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ORIGINAL RESEARCH

The Effect of Spinach (*Amaranthus hybridus*) on the Pharmacokinetic and Pharmacodynamic Profile of Warfarin in New Zealand White Rabbits

Norisca Aliza Putriana[1], Taofik Rusdiana[1], Tina Rostinawati[2], Irma Rahayu Latarissa[3]

[1]Department of Pharmaceuticals and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia; [2]Department of Biology Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia; [3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia

Correspondence: Norisca Aliza Putriana, Department of Pharmaceuticals and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Padjadjaran, Jl. Raya Bandung Sumedang KM. 21, Jatinangor, West Java, 45363, Indonesia, Email norisca@unpad.ac.id

Introductions: Spinach (*Amaranthus hybridus*) is a green vegetable containing 380 µg/100 g of vitamin K, while warfarin serves as an antagonist in inhibiting vitamin K epoxide reductase subunit C1 (VKORC). In this context, the co-administration of warfarin and spinach is frequently encountered among Indonesian patients, potentially leading to drug-food interactions. This study aimed to investigate the effect of concomitant administration of spinach on the pharmacokinetic and pharmacodynamic profile of warfarin in New Zealand White rabbits.

Methods: A total of 24 New Zealand White rabbits weighing about 1.5–2 kg were used in this study. For 16 days, these rabbits were given oral warfarin at a dose of 0.4 mg/kg BW by 10.00 am. Subsequently, 3 mL of blood samples were withdrawn in the lateral vein of the ear on the 13th and 16th days. The Prothrombin Time-International Normalized Ratio (PT-INR) is used to evaluate the pharmacodynamic profile, while the plasma concentration of S(R)-warfarin (Cp (AV)), half-life ($t_{½}$), area under the curve (AUC), volume of distribution (Vd), and clearance (C_L) are analyzed to determine the pharmacokinetic effects of warfarin.

Results: In the Fluconazole (FZ) group, there was a significant increase in the area under the curve (AUC) at maximum concentration (Cmax) after treatment, with a p-value of < 0.05. In the *Amaranthus hybridus* dose 1 (AH-1) and *Amaranthus hybridus* dose 2 (AH-2) groups, AUC and plasma drug concentration (Cp (AV)) were higher after treatment but the results of statistical analysis were not significant.

Conclusion: There was no pharmacokinetic or pharmacodynamic interaction between spinach (*Amaranthus hybridus*) and warfarin. Additionally, patients subjected to warfarin therapy could consume spinach with a recommended portion size below 100 grams per day.

Keywords: spinach, warfarin, vitamin K, New Zealand White rabbits, area under curve

Introduction

Warfarin is a class of anticoagulant drugs used widely to treat diseases associated with thromboembolism, such as atrial fibrillation, deep vein thrombosis, and pulmonary thromboembolism.[1,2] The main problem with the administration is a high variation in patients'responses, increasing the difficulty in determining the initial dose and drug-related problems (DRP) cases in the form of adverse reactions.[3] This phenomenon arises due to the narrow therapeutic index of warfarin and instances of underdosing can result in ineffective disease treatment or even complications. Conversely, overdosing may induce cerebral hemorrhage (stroke), or minor occurrences, including ocular bleeding.[1]

The COVID-19 pandemic has further complicated warfarin therapy due to its impact on coagulation abnormalities and increased thromboembolic risk in infected patients. Changes in inflammatory markers, metabolic alterations, and potential drug interactions with antiviral or supportive treatments necessitate closer monitoring of warfarin dosing.[4–6] These factors highlight the importance of individualized anticoagulation management to ensure optimal therapeutic outcomes.[7]

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Different factors affecting pharmacokinetic and pharmacodynamic parameters from patient demographics, diet, genetic factors, and interaction factors with drugs, foods, or herbal supplements should be studied to maintain the effectiveness and safety of using warfarin. Many studies have examined the interaction between warfarin, herbal medicines, and foods to determine changes in responses.[8,9] Based on previous studies, plants or herbs containing vitamin K potentially affect the pharmacokinetic and pharmacodynamic parameters of warfarin.[10] In a 1999 study, green tea had an antagonistic effect on warfarin by inhibiting the drug in preventing blood clots because the vitamin K content was 1.428 mg per 100 grams.[11] Jiang et al reported that warfarin co-administration with St. John's wort caused a significant increase in R- and S-clearance, which resulted in a decrease in the pharmacological effect.[12] However, co-administration with ginseng did not significantly affect the pharmacokinetic and pharmacodynamic changes of warfarin.[13]

The interaction between warfarin and foods containing vitamin K is very large due to the wide availability in the daily diet of patients. According to the American Heart Association (AHA) and American College of Cardiology (ACC), the use can reduce the anticoagulant effect, but these results refer to the consumption of vitamin K supplements.[14] A study reported that increasing the consumption of foods containing vitamin K by 100 µg for 4 days reduced International normalized ratio (INR) by 0.2.[15] Study on healthy subjects consuming warfarin and foods containing vitamin K such as spinach, broccoli, cheese, and natto showed a decrease in INR.[16] Meanwhile, spinach is a green vegetable containing a high vitamin K (Phylloquinone) of 0.380 mg/100 g.[17] The nutrition includes carbohydrates, fat, protein, calcium, phosphorus, iron, selenium, magnesium, zinc, potassium, water, vitamin A, vitamin B, vitamin C, and vitamin K.[18] The use of warfarin is very common in medical practice, and spinach is an often consumed vegetable by the Indonesian people. Therefore, the effects of the co-administration on pharmacokinetic (half-life ($t_{1/2}$), volume of distribution (Vd), area under curve (AUC), and clearance (C_L)) and pharmacodynamic (INR) responses of warfarin should be analyzed.

Materials and Methods

Materials

The materials used were warfarin United States Pharmacopeia (USP) Reference standard and ethyl carbamate (Wako, PT. Nebelin, Indonesia), warfarin pharmaceutical grade (PT. Fahrenheit, Indonesia), *Amaranthus hybridus* (Manoko, Lembang), Fluconazole (Sigma Aldrich, Japan), Pulvis Gom Arabicum, PGA (PT. Bratachem, Bandung, Indonesia), heparin (Wako, Japan), 1.5 mL anticoagulant-containing tube, SP10, SP31 tubing, 23 G needle, 1 mL and 3 mL syringe, string as well as a set of surgery tools. The botanist who formally identified the plant *Amaranthus hybridus* is Mega Dwi Puspa, S.Si. The plant specimen utilized in this study has been deposited in the Herbarium Jatinangor, Plant Taxonomy Laboratory, Department of Biology, FMIPA, Universitas Padjadjaran, under voucher number 29/HB/09/2019. The species was identified as *Amaranthus hybridus* L., with the sample collected from Lembang, West Java, Indonesia, on September 16, 2019. The specimen, consisting of leaves, was gathered by the research team from Universitas Padjadjaran. This voucher serves as a reference to ensure the authenticity and traceability of the plant material used in the research. We confirm that all necessary approvals and permits to conduct research with plant material have been obtained in accordance with institutional and local regulations. The research was carried out in compliance with ethical guidelines and local laws governing the collection and use of plant materials.

Animal and Study Design

A total of 24 New Zealand White rabbits were divided into 4 groups of Control, Fluconazole (FZ), *Amaranthus hybridus* dose 1 (AH-1), and *Amaranthus hybridus* dose 2 (AH-2), weighing about 1.5 −2 kg. The rabbits were acclimatized in cages at room temperature (25°C) for 1 week before testing. For 16 days, oral warfarin was administered at a dose of 0.4 mg/kg BW by 10.00 am. On the 14th, 15th, and 16th days, the application was carried out with 1% of PGA (control), 21 mg/kg BW of FZ group, 2 g/kg BW of spinach (AH-1 group), 7 g/kg BW of spinach (AH-2 group)/normal dose. The doses were given twice a day (07.00 and 16.00) and determined by 100 grams of daily consumption in humans (equivalent to a 7 g dose in rabbits). All experimental procedures and protocols were ethically reviewed and approved by The Research Ethics Committee of Universitas Padjadjaran Hospital, Bandung, Indonesia (No. 1342/UN6/KEP/EC/

2019). The guidelines followed for animal welfare are based on "Guidelines and Ethical Standards for Health Research and Development" issued by the National Health Research and Development Ethics Committee, Ministry of Health, Republic of Indonesia, 2021.

Blood Sampling

Blood samples (±3 mL) were withdrawn from the lateral vein of the rabbit ear on the 13th and 16th day at the time of minimum (Cmin) and maximum concentration (Cmax) before the next administration of warfarin and based on tmax (ss) at steady state [Cmax (ss)]. Blood samples were collected in a 4 mL tube containing heparin and centrifuged for 15 minutes at 2800 rpm. Therefore, ±400 µL plasma was obtained and stored in a refrigerator at −80°C for further analysis of warfarin levels using High Performance Liquid Chromatography (HPLC). For Prothrombin Time-International Normalized Ratio (PT-INR) analysis, 1.8 mL of blood was collected in a tube containing 3.2% citrate plasma anticoagulant (blue tube), and centrifuged at 2500 rpm for 15 minutes. Additionally, the plasma persisted for 8 hours at a temperature of 20–25°C, and the PT-INR value was determined using the STAGO Kit.

Determination of Warfarin Enantiomers' Concentration in Rabbit's Plasma

The measurement of S- and R-warfarin levels in blood was performed by using HPLC (Waters e2695 Separation Module) with a fluorescence detector (Waters Separation 2475 FLR Detector). A Chiralcel OD-RH column (4.6 x 150 mm, 5 µm; Daicel Chemical Industries, Tokyo, Japan) and an OD guard column -RH (4.0 x 10mm, 5 µm; Daicel Chemical Industries, Tokyo, Japan) were used and maintained at 40°C. Moreover, the mobile phase was 20 mmol/L potassium phosphate buffer (pH 2.0)/ acetonitrile (60/40 [v/v], which was pumped at a flow rate of 1.0 mL/min. Sample preparation was carried out by adding 100 µL of plasma to 10 µL of 0.5 g/mL Griseofulvin as an Internal Standard (IS) solution. In addition, 500 µL of cold acetonitrile was added and vortex-mixed after vortex-mixing. The mixed solution was allowed to stand in the refrigerator for 15 minutes followed by centrifugation at 13,000 rpm for 4 min in cold conditions (4°C). The supernatant was transferred to another tube and evaporated for drying under nitrogen gas at 40°C. Subsequently, it was reconstituted to 100 µL with mobile phase solution and centrifuged for 4 min at 13,000 rpm. At an excitation wavelength of 310 nm and emission of 350 nm, 100 µL of supernatant was injected into the HPLC system.

Measurement of Prothrombin Time (PT)

The water bath was set and regulated at 37°C and 100 µL of plasma was added into the respective tubes to stand for 3–5 minutes. Furthermore, 200 µL of SP normoplastin was added to each tube and simultaneously started a stopwatch. The tube was gently tilted, and the time in seconds was noted after the appearance of the initial fibrin strand. The test was repeated 2–3 times for the average value and the INR was derived from the PT. This was calculated as the ratio of the PT during the test to the normal PT raised to the power of the international sensitivity index (ISI).

$$INR = \left(\frac{PT\,(test)}{PT\,(normal)}\right)^{ISI}$$

Calculation of Pharmacokinetic Parameters

After the R- and S-Warfarin levels were obtained in the blood sample, a pharmacokinetic plot of plasma concentrations versus time in each rabbit was plotted. In addition, the pharmacokinetic model and parameters including plasma drug concentration (Cp (AV)), biological $t_{1/2}$, AUC, Vd, and C_L were determined using Excel MS-office 2013 and verified by PK solver application.

Statistical Analysis

A difference between the pharmacokinetic parameters of R- and S-Warfarin in the control and treated groups was analyzed through Paired sample *t*-test or Wilcoxon test method, with a level of significance at $\alpha = 0.05$ by using SPSS version 21.

Results

HPLC Method Validation

For R-warfarin analysis, limit of detection (LOD) and limit of quantification (LOQ) values were 18.6 ng/mL and 62.01 ng/mL. Meanwhile, for S-warfarin, LOD and LOQ values were 18.61 ng/mL and 62.04 ng/mL. A linearity was also reported in the concentration range of 100–2500 ng/mL with the equation y = 0.0272x + 0.0107, r^2 = 0.9969 and y = 0.0271x + 0.0116, r^2 = 0.9991 for R-warfarin and S-warfarin, respectively. This method met the requirements of selectivity, accuracy, and precision for within and between runs with the % RSD and % diff values less than 15%, as well as the recovery value > 80%.

Effect of Spinach on Pharmacokinetic Parameters of R-Warfarin

The concentrations of R-Warfarin in rabbits before and after spinach administration during warfarin treatment of PGA (control), FZ (positive control), AH-1, and AH-2 (treated sample) are presented in Table 1. S-Warfarin concentrations cannot be presented due to the absence of certain groups and the incompleteness of the dataset. The curve shows that the FZ group has the highest concentration compared to the control, AH-1, and AH-2. The pharmacokinetic parameters including Vd, Cp (AV), AUC, and C_L, for R-Warfarin in the four groups of samples, are shown in Table 2.

Table 1 The Concentration of R-Warfarin Before and After Spinach Co-Administration

Group	Before Treatment		After Treatment	
	C_{min} (µg/mL)	C_{max} (µg/mL)	C_{min} (µg/mL)	C_{max} (µg/mL)
Control (n=6)	0.61±0.21	0.89±0.69	0.53±0.40	0.62±0.41
FZ (n=6)	1.49±0.37	1.65±0.42	2.52±0.33	3.01±0.41
AH-1 (n=6)	0.62±0.20	1.03±0.35	0.86±0.11	1.24±0.35
AH-2 (n=6)	0.74±0.37	1.67±0.39	0.88±0.33	2.04±0.54

Notes: Control, 1% PGA; FZ, Fluconazole at a dose of 21 mg/kg BW; AH-1, *Amaranthus hybridus* infusion at a dose of 2 g/kg BW; AH-2, *Amaranthus hybridus* infusion at a dose of 7 g/kg BW, values are presented as mean ± SD.

Abbreviation: SD, StandardDeviation; Cmin, minimum concentration; Cmax, maximum concentration; n, number sample (rabbit).

Table 2 Pharmacokinetic Parameters of R-Warfarin Before and After Spinach Treatment

Para-meter	Unit	Before Treatment								After Treatment							
		C_{min}				C_{max}				C_{min}				C_{max}			
		FZ	PGA	AH-1	AH-2	FZ	PGA	AH-1	AH-2	FZ	PGA	AH-1	AH-2	FZ	PGA	AH-1	AH-2
Vd	L	0.34	0.85	0.83	0.70	0.31	0.58	0.50	0.31	0.20	0.97	0.60	0.58	0.17	0.84	0.42	0.25
Cp (AV)	µg/mL	1.19	0.49	0.49	0.59	1.32	0.72	0.82	1.342	2.02	0.42	0.69	0.70	2.42	0.50	1.00	1.64
AUC	mg.h/L	28.74	11.77	11.96	14.27	31.83	17.2	19.87	32.21	48.61	10.22	16.59	16.97	58.10	12.00	23.90	39.35
C_t (10^{-3})	L/h	1.04	2.55	2.51	2.10	0.94	1.75	1.51	0.93	0.62	2.93	1.81	1.77	0.51	2.51	1.25	0.76

Abbreviations: Control, 1% PGA; FZ, Fluconazole at a dose of 21 mg/kg BW; AH-1, *Amaranthus hybridus* infusion at a dose of 2 g/kg BW; AH-2, *Amaranthus hybridus* infusion at a dose of 7 g/kg BW; Cmin, minimum concentration; Cmax, maximum concentration; n, number sample (rabbit); Vd, volume of distribution; Cp (AV), average of plasma drug concentration; AUC, area under curve; C_L, clearance; values are presented as mean.

Table 3 PT INR Value

Group	Before Treatment		After Treatment	
	PT (minute)	INR	PT (minute)	INR
Control (n=6)	21.98±0.31	1.62	20.07±0,34	1.57
FZ (n=6)	21.37±0.25	1.64	35.63±0,45	2.71
AH-1 (n=6)	21.81±0.17	1.74	18.13±0,65	1.52
AH-2 (n=6)	22.63±0.28	1.78	16.37±0,22	1.45

Notes: Control, 1% PGA; FZ, Fluconazole at a dose of 21 mg/kg BW; AH-1, AH infusion at a dose of 2 g/kg BW; AH-2, AH infusion at a dose of 7 g/kg BW; Cmin, minimum concentration; Cmax, maximum concentration; n, number sample (rabbit); values are presented as mean ± SD. SD, Standard Deviation.

Effect of AH on Coagulation Parameters (PT-INR)

PT-INR values at the minimum concentration in rabbits before and after treatment of PGA (control), FZ (positive control), AH-1, and AH-2 (treated samples) are presented in Table 3. FZ group shows an increase in PT-INR values reporting that the coagulation effect occurs when 21 mg/kg BW of the drug is co-administrated with spinach.

Discussion

Warfarin functions as an anticoagulant by inhibiting the action of vitamin K and the drug possesses a narrow therapeutic index with high intersubject variability to induce undesirable effects.[19,20] Variations in individual responses can be attributed to various factors, including interactions with food. Numerous studies have reported that dietary intake of vitamin K-rich foods decreases the INR and impedes the efficacy of warfarin.[8–12,15] Among these foods, spinach has high vitamin K content[17,18] and harbors other bioactive compounds, namely flavonoids and polyphenols. These flavonoids have been documented to inhibit the activity of the Cytochrome P2C9 (CYP2C9) enzyme, an important catalyst in the metabolism of warfarin. Similarly, polyphenols have inhibitory effects on Cytochrome P450 (CYP450) enzyme, increasing the anticoagulant potency of warfarin and the metabolism. The concurrent consumption of spinach with warfarin results in reduced efficacy of the anticoagulant.[21–23]

In this study, rabbits were orally administered warfarin at a dose of 0.4 mg/kg BW for a duration of 16 days. Subsequently, the administration was continued with Fluconazole at a dose of 21 mg/kg BW, serving as the positive control, with spinach (*Amaranthus hybridus*) at a standard and high dose of 2 g/kg BW and 7 g/kg BW, constituting the test group. PGA 1% was used as the control group and the treatment protocol was implemented on the 14th, 15th, and 16th days. The treatment was carried out to assess the potential impact of spinach on the pharmacokinetic and pharmacodynamic profile of warfarin. Fluconazole was selected as the positive control due to the documented ability to increase the concentration of warfarin when co-administered. This was achieved through the inhibition of CYP2C9 enzyme activity, increasing the concentration of warfarin in the systemic circulation.[3,24,25] The enzyme played a crucial role in the metabolism of warfarin, particularly concerning the S-Warfarin enantiomer. By decreasing the activity responsible for warfarin metabolism, the concentration in the body was expected to increase.[2,24]

In addition, PGA was used as a control group because the administration did not affect the action of warfarin as well as the pharmacokinetic and pharmacodynamic profiles.[2] The results were obtained in the form of a pharmacokinetic profile in the form of concentration, AUC, Vd, and C_L. The pharmacokinetic profile was only the R-Warfarin enantiomer because S-warfarin was not detected by the HPLC measurement due to the differences in the rate of metabolism.[26,27] S-warfarin is 3–5 times more potent than R-warfarin in inhibiting the vitamin K epoxide reductase complex, its primary target. The two stereoisomers are metabolized by different Phase 1 enzymes. Specifically, S-warfarin metabolism is predominantly mediated by CYP2C9, leading to faster clearance from the bloodstream. R-warfarin is metabolized more slowly via CYP3A4, with contributions from other enzymes such as CYP1A1, CYP1A2, CYP2C8, CYP2C18, and CYP2C19, as described in the Warfarin Pharmacokinetics Pathway. Due to the rapid metabolism and elimination of

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S-warfarin, its concentrations in the blood were lower and, in several cases, below the detection limit in this study. Therefore, S-Warfarin concentration in the blood sample was very low and depleted by metabolism.

The results showed that the concentration of R-Warfarin in the fluconazole group had higher Cmin and Cmax values after treatment. This was consistent with another study where fluconazole had a mechanism to increase the concentration of warfarin by inhibiting the activity of the CYP2C9 enzyme.[2,24] In contrast, the Cmin and Cmax values of the PGA test group (control) before treatment were higher than after treatment. This was because the normal concentration of warfarin in the body decreased over time. For the low and high concentrations of spinach test groups, the values of Cmin and Cmax increased after treatment, as shown in Table 1. Other pharmacokinetic profiles showed that the fluconazole group had a higher AUC after treatment, while Vd and C_L parameters were lower. The PGA group had higher Vd and C_L after treatment, while the concentration and AUC parameters were lower than before treatment. In the low and high spinach test preparation groups, Vd and C_L were lower after treatment but other parameters were higher, as shown in Table 2. Based on statistical testing, there was no significant effect of low and high spinach concentration co-administration on warfarin level because the value obtained was $p > 0.05$.

The pharmacodynamic profile can be seen from the PT-INR value at the time of minimum concentration in rabbits before and after administration of PGA (control), FZ (positive control), AH-1, and AH-2. The PT-INR value after treatment in the FZ group increased, while in the AH-1 and AH-2 groups, there was a decrease, as shown in Table 3. Therefore, Fluconazole increased the concentration of warfarin with decreased PT-INR value. In this context, high and low spinach groups (AH-1 and AH-2) decreased the warfarin action due to vitamin K in spinach. The presence of high levels of vitamin K interferes with warfarin action to inhibit vitamin K epoxide reductase (VKOR) enzyme and decrease blood clotting.[28,29] The highest spinach dose was 7 g/Kg BW in rabbits equivalent to 100 g in humans. This shows that the ingestion of spinach up to a maximum of 100 g per day is safe when administered with warfarin. Despite observing a reduction in PT-INR levels in the AH-1 and AH-2 groups, the results of various tests showed no statistically significant variance ($p > 0.05$).

In another study, the consumption of foods containing vitamin K1 such as broccoli and spinach in patients who take oral anticoagulants results in a lower response compared to synthetic vitamin K1 supplements.[16] According to Karlson et al, patients subjected to the administration of warfarin with 250 g of spinach (equivalent to 1000 µg of vitamin K1) and 500 g of broccoli (equivalent to 500 µg of vitamin K1) reported a response characterized by an increase in Thrombotest values. However, these values remained within the confines of the therapeutic window limit and showed minimal influence.[30] The results are consistent with the studies mentioned above since spinach had little effect on the work of warfarin in rabbits. Additionally, there were no significant differences in the INR and pharmacokinetic profile. The patients subjected to warfarin therapy safely incorporated spinach into diet, provided the portion size did not exceed 100 g per day.

Conclusion

In conclusion, there were no pharmacokinetic and pharmacodynamic interactions between Spinach (*Amaranthus hybridus*) and warfarin. The patients subjected to warfarin therapy safely incorporated spinach into diet, provided the portion size did not exceed 100 g per day. Consistently consuming this leafy vegetable in the same quantity daily may help maintain the stability of warfarin's effects.

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Disclosure

The authors report no conflicts of interest in this work.

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