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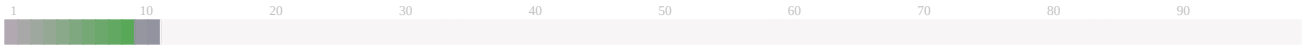

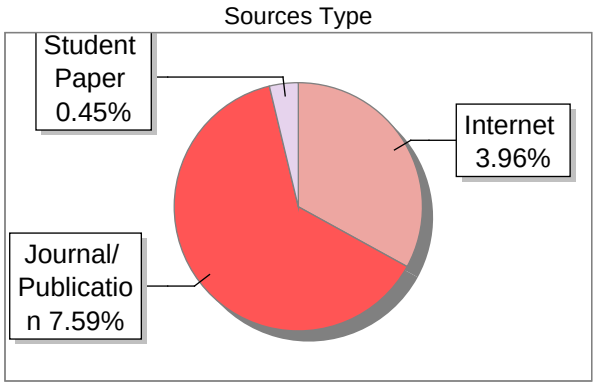

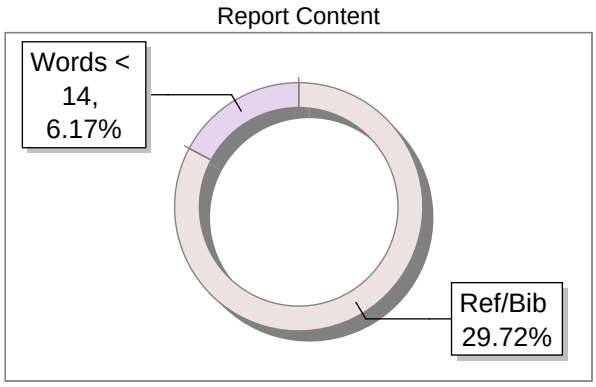

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Effects of *Piper crocatum* leaf extract-based ointments on bacteria associated with diabetic ulcers: an *in vitro* study

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ABSTRACT

Diabetic patients with poor blood glucose control are highly susceptible to developing secondary infections, which can lead to the development of prolonged diabetic ulcers. Therefore, a suitable medication that may effectively prevent the occurrence of secondary infections is crucial to shorten the closure of diabetic ulcers. Red betel leaf (*Piper crocatum* Ruiz & Pav) is reported to possess antimicrobial activity due to the presence of flavonoids. This study aimed to evaluate the effect of ethanolic extract of red betel leaf (EERBL) ointments against the most prevalent bacteria associated with diabetic foot ulcers (DFU): *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The EERBL was prepared by macerating powdered red betel leaf with 96% ethanol and was screened for the presence of flavonoids and the determination of total flavonoid content (TFC) by thin layer chromatography and UV-Vis spectrophotometry, respectively. This study examined three hydrophilic-based ointments containing 10%, 20%, and 30% EERBL, respectively, followed by characterization for pH, spreadability, adhesivity, and viscosity. The EERBL ointments' effect on the bacteria was evaluated using the well-diffusion method by observing inhibition zone formation after 24-hour incubation. The results showed that varying the EERBL concentrations in the formulations led to different spreadability, adhesivity and viscosity ($p<0.05$). Furthermore, all EERBL ointments demonstrated the formation of an inhibition zone on cultured media, indicating the presence of antimicrobial activity. The ointment with 30% EERBL had the largest diameter of the inhibition zone against both bacteria ($p<0.05$). The findings suggest a higher antimicrobial activity was observed with an increase in the concentration of EERBL within the ointments.

Keywords: Red betel leaf (*Piper crocatum*), flavonoids, ointment, antimicrobial activity, diabetic ulcers

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INTRODUCTION

In 2021, Indonesia was among the top ten countries worldwide with the highest occurrence of diabetes with approximately 11 million cases (Hidayat et al., 2022; Tanoey & Becher, 2021). Diabetes is a chronically persistent disease that ranked as the third leading cause of mortality in Indonesia, as reported in 2017 (Hidayat et al., 2022). Inadequate management and poorly controlled diabetes over a long period can result in several severe complications, such as macroangiopathy, diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy, including diabetic foot ulcers (Beulens et al., 2021; Farmaki et al., 2020).

Diabetic foot ulcer (DFU) is considered the most severe complication of diabetes mellitus that is linked to significant illness, death, and a decrease in quality of life, which continues to increase in prevalence over time (Cho et al., 2018). A diabetic foot ulcer is described as a deep wound located below the ankle in individuals with diabetes and may lead to a devastating long-term complication of non-traumatic lower limb amputation, especially in diabetic patients with poorly managed, chronically infected diabetic foot ulcers (Jupiter et al., 2016). Patients diagnosed with diabetes have a one-fourth probability of developing Diabetic Foot Ulcers (DFU) during the course of their lives, and the prevalence of DFU-related amputation is virtually every half minute globally (Chammas et al., 2016). Diabetic foot ulcers (DFU) are a prevalent reason for hospitalization among diabetic patients and have a notable socioeconomic effect (Ha et al., 2021). Patients with DFU experience a mortality rate that is more than double that of diabetic patients without ulcers, with approximately 40% of the five-year mortality rates after ulceration (Jupiter et al., 2016; Rubio et al., 2020). Additionally, the DFU and its long-term consequences are responsible for both direct healthcare expenses and extended periods of disability (Ha et al., 2021; Rubio et al., 2020).

Diabetic patients with ulcers are susceptible to developing secondary infections DFU due to delayed wound healing (Matheson et al., 2021; Sadeghpour et al., 2019). Gram-positive bacteria, such as Staphylococcus aureus, and gram-negative bacteria, including Pseudomonas aeruginosa, are the most responsible colonies that cause secondary infections in DFU, as observed from the bacterial cultures of patient samples globally (Datta et al., 2019; Sadeghpour et al., 2019). These bacteria on the surface wound create a favorable niche for further invasion, resulting in chronic infected DFU (Li et al., 2022). This infection increases the likelihood of multi-drug resistance and ultimately prolongs the wound-healing process (Datta et al., 2019; Yan et al., 2022).

Piper crocatum Ruiz & Pav (*P. crocatum*), or red betel, is a well-known traditional herbal remedy from Indonesia (Setyawati et al., 2023). This easily cultivated plant is readily available, and its leaf has been traditionally and empirically used as herbal medicine to treat wounds for generations in Indonesia (Suri et al., 2021). According to numerous studies, red betel leaves are composed of tannins, polyphenols, saponins, and flavonoids (Januarti et al., 2019; Rahma, 2022; Setyawati et al., 2021; Suri et al., 2021). In addition, flavonoids reportedly exhibit antibacterial properties and may serve as a viable alternative source of treatment for infections manifested in diabetic ulcers (Farhadi et al., 2019; Ibrahim et al., 2018; Shamsudin et al., 2022).

The current study focuses on formulating and characterizing topical ointments containing ethanolic extract of red betel leaf (EERBL) using a hydrophilic ointment base with variations of EERBL concentrations of 10%, 20%, and 30% b/b. The ointment base is selected due to its ability to form a physical barrier, which enables protection from potential bacterial infections in the wound area and provides moisture to facilitate growth factors to migrate and diffuse to the wound during the closure (Hoekstra et al., 2017; Taddese et al., 2021). This study aims to assess the effects of the EERBL ointments against the two most prevalent DFU-associated bacteria, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*, as compared to positive control (mupirocin topical cream) and placebo (the ointment base). Ultimately, the EERBL ointment with the largest inhibitory zone diameter, which signifies antimicrobial activity, will be selected for further in vivo investigation using diabetic animal models in future experiments.

MATERIALS AND METHOD

Materials

Piper crocatum (red betel leaves) was obtained from Bantul while ethanol 96%, toluene, ethyl acetate, formic acid, $AlCl_3$, $FeCl_3$, Na-acetate, NaOH, HCl, and silica gel 60 254 were purchased from Merck®. In addition, methyl paraben, propyl paraben, stearyl alcohol, and white vaseline were purchased from PT Brataco whereas sodium lauryl sulphate, propylene glycol, quercetin standard, and Dragendorff reagent were from Sigma Aldrich®. Furthermore, nutrient broth and Mueller Hinton were acquired from Oxoid®, and distilled water, Mayer's reagent, NaCl 0.9%, Mupirocin ointment were purchased from PT Widatra, Labchem®, Otsuka®, and PT Etercon Pharma, respectively. For antimicrobial activity evaluation, *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were employed in this study.

Methods

Sample preparation

Red betel leaves (*Piper crocatum* Ruiz & Pav) were collected from Bantul and were initially subjected to a determination test for plant identification at the Biology Laboratory, Faculty of Applied Science and Technology, the University of Ahmad Dahlan, Yogyakarta, Indonesia (ID no:142/Lab.Bio/B/III/2024). The red betel leaves were freshly sorted and rinsed to remove dirt and dust by flowing water before being dried at room temperature. The dried leaves were then blended and milled into small pieces and fine powder (Safithri et al., 2023).

Extract preparation and characterization

Ethanolic extract of red betel leaf (EERBL) was prepared by weighing the powdered red betel leaves for 350 g and macerated using 1400 mL of 96% ethanol in a glass jar for 24 hours with occasional stirring. After 24 hours, the filtrate and the residue were separated; the latter was re-macerated for another 24 hours with a similar protocol for two rounds. The filtrates from maceration and re-maceration were collected and evaporated for one hour at 60°C in a rotary evaporator (Heidolph®). Following this, the filtrates were then dried on a waterbath (Memmerth®) until a viscous extract was observed (Navirius et al., 2023; Puspita et al., 2019). The crude extracts were afterward characterized for organoleptic properties, % yield, and water content by the toluene distillation method with the equation (1) (Kemenkes RI, 2017):

$$\text{Water content (\%)} = \frac{\text{Water Volume (mL) x 1 gram/mL}}{\text{Sample weight (gram)}} \; x \; 100\% \quad \text{.....(1)}$$

Phytochemical screening

A qualitative phytochemical screening test was performed on the concentrated EERBL for flavonoids, alkaloids, saponin, tannin, triterpenoids, and steroids (Puspita et al., 2019). In addition, thin-layer chromatography (TLC) was employed to ensure the presence of flavonoids within the ethanolic extracts with quercetin as a biomarker according to the Indonesian Herbal Pharmacopeia Edition II (Kemenkes RI, 2017). A mixture of toluene P, ethyl acetate P, and formic acid P (7:2.5:0.5) was used as a mobile phase while a silica gel 60 F254 was used as a stationary phase (Kemenkes RI, 2017).

Determination of total flavonoid content

Total flavonoid content was determined by UV-Vis spectrophotometry (Shimadzu®) using a quercetin standard. The standard solutions were prepared at 5, 10, 25, 50, 75, and 100 mg/L dissolved in ethanol with the addition of 0.1 mL of 10% $AlCl_3$ and 1 mL of 1M Na-acetate prior to measurement at a wavelength of 438 nm. For sample analysis, 0.5 mL of each concentrated ethanolic extract was added with 1.5 mL of ethanol, 0.1 mL of 10% $AlCl_3$, and 1 mL of 1M Na-acetate and was read at the

same wavelength as the standards (Nerdy et al., 2022). The flavonoid content was calculated and expressed as quercetin equivalent (mg QE/g) with the equation (2) (Kemenkes RI, 2017):

$$\text{Total Flavonoid (QE)} = c\,(V/m) \quad \ldots\ldots\ldots\ldots (2)$$

QE = quercetin equivalent
c = total flavonoid concentration from quercetin standard curve (mg/l)
V = volume of sample
m = weight of sample (gram)

Preparation of ointment of ethanolic Extract of Red Betel Leaf (EERBL Ointment)

An ointment is generally a favorable topical preparation for diabetic ulcer treatment (Agharazi et al., 2022; Salahi et al., 2024; Zhao et al., 2023). In this study, the EERBL ointments were prepared through a fusion method. Firstly, methylparaben, propylene glycol, sodium lauryl sulfate, and water were mixed in an evaporating porcelain dish and heated at 60°C until partly melted; this mixture was referred to as mixture one. Additionally, a second mixture consisting of white vaseline, propylparaben, and stearyl alcohol was prepared and subjected to the same temperature as the previous mixture; this mixture was referred to as mixture two. Subsequently, mixture one was introduced into mixture two; both mixtures were homogeneously mixed. Ultimately, the EERBL was added and constantly stirred using a stirring rod until a homogenous ointment was formed (USP, 2007). All ointments were prepared according to the following formulations (Table 1).

Table 1. Formulations of ethanolic extract of red betel leaf ointments

Ingredients	Ointment Formulations (gram)		
	F1	F2	F3
Ethanolic extract of Red Betel Leaf (EERBL)	10	20	30
Methyl paraben	0.025	0.025	0.025
Propyl paraben	0,015	0.015	0.015
Sodium Lauryl Sulfate	0.7	0.7	0.7
Propyleneglycol	8.5	8,5	8.5
Stearyl alcohol	15	15	15
White vaseline	20	20	20
Aquadest (distilled water)	ad 100	ad 100	ad 100

Characterization of ointment

The EERBL ointments were subjected to characterization for pH, spreadability, adhesivity, and viscosity (Maulina & Sugihartini, 2015). Firstly, the pH was measured by mixing 2.5 grams of the ointment with 50 mL of aquadest. The mixture was then heated up to 60-70°C until homogenously mixed, following measurement with a digital pH meter. Secondly, the spreadability was determined by applying 0.5 grams of the ointments onto a round glass plate, with a second glass plate was placed on top of it. A weight of 100 grams was allowed to remain on the top glass plate for one minute. The diameter of the circle was measured after the ointment was widely spread. Thirdly, the adhesivity was conducted by placing one gram of the ointment on a glass plate that was subsequently covered with another glass plate, with a weight of one kg was added on top of it for 5 minutes. Afterward, the squeezed glass was released, and the adhesiveness was evaluated by measuring the time required to separate both glasses using a stopwatch. Lastly, the viscosity test was carried out using Rheosys Merlin VRII (Scientex®) using a cone and plate 2°/30mm spindle with 0.5 gram of sample of each run.

Antibacterial activity

Nutrient Broth (NB) media was used to propagate pure cultures of DFU-associated bacteria: *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853). The cultured media was placed into an incubator (Binder® Series E/B 28) for 24-hour incubation. Following this, 0.9% NaCl was mixed with bacterial cultures on the NB media, and the level of turbidity was standardized using Mc Farland 0.5. The standardized turbidity level bacterial cultures were uniformly swabbed on the surface of a Mueller Hinton Agar (MHA) media and allowed to sit for 5 minutes. The antibacterial activity assay was performed by means of the well-diffusion method by filling the reservoirs in the MHA media with 0.02 grams of 10%, 20%, and 30% EERBL ointments (F1, F2, and F3), 2% mupirocin ointment (positive control) and placebo (ointment base only). The MHA media was incubated at 37°C for 24 hours. After incubation, a calliper was used to measure the formation of the inhibition zone diameter (Balouiri et al., 2016; Blando et al., 2019; Puspita et al., 2019).

Data Analysis

The data collected from this study were statistically analyzed utilizing the one-way ANOVA and the Tukey test with a significance level of $p<0.05$.

RESULT AND DISCUSSION

Characterization of EERBL

The concentrated ethanolic extract of red betel leaves (*Piper crocatum* Ruiz & Pav) (EERBL) was subjected to characterization, including organoleptic properties, % yield, and water content. The crude extracts were observed as a thick, dark brown-reddish-colored extract with a bitter flavor and typical aroma of red betel leaf. Furthermore, the total weight of the crude concentrated EERBL was 98.12 grams from 350 grams of powdered red betel leaf used, resulting in a yield of 28.03%. Subsequently, the water content was also determined to ensure the risk of potential bacterial or microorganism growth during the storage is low, thereby not affecting the quality and safety of the product. The water content was 7.51% ± 0.286, as determined by the toluene distillation method (Kemenkes RI, 2017). All these evaluated characteristics were in line with the compendial criteria suggested in the Indonesian Herbal Pharmacopeia Edition II and are presented in Table 2.

Table 2. Attributes of crude ethanolic extract derived from red betel leaves

Parameter	Result	Requirement*
Color	Dark brown-reddish	Dark brown-reddish
Taste	Bitter	Bitter
Odor	Distinctive aroma of red betel leaf	Distinctive aroma of red betel leaf
Yield	28.03%	> 17%
Water Content	7.51% ± 0.286**	< 10%

*Compendial requirement as described in the Indonesian Herbal Pharmacopeia Edition II for red betel leaf extract
**data are shown as mean ± SD (n=3)

Phytochemical screening & total flavonoid content

Preliminary phytochemical screenings were performed on the extracts prior to ointment preparations (Puspita et al., 2019). According to the Indonesian Herbal Pharmacopeia Edition II, the red betel leaf extract is known to contain flavonoids with quercetin used as a marker to identify the flavonoid content in the extract (Kemenkes RI, 2017). The results suggest the presence of flavonoids, alkaloids, phenols, steroids, triterpenoids, and tannins with an absence of saponins in the extracts as screened through a qualitative phytochemical screening test (Table 3). These findings are in line with previous studies (Heliawati et al., 2022; Puspita et al., 2019; Suri et al., 2021). In addition, a thin-layer chromatography test on the extract was also performed to ensure the flavonoid content as compared to the quercetin standard with a theoretical Rf value of 0.38 for identification (Kemenkes RI, 2017). The result showed that both the extract and the quercetin standard had the same Rf value at around 0.38,

establishing the presence of quercetin in the extract (Figure 1). Ultimately, total flavonoid content (TFC) in EERBL was also determined via UV-Vis spectrophotometry using a quercetin standard with a result of 78.14 ± 7.63 mg QE/g (n=5).

Figure 1. Thin layer chromatography analysis of quercetin standard (A) vs EERBL (B) with identical RF values, suggesting the presence of flavonoid quercetin in the EERBL

Table I. Preliminary phytochemical screenings on the red betel leaf ethanolic extracts

Secondary Metabolite	Test	Result
Flavonoids	Bate Smite-Metcalfe's test	+
	NaOH 10%	+
Alkaloids	Deagendorff's test	+
	Mayer's test	+
Saponins	Frothing test	-
Tannins	Ferric Chloride test	+
Triterpenoids	Liebermamn's test	+
Steroids	Liebermamn's test	+
Phenols	Ferric Chloride test	+

Key = + present, - absent

Preparation and characterization of ointment

Three different formulations were employed for preparing the hydrophilic-based ointments with variations on the concentration of ethanolic extract of red betel leaf (EERBL): 10%, 20%, and 30%, respectively. A hydrophilic base was selected as the ointment base due to its ability to facilitate good absorption while providing optimum viscosity for better spreadability and adhesivity to the skin upon application (Shigeyama et al., 1999).

Table 4. Characterization data of the red betel leaf ethanolic extracts ointments

Parameter	F1	F2	F3
pH	4.81 ± 0.14^{a}	5.12 ± 0.21^{ab}	5.28 ± 0.18^{b}
Spreadability (cm)	5.52 ± 0.24^{a}	4.89 ± 0.19^{b}	4.37 ± 0.19^{c}
Adhesivity (seconds)	32.43 ± 1.8^{a}	54.18 ± 2.6^{b}	63.71 ± 1.4^{c}
Viscosity (cP)	8367 ± 373^{a}	$10,453 \pm 910^{b}$	$13,551 \pm 794^{c}$

Key: EERBL = Ethanolic Extract of Red Betel Leaf; data are shown as mean ± SD (n=3); values within a row with different superscripts are significantly different according to the Tukey test ($p < 0.05$)

After preparation, the EERBL ointments were characterized for pH, spreadability, adhesivity, and viscosity. The measured pH of the ointments ranged between 4.81 to 5.28. The spreadability of the ointments was at 4.65 to 5.20 cm, while the adhesivity ranged from 32 to 63 seconds among the three formulations, respectively. Ultimately, the viscosity of the ointments was also evaluated with Rheosys Merlin VRII (Scientex®) using a cone and plate 2°/30mm spindle with a 0.5 gram sample for each measurement. The data of the ointment's characterization is presented in Table 5.

From Table 4, the pH of the ointments met the criteria of the ideal pH ointment for human skin, which is 4.5-6.5. For spreadability, Formulation 1 showed the highest value with an average spread of 5.22 cm, while for the adhesivity parameter, Formulation 3 had the longest adherence with 63.71 seconds, slightly longer than Formulation 2. The same trend was also observed in the viscosity, where Formulation 3 demonstrated the most viscous ointment with 11,851 cP. The viscosity of all groups was still within the range of compendial viscosity for an ointment, which is around 2000 to 50,000 cP (Anonim, 2020). Consistency of the ointments might be responsible for these findings as a result of different ratios between aqueous and non-aqueous components in each formulation which determines the liquidity of the ointments, leading to different spreadability, adhesivity, and viscosity (Conti-Silva et al., 2018; Herbig et al., 2023). Of the three formulations, Formulation 1 contains the highest amount of water within its formulation, while Formulation 3 is the opposite. This implies that Formulation 1 became easily spread ointment while Formulation 3 was the stickiest and most viscous ointment. Despite these results, all the parameters above follow the ideal criteria for ointments.

Antibacterial activity

Previous studies showed that ethanolic fraction of red betel leaf extract was able to exert antimicrobial activity against several bacteria (Candrasari, et al., 2012; Purba et al., 2022; Puspita et al., 2019; Rachmawaty et al., 2018). Therefore, this study focuses on evaluating the antimicrobial activity of the EERBL with different concentrations within the ointment. The antibacterial activity of the EERBL ointments was evaluated by means of determining the inhibition zone diameter on the two most common bacteria associated with diabetic foot ulcer (DFU): *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Sadeghpour et al., 2019). Mupirocin ointment was used as a positive control as it is widely used clinically as a broad-spectrum topical antibiotic for ulcers (Dallo et al., 2023; Ishikawa & Horii, 2005). The diameter zone of inhibition was examined using the well-diffusion method. Data on the diameter of the inhibition zone is displayed in Table 5.

Table 5. Inhibition zone diameter of the red betel leaf ethanolic extracts ointments

Formulation	Diameter of Inhibition Zone (mm)	
	Staphylococcus aureus	***Pseudomonas aeruginosa***
F1 (10% EERBL)	10.4 ± 0.60^{a}	8.1 ± 0.40^{a}
F2 (20% EERBL)	12.6 ± 0.63^{b}	9.8 ± 0.35^{b}
F3 (30% EERBL)	14.8 ± 0.98^{c}	12.7 ± 0.55^{c}
Positive Control*	36.4 ± 0.65^{d}	32.1 ± 0.93^{d}
Placebo**	0 ± 0^{e}	0 ± 0^{e}

Key = EERBL = Ethanolic Extract of Red Betel Leaves; *Mupirocin cream (2%); **ointment base only; Data are shown as mean ± SD (n=3); values within a column with different superscripts are significantly different according to the Tukey test ($p < 0.05$)

The antimicrobial activity in this study was determined by the ability to form an inhibition zone in the cultured media after 24-hour incubation. As depicted in Table 5, all placebo groups - containing only ointment base - showed no inhibitory activity, while positive controls exhibited the largest inhibition zone diameter in both groups. This is reasonable since mupirocin is a broad-spectrum topical antibiotic that can clinically constrain a wide range of bacterial growth by blocking bacterial RNA and protein synthesis, including Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* (Dallo et al., 2023; Erwin, 2024; Ishikawa & Horii, 2005). In addition, the

formation of an inhibition zone was observed in all EERBL ointment groups. Formulation 1 exhibited a weak antimicrobial activity in both bacteria while Formulation 3 had the largest diameter with a moderate activity against both bacteria. Even though the magnitude of the constrained activity was approximately a third lower than that of the control group, all EERBL ointments (10-30%) were able to exert antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the most common bacteria isolated from diabetic foot ulcer samples (Sadeghpour et al., 2019). The statistical analysis of ANOVA and Tukey test revealed that all groups were significantly different ($p<0.05$).

As shown in Table 5, the antimicrobial activity is higher with an increase in the concentration of EERBL within the ointments. Such inhibition activity is likely due to the flavonoid content of EERBL in the EERBL ointments. In general, the inhibitory activity of the EERBL ointments against *Staphylococcus aureus* is slightly greater than *Pseudomonas aeruginosa* as evaluated by the inhibition diameter zone. These results are in agreement with other studies that report similar findings (Hartini & Nugroho, 2020; Puspita et al., 2019; Rachmawaty et al., 2018). Flavonoids are effectively able to affect bacterial cell membrane integrity and biofilm formation, leading to bacterial growth suppression (Heliawati et al., 2022; Kaul et al., 2013; Puspita et al., 2019; Shamsudin et al., 2022).

CONCLUSION

The EERBL ointments exhibit antimicrobial activity by forming an inhibitory zone against DFU-associated bacteria: *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The EERBL ointment with 30% EERBL content - provides the second-largest inhibition zone diameter after the control group with moderate activity to both bacteria. Diabetic patients with poor blood glucose management are at high risk of acquiring secondary infections that may delay wound closure. Such delay in wound healing may develop minor injuries into diabetic ulcers if not properly treated. Regarding this, therefore, the EERBL ointment is likely to be developed as a candidate for DFU topical treatment as it exerts flavonoid-related antimicrobial activity that may prevent secondary infection, leading to shortened closure of DFU. However, further experiments are needed to evaluate the effectiveness of the EERBL ointment for DFU in diabetic animal models.

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