Journal of Applied Pharmaceutical Science Vol. 14(01), pp 291-296, January, 2024
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2024.141692
ISSN 2231-3354

Effects of *Erythrina subumbrans* (Hassk.) Merr. leaves extract on RBCs membrane stability and egg white-induced edema in rats

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ARTICLE HISTORY

Received on: 24/06/2023
Accepted on: 27/10/2023
Available Online: 04/01/2024

Key words:
Anti-inflammatory, *Erythrina subumbrans*, human red blood cells methods, proinflammatory cytokines.

ABSTRACT

Erythrina subumbrans, an Asian plant, has been traditionally used to treat fever and edema. Studies about *E. subumbrans* are very limited; however, alkaloids, pterocarpans, flavanones, and triterpenes have been reported to be present in the barks, twigs, and roots. These metabolites have been explored for their antidiabetic and antimicrobial activity. The leaves have been studied on carrageenan-induced Wistar rats. Our work explored the effects of *E. subumbrans* leaves extract (ESE) on the membrane stability of human red blood cells (HRBC) and in egg white-induced edema of 25 male Wistar rats. The rats were randomly assigned to five groups: the normal control group (treated with sodium-carboxymethylcellulose 0.5%); the positive control group (treated with sodium diclofenac 4.5 mg /kg BW); and three test groups (treated with ESE 100 mg/kg body weight, 200 mg/kg body weight, and 400 mg/kg body weight). All groups were edema-induced using egg white 1%, and the volume of edema was measured every 30 minutes until 180 minutes. The phytochemical screening of ESE indicated the presence of flavonoids, alkaloids, and tannins. The *in vitro* study confirmed that ESE inhibits hemolysis in HRBC with an IC_{50} of 75.61 ± 0.366 µg/ml, while the IC_{50} of sodium diclofenac is 49.97 ± 0.001 µg/ml. The *in vivo* study revealed that all doses of ESE and sodium diclofenac significantly reduced edema in rats' paws at 180 minutes. These findings suggested that ESE might be further developed as an anti-inflammatory drug candidate.

INTRODUCTION

Acute inflammation starts after the presence of inducers that eventually triggers the release of cytokines, acute-phase proteins, and chemokines to initiate the migration of neutrophils and macrophages to the site of inflammation [1]. Allergens are categorized as the inducers of inflammation. At the initial stage of inflammation, the cells are activated and liberate proinflammatory proteins. Egg white, which contains approximately 10% of protein [2], among those are allergens [3], could significantly reduce serum immunoglobulin E (IgE) levels, elevate interleukins and tumor necrosis factor-alpha, and activate B lymphocytes in allergy-sensitized mice [3]. The egg white was reported prospective to be used as an inducer for animal models of inflammation [4].

Discovering plant-based anti-inflammatory drugs is challenging yet worth exploring. Studies have been carried out to obtain potential plants [5–9]. *Erythrina subumbrans* (local name *dadap serep*) has been traditionally used by the people in Ciamis, West Java, Indonesia, to treat fever and edema. The barks, twigs, and roots of this plant have been reported to contain alkaloids, pterocarpans, flavanones, and triterpenes [10–12]. These metabolites have been explored for their antidiabetic and antimicrobial activity [12]. The leaves have been studied on carrageenan-induced edema in paw rats and revealed that a dose of 400 mg/kg body weight possessed the strongest activity in reducing edema [13]. Although very limited pharmacological descriptions of *E. subumbrans* are provided, several studies on

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the leaves and barks of *E. variegate*, a plant of the same genus, have confirmed its analgesic and anti-inflammatory activity [14–16]. Moreover, flavanones, prenylated flavonoids, prenyl isoflavones, and pterocarpanes isolated from the *Erythrina* genus have demonstrated anti-inflammatory activity by various mechanisms [17–19]. For that reason, our work aimed to study the effects of *E. subumbrans* leaves extract (ESE) on the membrane stability of human red blood cells (HRBC) and in egg white-induced edema of male Wistar rats.

MATERIALS AND METHODS

Study area

The *in vitro* study was carried out in the Biology Pharmacy Laboratory, and the animal study was performed at the Pharmacology Laboratory of Universitas Bhakti Kencana, Bandung, West Java, Indonesia. The duration of the experiment was 8 months (November 2021-July 2022).

Plant materials

The leaves of the plants were collected from Purwajaya Village, Ciamis, West Java, Indonesia, and taxonomically identified by a botanist at the Biology Herbarium Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia. The plant samples were confirmed as *E. subumbrans* (Hassk.) Merr. (Fabaceae) (Letter No. 21/HB/11/2021). The characteristics of the plant samples matched those described in Naturalis Biodiversity Center (NL)-Botany (https://www.gbif.org/occurrence/2517594190).

Instruments and chemicals

Instruments used were digital analytical balance (Shimadzu), digital rotavapor RV 10 (IKA®), centrifuge (Beckman J2-HS), evaporator glass-ware (Pyrex®), chemical glass-wares (Pyrex®), micropipette (Dragon Lab), water plethysmometer (PanLab), and double-beam UV-visible spectrophotometer (Hitachi U-2900).

Chemicals were ethanol 96% (Brataco-Chemika, Indonesia), egg white 1% (contained albumin, prepared by dissolving 1 ml of freshly taken hen's egg white in 100 ml of distilled water), sodium diclofenac, sodium carboxymethylcellulose, phosphate buffer solution pH 7.4, Alsever′s solution (A3551 Sigma-Aldrich) to prevent coagulation of blood (which is composed of 2.05% dextrose, 0.8% sodium citrate, 0.055% citric acid, and 0.42% sodium chloride).

Collection of human whole blood

Human whole blood was obtained from the Blood Transfusion Unit of the Indonesian Red Cross Society (https://www.pmikotabandung.org/pendaftaran_online/), postal address at Jl. Aceh 79, Bandung, West Java, Indonesia. The protocol of this study has been approved by Padjadjaran Research Ethics Committee (https://kep.unpad.ac.id/) with document no. 506 / UN6.KEP/EC/2022.

Preparation of *E. subumbrans* extract (ESE)

The fresh leaves were cleaned from dust and dirt, washed under tap water, and dried in a drying cabinet at room temperature for 5 days until constant weight. The dried leaves (weight 2,045 g) were ground and sieved (mesh 4/18). 250 g of the leaves powder was soaked in 1,500 ml of ethanol 96% for 3 × 24 hours at 25°C–26°C. The extract (313.28 g) was filtered, and the solvent was rotary-evaporated at 50°C–60°C. The viscous extract yielded 15.3 % *w*/*w*.

Phytochemical screening

The phytochemical screening was done by following a previous procedure described elsewhere [20] as follows:

Flavonoid assay

0.1 g of ESE was added with 10 ml of distilled water and filtered. The filtrate was added with a small quantity of magnesium powder, a few drops of concentrated hydrochloric acid, and 1 ml of amyl alcohol. The mixture was shaken vigorously. Flavonoids were confirmed present in ESE as indicated by the reddish color in the amyl alcohol layer.

Alkaloid assay

0.1 g of ESE was dissolved in 10 ml of chloroform and added with four drops of ammonium hydroxide to alkaline. The mixture was added with 10 drops of sulfuric acid 2M. The water layer was added with three drops of freshly prepared Mayer reagent (a mixture of 1.36 g of mercuric chloride and 5 g of potassium iodide in 100 ml of water). Alkaloids were present in ESE as indicated by a cream-colored precipitate.

Tannins assay

0.1 g of ESE was added with 10 ml of distilled water and a few drops of ferric chloride 1% solution. Tannins were present in ESE, as indicated by the dark blue color of the solution.

The effect of ESE on the membrane stability of HRBC

The reagents were prepared as described previously [16]. Human whole blood 10 ml was mixed with 10 ml of Alsever's solution and centrifugated at 27°C for 10 minutes at 3,000 rpm. The supernatant was separated, the packed erythrocyte cells were washed using an isotonic saline solution, and the solution was recentrifugated until a 10% HRBC suspension was obtained [21, 22]. The HRBC (erythrocyte) suspension was incubated at 4°C for further use.

The negative control sample consisted of 0.5 ml HRBC suspension mixed with 1 ml hypotonic saline alone. ESE sample solutions (20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) were prepared in a phosphate buffer saline. 1 ml of sample was mixed with 0.5 ml HRBC suspension and incubated at 37°C for 30 minutes. After incubation, mixtures were centrifuged at 3,000 rpm for 20 minutes, and the supernatant was measured at 560 nm [21]. Diclofenac sodium solutions (20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) were used as the positive control drug.

% inhibition of hemolysis (represents the membrane stability) was calculated by following this formula:

$$\%\ \text{Inhibition of hemolysis} = \left(\frac{A1 - A2}{A1}\right) \times 100\%,$$

where A1 is the absorbance of phosphate buffer saline and A2 is the absorbance of ESE or sodium diclofenac.

Animals

Twenty-five male Wistar albino rats were purchased from an official animal breeding farm in Majalaya-Bandung, West Java, Indonesia, aged 10–12 weeks and weighing 200–300 g. The rats were acclimatized at 25°C–26°C under a 12 hours light, 12 hours dark cycle, 55% relative humidity, with standard food (composed of carbohydrate 274 g, vegetable protein 706 g, vegetable fat 20 g) and water freely for 1 week. The rats were randomly assigned to five groups. Animal health and behavior were monitored daily during acclimatization week and *in vivo* study. Animal handling was approved by the Research Ethics Committee, Padjadjaran University, Indonesia (approval document No. 506/UN6. KEP/EC/2022). No animals were found dead during the study.

The effect of ESE in egg white-induced edema rats

The study was conducted by following a previous procedure described elsewhere [23].

The rats were randomly assigned to five groups (5 rats/cage, dimension of the cage, 60 cm length × 40 cm width × 38 cm height): (1) the negative control group (treated with sodium-carboxymethylcellulose 0.5%); (2) the positive control group (treated with diclofenac sodium 4.5 mg /kg body weight); and three test groups treated with (4) ESE 100 mg/kg body weight; (4) ESE 200 mg/kg body weight, and (5) 400 mg/kg body weight, all diluted in distilled water, respectively. The administration of ESE to the rats was done using an oral gavage needle, which directly transfers the extract into the esophagus of the rats [8,24,25]. Diclofenac sodium was chosen as the positive control drug because this drug inhibits the biosynthesis of prostaglandin synthesis [26].

All groups of rats were edema-induced using an intraplantar injection of 0.1 ml egg white 1% on their right limb as described previously with a few modifications [27]. The edema volume was measured every 30 minutes until 180 minutes using a plethysmometer.

Statistical analysis

Data were analyzed using IBM SPSS (International Business Machines Corporation Statistical Product and Service Solutions) Statistics version 20.0 (https://www.ibm.com/spss). The experiments were performed in triplicates, and all values are expressed as mean ± SD. Data obtained from the *in vivo* study were analyzed by one-way analysis of variance (ANOVA), continued with the least significant difference (LSD) Test to compare between groups and time. The LSD is used when the difference between the population means is significant [28]. Further analysis was done using Tukey and Bonferroni Tests. The mean difference is statistically significant at the *p*-value < 0.05. The one-way ANOVA and the independent paired-samples *t*-test were employed for the *in vitro* study.

RESULTS AND DISCUSSION

The phytochemical screening of ESE confirmed the presence of flavonoids, alkaloids, and tannins. Compared to our results, a previous study reported the presence of alkaloids, flavonoids, and pterocarpans in *E. subumbrans* barks, twigs, and roots [4–6]. Minor differences in the chemical contents of the leaves compared to those on the bark and roots of another plant (*Cassia sieberiana*) were also reported [29].

ESE at concentrations of 20–100 µg/ml protected the human erythrocyte membrane against lysis induced by hypotonic solution. The percent hemolysis inhibition diagram for ESE (Fig. 1) revealed a good inhibition of hemolysis in the HRBC membrane. The concentration at which 50% of the blood cells were inhibited from hemolysis (IC_{50}) was determined by

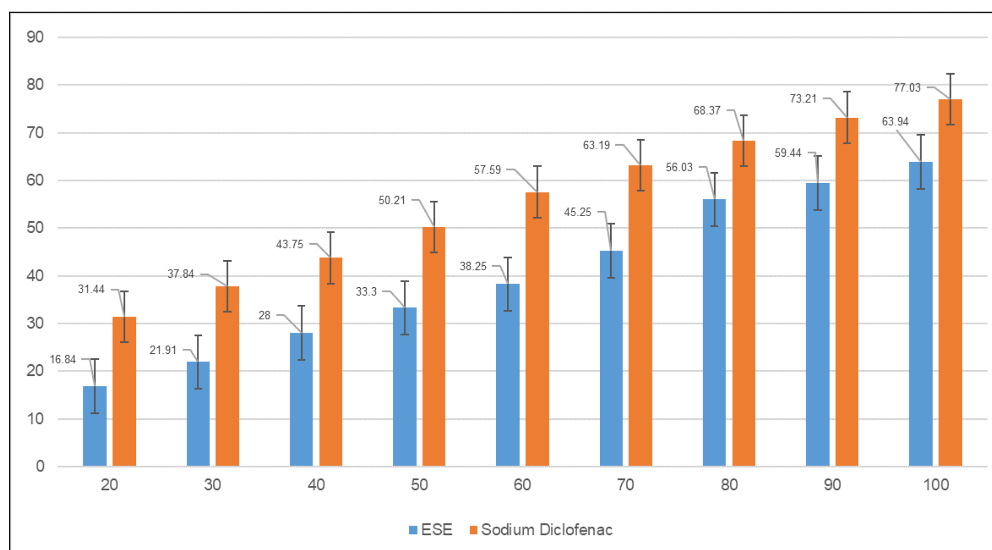

Figure 1. Diagram of % hemolysis inhibition on HRBC membrane by ESE (blue) and sodium diclofenac (red). IC_{50} of ESE = 75.61 ± 0.366 µg/ml, IC_{50} of sodium diclofenac = 49.97 ± 0.001 µg/ml. The *x*-axis represents the concentration in µg/ml; the *y*-axis represents the % hemolysis inhibition. Error bars represent the SDs.

Table 1. The effect of ESE on the edema volume of egg white-induced male Wistar rats (mean ± SEM) (n = 5).

Treatment Group	Mean volume (ml) ± SD						
	T0	T30	T60	T90	T120	T150	T180
Negative Control (egg white 1%)	0.27 ± **0.06**	0.40 ± 0.00	0.50±0.00	0.50 ± 0.00	0.43 ± 0.06	0.40 ± 0.00	0.40 ± **0.00**
Diclofenac sodium (4.5 mg/kg BW)	0.28 ± **0.05**	0.40 ± **0.00**	0.47 ± 0.08	0.40 ± **0**.00	0.37 ± 0.06	0.30 ± 0.00*	0.20 ± **0.00***
ESE 100 mg/kg BW	0.28 ± **0.05**	0.50 ± 0.00	0.40 ± 0.00	0.38 ± **0.06**	0.38 ± 0.05	0.33 ± 0.06	0.28 ± 0.05*
ESE 200 mg/kg BW	0.28 ± **0.05**	0.43 ± **0.06**	0.43 ± 0.06	0.37 ± **0.05**	0.37 ± 0.05	0.30 ± 0.00*	0.28 ± 0.05*
ESE 400 mg/kg BW	0.28 ± **0.05**	0.43 ± **0.05**	0.40 ± 0.06	0.33 ± 0.05	0.30 ± 0.05*	0.30 ± 0.00*	0.27 ± 0.00*

Statistical analysis was done using IBM SPSS Statistics version 20.0 for Windows, with one-way Analysis of Variance (ANOVA), continued with the Least Significant Difference (LSD) Test to compare between groups and time. Further analysis was done using the Tukey and Bonferroni tests. The mean difference is statistically significant at the p-value < 0.05.
*Significant difference (p < 0.05) compared to the negative control group.

plotting concentration against percentage inhibition, keeping the hemolysis produced within the negative control group at 100% (IC_{50} of ESE = 75.61 ± 0.366 µg/ml); however, compared to that of diclofenac sodium (IC_{50} = 49.97 ± 0.001 µg/ml), the inhibitory activity of ESE towards hemolysis is weaker.

The membrane of the red blood cells comprises a cytoskeleton and a lipid bilayer. Impairments in the membrane may alter the integrity of the cells and lead to cell hemolysis [30]. HRBC membranes are much like lysosomal membranes; thus, hypotonicity-induced HRBC membrane lysis may be adopted to assess the anti-inflammatory activity of drugs [31. A previous study reported that the release of phospholipase A2 (PLA2) can be prevented by stabilizing the erythrocyte membrane. PLA2 is an enzyme that catalyzes the conversion of phospholipid in the cell membrane to arachidonic acid, which is the substrate of cyclooxygenase enzymes. Thus, preventing the release of PLA2 leads to reducing the inflammation process [32].

Inflammation induced by egg white 1% and the anti-inflammatory test resulted in changes in the paw volume of each group (Table 1). In the negative control group, the edema volume increased from T0 to T90 and showed a slightly different trend compared with the other groups due to no inhibition of the inflammatory process. However, the positive control group treated with diclofenac sodium and the ESE groups revealed a diminishing trend starting at T90 (although not significantly compared to the negative control group), which indicated the occurrence of inhibitory activity by the drug and the extracts. Diclofenac sodium significantly reduced the edema volume in rat's paw at T150 (p < 0.05), similar to that of ESE doses of 100 mg/kg BW and 200 mg/kg BW. Interestingly, the ESE dose of 400 mg/kg BW could significantly reduce the edema at T120. These findings confirmed that ESE could reduce egg white-induced edema. Thus, it is predicted to inhibit inflammation by blocking the liberation of histamine and serotonin, allergenic mediators, which are activated by egg white [33].

Egg white contains proteins, e.g., ovalbumin (OVA), ovotransferrin, ovomucin, lysozyme, and avidin. These proteins reveal antibacterial and immunoprotective activities; in fact, they are also capable of activating unfavorable proinflammatory responses, such as allergens [2–4, 34–37] that are responsible for inducing edema [37, 38] as a result of type 1 hypersensitivity reaction [39]. Type 1 hypersensitivity involves the release of IgE antibodies against the exposure of antigens (allergens). Allergens activate and bind to antigen-presenting cells, eventually releasing peptides. With the aid of T-helper cells, the allergens stimulate B cells to produce IgE antibodies. The IgE then attaches to Fc receptors on the surface of the mast cells and activates the cells, which results in degranulation and the release of histamine and other inflammatory mediators [39]. Inflammation, in this case, edema, often occurs after intraplantar injection of inducers in the hind paw of rats. A previous study reported a strong infiltration of neutrophils and eosinophils in the Wistar rat paws after an inflammation induced by OVA [40].

Our present study discloses the effect of ESE on the inhibition of hemolysis in hypotonicity-induced human red blood cell membranes by the spectrophotometric method and investigates the *in vivo* effect of ESE on the egg white-induced paw of male Wistar rats. In conclusion, ESE revealed anti-inflammatory activity as confirmed by both the *in vitro* (IC_{50} of ESE in inhibiting hemolysis in human red blood cell membranes = 75.61 ± 0.366 µg/ml) and *in vivo* by reducing the edema volume of rat's paw at T90. ESE doses of 200 mg/kg BW and 400 mg/kg BW significantly reduced the edema volume in rat's paw at T150. These findings provide evidence that ESE is the potential to be developed as an anti-inflammatory agent. However, this study still lacks the molecular mechanism of ESE in inhibiting hemolysis in human red blood cell membranes and edema in rats' paws; therefore, it is challenging to carry out further studies.

AUTHOR'S CONTRIBUTIONS

Sri Adi Sumiwi, Jutti Levita, and Yasmiwar Susilawati were responsible for the conception and design of the study and supervised the project. Elis Susilawati, Widhya Aligita, Marita Kaniawati, and Divi Ade Liani contributed to the extraction process, *in vitro* study, animal handling, and *in vivo* study and confirmed the authenticity of all the raw data and statistical analysis. Sri Adi Sumiwi and Jutti Levita contributed equally to the acquisition and interpretation of the reported data. Elis Susilawati and Jutti Levita contributed to the writing and revising of the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declared no potential conflicts of interest.

FUNDING

This work was funded by Universitas Bhakti Kencana via the Center of Research and Community Service. The publication fee is funded by Universitas Padjadjaran via the Directorate of Research and Community Engagement in the scheme of the Academic-Leadership Grant 2022 of Prof. Dr. Jutti Levita.

ETHICS APPROVALS

Animal handling and human whole blood procedures were performed as approved by the Research Ethics Committee (https://kep.unpad.ac.id/; approval document No. 506/UN6. KEP/EC/2022), Universitas Padjadjaran, Indonesia (recognized by Forum of Ethics Review Committee in Asia & Western Pacific Region).

DATA AVAILABILITY

The datasets used and/or analyzed during the present study are available from the first author upon reasonable request.

PUBLISHER'S NOTE

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How to cite this article:
Susilawati E, Aligita W, Kaniawati M, Liani DA, Levita J, Susilawati Y, Sumiwi SA. Effects of *Erythrina subumbrans* (Hassk.) Merr. leaves extract on RBCs membrane stabilityand egg white-induced edema in rats. J Appl Pharm Sci. 2024;14(01):291–296.