Northwestern Med J. 2022;2(3):138-144
doi: 10.54307/NWMJ.2022.25744

Original Article

Subchronic effects of bay leaves (*syzygium polyanthum*) extract on body weight, physical changes and mortality in female wistar rats with low iron diet

Düşük demir içerikli diyetle beslenen dişi wistar ratlarda szygium polyanthum ekstresinin vücut ağırlığı, fiziksel değişiklikler ve mortalite üzerine kronik etkileri

Suharni Ani, **Dono Indarto**, **Risya Cilmiaty**

Atıf/Cite as: Ani S, Indarto D, Cilmiaty R. Subchronic effects of bay leaves (syzygium polyanthum) extract on body weight, physical changes and mortality in female wistar rats with low iron diet. Northwestern Med J. 2022;2(3):138-144.

ABSTRACT

Introduction: Anemia is a globally nutritional problem and taking iron tablets regularly is recommended. Meanwhile, bay leaves are widely used for food flavoring, but only limited for anemia treatment. This study aimed to investigate the subchronic effects of bay leaves extract on body weight (BW), physical changes, and mortality in female Wistar rats with low iron diet.
Methods: Thirty female Wistar rats, aged 8-10 weeks and weighing 160-200 g, were used in this research study. They were randomly divided into 5 groups: the positive control (PC) received an AIN 93 M standard food, supplemented with 1.36 mg/100 g Fe mineral mix (low iron diet), and 3.7 mg/kg BW iron tablet, while the treatment (T1-4) groups received the low iron diet, supplemented with 300; 1.000; 2.000; and 5.000 mg/kg BW bay leaves extract respectively for 28 days. Furthermore, BW measurement was carried out in day 0, 14, and 28 and physical changes were regularly observed 5 times using a checklist. Data were analyzed using one way ANOVA and repeated measures tests.
Results: The average of BW in all rat groups increased slightly except in PC group. The PC group had the highest average of BW (184.2±5.54g) on day 28 of treatment, but it was not significantly compared to other groups (p=0.123). Rats in groups C and T showed normal physical performances, somatomotor activities, and zero death.
Conclusion: Oral administrations of four doses of bay leaves extract to female Wistar rats with low iron diet showed no observed adverse effects.

Keywords: Bay leaves extract, subchronic toxicity, anemia, female wistar rats

ÖZ

Giriş: Anemi, dünya çapında bir beslenme sorunudur ve düzenli olarak demir tabletleri alınması önerilir. Defne yaprağı, gıda aroması için yaygın olarak kullanılır, ancak anemi tedavisi için sınırlıdır. Bu çalışma, düşük demir diyetli dişi Wistar sıçanlarda defne yaprağı ekstraktının vücut ağırlığı (BW), fiziksel değişiklikler ve mortalite üzerindeki subkronik etkilerini araştırmayı amaçladı.
Yöntem: Bu laboratuvar deneyinde 8-10 haftalık ve 160-200 g ağırlığında 30 dişi Wistar sıçan kullanıldı ve rastgele 5 gruba ayrıldı: pozitif kontrole (PC) AIN 93 M standart gıda, 1.36 ile ilave edildi. mg/100 g Fe mineral karışımı (düşük demir diyeti) ve 3.7 mg/kg VA demir tableti tedavi sırasında (T1-4) gruplarına düşük demir diyeti verildi ve 300; 1000; 2.000; 28 gün boyunca sırasıyla 5.000 mg/kg BW defne yaprağı özütü. 0, 14 ve 28. günlerde VA ölçümü yapıldı ve bir kontrol listesi kullanılarak düzenli olarak 5 kez fiziksel değişiklikler gözlendi. Veriler tek yönlü ve tekrarlanan ölçümler ANOVA testleri kullanılarak analiz edildi.
Bulgular: PC grubu hariç tüm sıçan gruplarında ortalama vücut ağırlığı biraz arttı. PC grubu 28. günde tedavide en yüksek ortalama vücut ağırlığına (184.2±5.54g) sahipti, ancak diğer gruplarla karşılaştırıldığında anlamlı bir farklılık göstermedi (p=0,123). C ve T gruplarındaki sıçanlar normal fiziksel performans, somatomotor aktivite ve sıfır ölüm gösterdi.
Sonuç: Düşük demir diyeti olan dişi Wistar sıçanlarında dört doz defne yaprağı ekstraktının oral uygulamaları gözlenen herhangi bir yan etki göstermedi.

Anahtar kelimeler: Defne yaprağı ekstresi, subkronik toksisite, anemi, dişi wistar fareleri

Received: 24.03.2022
Accepted: 26.08.2022
Publication date: 01.10.2022

Corresponding Author:
D. Indarto
ORCID: 0000-0001-7420-5816
Department of Physiology and Biomedical Laboratory, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia
✉ dono@staff.uns.ac.id

S. Ani
ORCID: 0000-0001-7142-3383
Human Nutrition Sciences, Universitas Sebelas Maret, Surakarta, Indonesia

R. Cilmiaty
ORCID: 0000-0001-9351-5246
Department of Dentistry and Oral Health, General Hospital UNS, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

INTRODUCTION

Anemia is a nutritional disorder, which remains a health problem in both developing and developed countries. It is reported that about 2 billion patients are affected globally, and causes three-quarters of one million deaths per year in Africa and Southeast Asia (1). Anemia can be caused by many factors including blood loss, deficiency of iron, vitamin B12 and folic acid, blood diseases, infectious diseases, and inflammation (2) but 50% is caused by iron deficiency (3). In Indonesia, the prevalence of anemia in 15-24 years old increased from 37.1% in 2013 to 84.6% in 2018 (4) (5). The prevalence of iron deficiency anemia (IDA) is 23.9% in women, compared to 18.4% men (4). Furthermore, WHO has developed a global plan to decrease anemia by 25% in 2025 in reproductive women (6). Iron deficienc anemia IDA can occur due to lack of iron-rich intake, poor absorption, gastrointestinal diseases, and regular iron loss from the body (7,8). In addition, anemia is associated with impaired immune functions, resulting in an increased risk of developing infectious diseases (9). Anemia in school-age children have some negative effects such as low learning concentration, cognitive impairment, and impaired physical development (10). In adolescents and adults, it influences work productivity and performance (11).

To reduce the prevalence of IDA in Indonesia, the Ministry of Health issued a regulation on the national standard for iron tablets for reproductive and pregnant women (12). However, data from Basic Health Research (2018) indicated that the coverage of young women receiving iron tablets is only 22.9%, while pregnant women receiving tablets containing iron ≥90 elements is only 38.1% is (5). The root cause of non-adherence consumption of tablets is due to uncomfortable side effects such as nausea, vomiting, heartburn, constipation, and black stools (6). Therefore, patients are recommended to consume iron-rich food sources, which are usually found in animal food sources such as eggs, fish, liver and meat, but the price is more expensive and not quite affordable by most Indonesians (5). Alternatively, they can consume vegetables and fruits, which also have high iron levels but the content is in ferric ion that is firstly converted in to the ferro ion before absorption in the small intestine (13).

Bay leaves (*Syzygium polyanthum*) is a tropical plant that is widespread in Indonesia and often used as a spice in cooking and in traditional medicine. Many studies explored bay leaves extracts for treatment of certain human diseases, but its usage for anemia treatment is limited. From a nutritional perspective, it contains protein, iron, vitamin C and B2 (14,15). A recent study reported that 100g bay leaves flour contain 1.48 and 29.42 mg iron and vitamin C, while its extract contain 30.09 and 9.39 mg of iron and vitamin C, respectively (16). Furthermore, the extract contain various secondary metabolites such as saponins, tannins, terpenoids, flavonoids, polyphenols and alkaloids (15). Subsequently, no well-tolerated maximum doses of bay leaves extract have been established, specifically changes in body weight (BW) and physical performance. Therefore, this study aimed to investigate the subchronic effects of bay leaves extract on BW, physical changes, and mortality in female Wistar rats with low iron diet.

MATERIALS AND METHODS

Extraction of Bay Leaves

Dried *S. polyantha* leaves were obtained from the Herbal Center of Merapi Farma, Pakem, Sleman Regency, Yogyakarta Province, Indonesia, and grounded at the Laboratory of Pharmacology, University of Gadjah Mada, Yogyakarta, to produce powder. The bay leaves powder was then macerated using 70% v/v ethanol solvent (Merck, Germany), with 1:5 ratio for 3 days at room temperature. Subsequently, the macerate was filtered using 0.45 µm membrane paper and concentrated using a *rotary vacuum evaporator* at 60°C, 80 rpm for 60 minutes. The extract of bay leaves was then stored at 4°C before further analysis.

Experimental Design

This randomized control study with post-test only group design was conducted from August

to September 2021 at the Integrated Biomedical Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Central Java Province, Indonesia. The sample size was calculated using Federer formula (17): (r-1) (t-1) ≥ 15 (t=group treatment and r= number of replications). Furthermore, this experimental study was carried out using 5 groups, and 5 female groups were finally formed. To avoid drop out, 10% was added to each group, therefore, each had 6 female rats. This study protocol received an ethical clearance from the ethics committee, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Central Java Province, Indonesia, with number 29/UN27.06.6.1/KEP/EC/2021.

A total of 30 female Wistar rats, aged 8-10 weeks and weighing 160-200 g, were randomly divided into 5 groups. The positive control (PC) group was received a low iron-restricted food containing the American Institute of Nutrition (AIN) 93 maintenance diet (Dyets, USA) with Fe 1.36 mg/100 g mineral mix and added with 3.7 mg/kg BW iron tablet. The treatment (T1-4) groups received the low iron-restricted food, supplemented with 300; 1.000; 2.000; and 5.000 mg/kg BW bay leaves extract respectively. The dose range of bay leaves extract was based on the toxicity test according to the Indonesian Pharmaceutical Food Regulatory Agency (18). The intervention was conducted for 28 days and all rats had free access to drinking water.

Body Weight (BW) Measurement and Physical Examination

The rats BW was regularly measured using a digital weight scale (Triple Beam, Europe) on day 0, 14 and 28 of intervention, while physical changes were regularly checked every week using an existing checklist. From the skin and fur, piloerection was reporting and some changes were observed any in the eyes such as clarity, blinking, inflammation, corneal and palpebral reflexes, lacrimation, nystagmus, photophobia, mydriasis or miosis. In the respiratory system, breath frequency and discharge from the nose was observed. The peripheral and central nervous systems were observed digital reflex, seizure, paralysis, torticollis and somatomotor activities. The behavior evaluation consisted of walking backward or using the stomach, shaking, salivation, dry mouth, urinating in the buttocks area, weakness, sleep and coma.

Data Analysis

All collected data were analyzed using the SPSS program version 25. Numerical data were presented as mean ± standard deviation and were verified using the Shapiro-Wilk for the xdnormality test. The average of rat BW in the groups was statistically analyzed using one way ANOVA and repeated measures analysis of variance tests. In addition, data for physical changes were presented as positive and negative without any statistical test. Significant differences were set at $p< 0.05$. Based on the results of one way ANOVA and repeated measures tests, there was no post hoc test because the significant value was > 0.05.

RESULTS

BW measurement is often used for evaluation of metabolic and toxicity effects of natural products. In Table 1, the average BW of rats before treatment was similar with p= 0.253, which was the highest BW in the T3 group (182.2±4.76 g) and the lowest

Table 1. The Average of BW in Female Wistar Rats Treated with Various Doses of Bay Leaves Extract.

	BW (g)			
Group	**0 day**	**14 day**	**28 day**	**p[b]**
PC	181.8±3.27	182.8±3.89	184.2±5.54	0.126
TI	177.80±4.06	178±4.69	178.8±5.11	0.426
T2	179±1.00	179.60±1.34	180±2.73	0.496
T3	182.2±4.76	183.2±3.27	183.8±2.49	0.519
T4	181.4±12.46	181.8±11.68	183±10.93	0.196
p[a]	0.253	0.142	0.123	

a) One Way ANOVA; b) Repeated Measures ANOVA

Table 2. Observation of Physical Changes in Female Wistar Rats Treated with Various Doses of Bay Leaves Extract.

Physical Observation	Group	Observation (Day)				
		1	2-9	10-15	16-21	22-28
Skin	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Fur	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Eyes	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
	T4	N	N	N	N	N
Behavior	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Tremble	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Salivation	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Sleep	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Weak	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Death	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N

was in the T1 group (177.80±4.06 g). During the 14 and 28-days treatments, the average BW in all rat groups increased slightly except in PC and T4 groups. In the T3 group, the average BW of rats (182.2±4.76; 183.2±3.27, and 183.8±2.49 g) remained higher than other T groups during 28 days treatment. Meanwhile, the PC group had the highest average BW at the end of treatment (184.2±5.54 g), although it was not significantly different from the other groups (p=0.123).

To further evaluate the toxic effect of bay leaves extract, regular examination was carried out on the skin, fur, eyes, behavior, somatomotor activities, respiratory system to the nervous system. Tables 2 and 3 indicated that control and treated rats had normal physical performance and activities, compared to the normal control rats and those that survived until the end of the treatment.

Table 3. Observation of Changing Respiratory and Nervous Systems in Female Wistar Rats Treated with Various Doses of Bay Leaves Extract.

		Observation (Day)				
	Group	1	2-9	10-15	16-21	22-28
Respiratory system	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Peripheral nervous system	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Central nervous system	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N
	T4	N	N	N	N	N
Somatomotoric activity	PC	N	N	N	N	N
	TI	N	N	N	N	N
	T2	N	N	N	N	N
	T3	N	N	N	N	N

DISCUSSION

In this study, it has been proven that the administration of the highest dose of bay leaves extract (5.000 mg/kg BW) in female Wistar rats with low iron diet for 28 days is safe in terms of BW, physical performances, activities and mortality, according to the Indonesian Food and Drug Supervisory Agency (18). The increase in BW is a sensitive indicator for evaluation of animal health status and the most visible indicator for the toxicity effect in rats (19). This findings are in line with the results of Djamaluddin and his colleagues, which reported that the administrations of 1.250; 2.500 and 5.000 mg/kg BW bay leaves extract in female and male rats for 14 days significantly increase the average BW of 38.33±4.63, 40.33±2.80, and 41.00±3.35 g, respectively, compared to the control group (34.00±2.35 g) (20). Additionally, this findings also confirm another study performed in both sexes of Wistar rats in which increased BW was observed in all Wistar rats treated with 100 and 400 mg/kg BW and in female Wistar rats treated with 1.000 mg/kg BW bay leaves extract (21).

In general, BW changes can be influenced by several factors including internal and external factors. Genetic factors are important determinants to control individual BW, which are passed down from parents to their children through the modulation of hormones and enzymes in the human body (20). In addition to the administration of phytochemicals, bay leaves extract contains alkaloid, terpenoids, flavonoid, saponin, steroid and tannins (22) including 3.522,63 ± 39.73 ppm tannins levels. These active compounds easily bind to proteins in the intestinal cell membrane, leading to reduction of food absorption (16,20). Additionally, bay leaves extract also contains terpenoids, which have a bitter taste, resulting in appetite reduction and inhibit the intestinal lipase enzyme, functioning as a preventative measure for obesity (23).

Secondly, all of the low-iron diet Wistar rats treated with 5.000 mg/kg BW bay leaves extract for 28 days showed normal performance and physical activity and zero death. These results provided new evidence that bay leaves extract has a broad spectrum of toxicity. This finding also strengthens previous studies that administrations of 1.000 mg/kg BW bay leaves extract in female and male Wistar rats for 90 days (21) and 2.000 mg/kg BW methanol extract of bay leaves for 28 days in Sprague Dawley rats (24) did not cause abnormal physical performances, physical activities and mortality. Although a different experiment used another species of *Syzygium*, rats treated with 1.500; 2.000 and 5.000 mg/kg BW methanol extract of *S. guineense* leaves for 28 days had the same results as treated with bay leaves extract (19). In contrast to this findings, oral administration of 200 and 600 mg/kg BW aqueous extract of *S. guineense* leaves in rats for 42 days resulted in two rats death on day 32 and 40 of the treatment (25). The different results are probably due to duration of treatment, study animal, species plant and extraction solvent.

Although experiment studies using bay leaves extract have been published in some previous journals, this study provides new insight in terms of subchronic toxicity during 28 days intervention and four doses of bay leaves extract (300; 1.000; 2.000; and 5.000 mg/kg BW). In this study, there are some limitations. At first, the wet weight of vital rat organs were not measured because the toxic effect of bay leaves extract can cause cell and organ damage, resulting in BW reduction. Additionally, histological staining were not performed on the organs to verify when inflammation and necrosis occurred. Liver and kidney function tests are important parameters to evaluate metabolism of the extract but were performed, which provide important data for other signs of toxicity. However, hematological test was carried out and some parameters of iron metabolism was evaluated but the data are not shown.

In conclusion, oral administrations of four doses of bay leaves extract on female Wistar rats with low iron diet shows increase BW, have normal physical performances, somatomotor activities and zero mortality. In the future, bay leaves extract can be administered to female Wistar rats with iron anemia deficiency using hematological and iron metabolism parameters.

ACKNOWLEDGEMENTS

The authors are grateful to the staff of Integrated Biomedical Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang, and Laboratory of Pharmacology, University of Gadjah Mada, Yogyakarta, for providing facilities and assistance during this study.

Ethics Committee Approval: The study protocol was approved by the Faculty of Medicine, Universitas Sebelas Maret Ethics Committee (29/UN27.06.6.1/KEP/EC/2021).

Conflict of Interest: The authors have declared that they have no conflict of interest.

Funding: The authors have declared that they have not received any financial support.

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