committee, Faculty of Medicine, Sebelas Maret University No: 17/UN27.06.6.1/KEP/EC/2021.

Observation of Clinical Manifestations and Behaviour Changes

The oral doses of ethanol extract of Jamaican cherries were calculated using the standard guidelines of repeated doses for a 28-day oral toxicity test (OECD, 407) and the Total Effective Dose (TED) standard from the Patel, et al study (2018). Clinical manifestations of subchronic toxicity such as changes in the skin, fur, eyes, mucous membranes, respiratory system, shivering, salivation, and defecation in female Wistar rats treated with ethanol extract of Jamaican cherries, were observed directly and were recorded the changes. Meanwhile, nervous system reflexes and behavior changes such as somatomotor activity, weakness, sleep, coma, and death were observed by looking at the response of rats. If the rat gave responses such as looking at the fingers and blinking, the rat response would be considered as a normal condition. Clinical manifestations and behavior changes were observed in the first 24 hours, 14 days, and 28 days. Additionally, the rat's body weights were regularly weighed on the same days using an Ohaus Triple Beam scale.

Data Analysis

Data of BW were presented as mean ± standard deviation (SD) and were statistically analyzed using the SPSS software ver. 25, while the clinical manifestation and behavior changes were classified into normal and abnormal conditions. The one-way ANOVA test was used to compare mean differences among groups in 0-, 14-, and 28-days treatments. We also used the ANOVA Repeated-Measure to analyze a serial time of treatment among groups. The significance value of statistical analysis was p-value <0.05.

Table 1.
Clinical Manifestations of Subchronic Toxicity in Female Wistar Rats Treated with Ethanol Extract of Jamaican Cherries Fruits

Parameters	Groups	1 day	14 days	28 days
Skin and fur	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal*	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Eyes	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Mucous membranes	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Respiratory system	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Shivering	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Salivation	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Defecation	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Behaviour	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal

Note. *One rat with red spots in the back skin

RESULTS AND DISCUSSION

In this study, we evaluated the sub-chronic toxicity of the use of Jamaican cherries ethanol extract in female Wistar rats, which comprises observations on clinical manifestation and behavior changes. Table 1 shows no clinical manifestations after administration of ethanol extract of Jamaican cherries for 1, 14, and 28 days. Nevertheless, one rat in the T2 group had a red spot on its back skin on day 14, which then disappeared on day 28.

The second evaluation of sub-chronic toxicity of Jamaican cherries ethanol extract is the effects on the nervous system of the rats. Table 2 showed that all rats responded well with both the central or peripheral nervous systems throughout the 28-day exposure with ethanol extract of Jamaican cherries fruits. However, another rat in the T2 group had torticollis from 14 to 28 days of administration.

Tabel 2.
Central, Pheripheral Reflexes and Behaviour Changes in Female Wistar Rats Treated With Ethanol Extract Of Jamaican Cherries Fruits

Parameters	Groups	24 hours	14 days	28 days
Peripheral nervous system - Palpebral reflex - Digiti reflex	N	Normal	Normal	Normal
	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Central nervous system	N	Normal	Normal	Normal
	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal*	Normal*
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Somatomotor activity	N	Normal	Normal	Normal
	PC	Normal	Normal	Normal
	T1	Normal	Normal	Normal
	T2	Normal	Normal	Normal
	T3	Normal	Normal	Normal
	T4	Normal	Normal	Normal
Weakness	N	-	-	-
	PC	-	-	-
	T1	-	-	-
	T2	-	-	-
	T3	-	-	-
	T4	-	-	-
Sleep	N	-	-	-
	PC	-	-	-
	T1	-	-	-
	T2	-	-	-
	T3	-	-	-
	T4	-	-	-
Coma	N	-	-	-
	PC	-	-	-
	T1	-	-	-
	T2	-	-	-
	T3	-	-	-
	T4	-	-	-
Death	N	-	-	-
	PC	-	-	-
	T1	-	-	-
	T2	-	-	-
	T3	-	-	-
	T4	-	-	-

Note. *One Rat with Tortikolis

In table 3, the average body weight among the rats in all groups increased during the 28-day administration with ethanol extract of Jamaican cherries except the rats in the T2 group. The average body weight in the PC group was increased by 0.5 g, while the average body weight in the T1, T3, and T4 groups was increased by more than ± 2g. In contrast, all rats in the T2 group had a slight decrease of average BW from 162.50 ± 8.10g to 162.25 ± 7.32g. However, the changes of BW among the groups of rats did not differ significantly.

The repeated-measures ANOVA was then used to evaluate the correlation between dose and duration of ethanol extract of Jamaican cherries exposure with rats' BW (Figure 1). It indicates that administration of ethanol extract of Jamaican cherries may increase the body weights of the rats, depending on dose and duration of exposure except for the rats in the T2 group, which the average BW tended to

decrease in the remaining 14 days of administration. Only the rats in the T1 group showed a significant increase in average BW during the intervention (p=0.012).

Table 3.
Body Weight Changes in Female Wistar Rats Treated WithEthanol Extract of Jamaican Cherries Fruits

Groups	Body Weight (Mean ± SD)			p[a]
	Before (g)	During (g)	After (g)	
PC	166.25 ± 4.11	167.25 ± 3.59	167.75 ± 1.89	0.178
T1	163.50 ± 4.79	164.50 ± 4.04	166.75 ± 5.67	0.012[*)]
T2	162.50 ± 8.10	162.00 ± 8.52	162.25 ± 7.32	0.897
T3	161.25 ± 7.50	162.75 ± 7.14	163.25 ± 6.89	0.125
T4	164.25 ± 6.50	165.25 ± 5.25	166.50 ± 4.50	0.131
p[b)]	0.842	0.750	0.584	

Notes. [a)] Repeated Measures ANOVA Test[b)] One Way ANOVA Test [*)] Test statistically significant if p-value less than 0.05

The observation for clinical manifestations of sub-chronic toxicity in female Wistar rats ministered with ethanol extract of Jamaican cherries showed no clinical manifestations after administration for 1, 14, and 28 days. Interestingly, all rats in the T4 group (rats ministered with high doses) showed normal behaviors with healthy respiratory and gastrointestinal systems. However, one rat in the T2 group had a red spot on the back skin on day 14 of intervention, which disappeared on day 28. In general, it indicates that the lower to higher doses of ethanol extract of Jamaican cherries are safe and non-toxic. The findings are similar to the previous study that tested 1,000 mg/kg BW ethanol extract of Jamaican cherries in male and female albino mice, which conveyed that the substance has no toxic and death risks (Karthyaini, 2012). However, the oral dose of the Jamaican cherries fruits extract was not equal between rats and mice. The clinical signs of sub-chronic toxicity are usually hyperresponsive to external stimuli, leading to panic responses, low activity, or depression. The most common indicators for disease development and decrease in health status are piloerection, frizz, hair loss, skin disorders such as wrinkles, signs of muscle wastage on the back, dehydration, and reduced body weight. Generally, rats' hair and skin are the most sensitive organs in the body, allowing them to interact directly with the environment. Hair loss and skin irritation are examples of the body's protective response to its environment (Koolhaas, 2010). It protects the entire body with a continuous protective outer coating and defends the body from numerous forms of bacterial, viral, and fungal infections that might change the body's metabolism (Saxena et al., 2014).

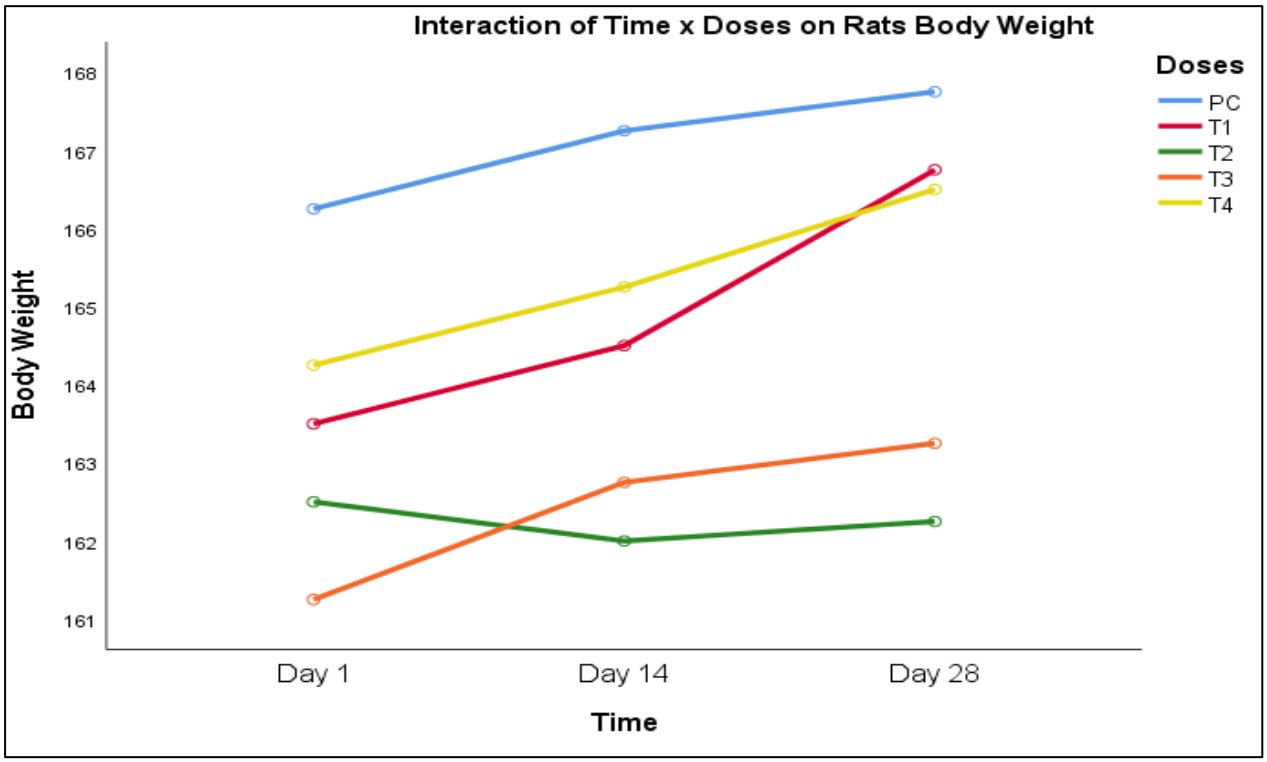

Figure 1. BW Changes in Female Wistar Rats Treated with Ethanol Extract of Jamaican Cherries Fruits. Each Line Represented 4 Female Rats. The Data Were Presented as Mean ± SD And Were Analyzed Using the Repeated Measure ANOVA.

On the other hand, the observation of the rats' central and peripheral reflexes and behavior changes showed that all rats responded well with either the central or peripheral nervous systems throughout the 28-day of administration. However, one rat in the T2 group had torticollis from day-14 to day-28 of intervention. The most probable diagnosis for this type of torticollis in rats is *Spasmodic Torticollis* (*dystonia*). This type is the most prevalent reason for neck stiffness in rats. Emotional stress, physical overload, or sudden movement are the most typical triggering causes. It is estimated that 90% of individuals will experience at least one episode of torticollis during their lives. The occurrence of torticollis in female rats is two times higher than in male rats (Cunha et al., 2021). This is the reason for using female rats in this study, because they are generally more sensitive than male Wistar rats in response to the reduction of minerals, such as iron. This condition mimics the condition of female adolescents with iron deficiency anemia (Stevani, 2016). In this study, the rat diagnosed with torticollis did not show any worsening symptoms, weight loss, and reduction of appetite, with only insignificant difficulties in moving. The rat's body undergoes reflex alterations in an attempt to retain body alignment. The vestibular (balance) system in the ear regulates body orientation. Hair cells in the vestibular system operate as stimulus receptors (receivers). If the hair cells are disrupted, the animal will have difficulties maintaining physical balance, especially if the damage occurs in the vestibular organ's peripheral nerve system (Zabolotnyi & Serhiivna Mishchanchuk, 2020). All rats showed no weakness, sleep, coma, or death. Based on this study, the use of higher doses of ethanol extract of Jamaican cherries fruits (>2000mg/Kg BW) was not toxic because all rats were still alive until the end of the intervention. As stated by the United Nations (2021), any substance is considered as a low hazard potential substance when the oral dose given is more than 2,000 mg/kg BW and the lethal toxicity among tested rats is less than 50%. Therefore, the administration of ethanol extract of Jamaican cherries in this study is proven to be safe.

To further evaluate the sub-chronic toxicity of ethanol extract of Jamaican cherries, we regularly measured the rats' BW because BW changes are the most visible and early sign of the toxic effect of any substance (Lukman & Christin, 2020). The effect of Jamaican cherries extract on rats' body weight during a 28-day period in this study indicates no toxic symptoms, because the increase in the rats' average body weight occurred at each dose is considered normal. The BW of the treatment group did improve more slowly than the normal control group, but this condition is usual in rats' intervention. The result findings were in accordance with the previous study, that tested 200mg/kg BW ethanol extract of Jamaican cherries in male and female albino mice that showed the weight of the mice which were treated was smaller than the control group (Sujono et al., 2021). In the toxicity study, changes in body weight should always be monitored to control the possibility of stress in rats. Stress in rats can result in decrease of BW (Sequeira-Cordero et al., 2019). Stress conditions are closely related to a decrease in appetite which results in changes in BW (Ningsih et al., 2017).

In this study, the administration of ethanol extract from Jamaican cherries contains a high source of Fe. Foods high in Fe have a role in regulating the body's metabolism, especially in energy metabolism, by transporting oxygen to cells and tissues throughout the body. The administration of Jamaican cherry extract in this study showed that up to a dose of 7500 mg/kg BW rats (Fe=1.56 g/day) still showed either an increase of normal body weight or no weight loss of 20-25%. Rats are considered to have symptoms of weight poisoning if showed signs of illness, such as weight loss of 20% or more for seven or more consecutive days followed by a decrease in appetite (Sulastra et al., 2020).

Overall, the female Wistar rats exposed with ethanol extract of Jamaican cherries showed normal conditions during a 28-day administration. In addition, increased average BW was observed in all rats treated with low to high doses of ethanol extract of Jamaican cherries. Thus, it suggests that ethanol extract of Jamaican cherries is safe and not toxic to body organs.

LIMITATION OF THE STUDY

The limitation of this research was that we did not present our data related to biochemical evaluation and did not perform a histopathological examination of vital organs to determine changes in rat's iron metabolism. Therefore, we could not assume if the normal condition and behavior in rats treated with ethanol extract of Jamaican cherries have the normal hematological status and iron metabolism.

CONCLUSION AND SUGGESTION

The administration of ethanol extract in Jamaican cherries up to 7,500 mg/Kg BW does not indicate clinical manifestations and behavior changes in female Wistar rats. Further investigation is required to investigate the effective dose of ethanol extract in Jamaican cherries to increase hemoglobin levels and to regulate iron metabolism in female Wistar rats with anemia.

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ETHICAL CONSIDERATIONS

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Conflict of Interest Statement

The author declares that there is no conflict of interest.

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