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Research Article

IN VITRO ANTI-INFLAMMATORY ACTIVITY TEST OF TINOCRISPOSIDE AND FREEZE-DRIED AQUEOUS EXTRACT OF *TINOSPORA CRISPA* STEMS ON HUMAN RED BLOOD CELL BY INCREASING MEMBRANE STABILITY EXPERIMENT

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ABSTRACT

Objective: This study was aimed to evaluate the anti-inflammatory effect of isolated tinocrisposide and freeze-dried aqueous extract of *Tinospora crispa* stems on human red blood cell (HRBC) by increasing membrane stability *in vitro* models.

Methods: Anti-inflammatory effect of tinocrisposide and FDAETCS was evaluated by *in vitro* HRBC membrane stabilization method. The study was separated into two steps which were a hemolytic and a membrane stabilization experiment. The hemoglobin that was released throughout the damaged erythrocytes membrane was then quantified at the wavelength of (λ) 560 nm.

Results: The hemoglobin in the HBRC supernatant that treated with tinocrisposide at concentration of 100, 200, 400, 600, 800, and 1000 µg/ml showed an absorbance at λ 560 nm of 0.060, 0.061, 0.071, 0.072, 0.075, and 0.0793, respectively, and the calculated hemolysis percentage was 0.032, 0.097, 1.203, 1.236, 1.641, and 2.079%, respectively. We found a linear correlation between concentration and hemolytic activity of tinocrisposide, with regression equation, y=0.0023x−0.1312 (r=0.929). Meanwhile, the HBRC supernatant that treated with FDAETCS at concentration of 100, 200, 400, 600, and 800 µg/ml showed an absorbance at λ 560 nm of 0.063, 0.064, 0.066, 0.067, and 0.077, respectively, and revealed the hemolytic percentage of 0.347, 0.473, 0.693, 0.992, and 1.896%, respectively. It also gave a linear correlation between FDAETCS concentration and hemolytic activity percentage, with regression equation, y=0.002x+0.0222 (r=0.895). Moreover, in HRBC membrane stability experiment, tinocrisposide concentration of 100, 200, 400, 800, and 1000 µg/ml gave absorbance at λ 560 nm of 0.818, 0.808, 0.798, 0.789, 0.773, and 0.761, respectively, and calculated HRBC membrane stabilization activity as much as 5.437, 6.533, 7.707, 8.748, 10.597, and 12.100%, respectively. Meanwhile, the positive control ibuprofen 25 µg/ml only exerted the membrane stability of 5.620%. It was found a linear correlation between tinocrisposide concentration and membrane stability percentage, with the regression equation, y=0.0072x+4.8312 (r=0.9932). Treated FDAETCS in the concentration of 100, 200, 400, and 800 µg/ml gave the absorbance at λ 560 nm of 0.802, 794, 0.777, 0.791, and 0.792, with stability membrane percentage of 7.283, 8.208, 10.944, 8.555, and 8.401%, respectively. It can be seen that the FDAETCS concentrations and its hemolytic percentage showed a parabolic relationship, which gave a maximum at a concentration of the extract of 400 mg/ml with membrane stabilizing of 10.944%.

Conclusion: It can be concluded that tinocrisposide and FDAETCS have an anti-inflammatory activity by increase the membrane stability of lysosome cell that has equal physiological properties with erythrocytes membrane cell and it has no hemolytic activity.

Keywords: Anti-inflammatory, Hemolytic, Human red blood cell, Membrane stabilization, Tinocrisposide, *Tinospora crispa*.

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INTRODUCTION

Tinospora crispa is a climber plant widely distributed from the Southwestern part of China to Southeast Asia including Indonesia, Malaysia, Vietnam, Thailand, and India. It naturally occurs in primary rainforests or mixed deciduous forests up to 1000 m above sea level [1]. *T. crispa* is well known as a bitter medicinal plant called Brotowali, Akar Seruntun, or Andawali in Indonesia, Akar Patawali in Malaysia, and Makabuhay in Philippine. A decoction of the stems has been traditionally used for the treatment of gout, diabetes, malaria, fever, wound healing, and hypertension, and scientifically reported as an analgesic and anti-inflammatory agents [2].

T. crispa has been demonstrated to possess antioxidant, antiproliferative [3], antinociceptive [4], anti-inflammatory [5], antibacterial [6], antifilarial, antimalarial, antipyretic [7], and antihyperglycemic activities [8]. In our previous study from methanol extract of dried pulverized *T. crispa* stems, tinocrisposide ($C_{27}H_{36}O_{11}$) has been isolated, a furanoditerpene glycoside with a very bitter taste (Fig. 1) [9]. Furthermore, in our previous research, various concentrations of tinocrisposide were tested against the LPS-stimulated RAW 264.7 cells, and the results showed the decrease of NO level production in concentration activity-dependent manner, with half-maximal inhibition concentration of 46.92 µM. Thus, it can be developed as anti-inflammatory candidate drug because NO is a reactive nitrogen species which is produced by NO synthase [10]. S. Chanda and A.R. Juvekar have tested *in vitro* anti-inflammatory activity of various concentrations of syringic acid by protein denaturation and human red blood cell (HRBC) membrane stabilization assay. The reference drugs used in experiments were aspirin and diclofenac sodium [11]. Meanwhile, Shamsi *et al*. studied anti-inflammatory activity of Joshanda, a polyherbal Unani formulation consists of seven plant ingredients by albumin denaturation inhibition experiment used aspirin as a reference compound [12]. In the current research, we are trying to prove the anti-inflammatory activity of tinocrisposide that can exert the increasing of HRBC membrane stability.

Inflammation is a normal protective response to tissue injury caused by physical trauma, destructive chemicals, or microbiological

agents [13]. It is characterized by an alteration of immune system [14]. The inflammatory process is very closely related to wound healing. Inflammation and repair are a continuous process in wound healing involving inflammatory cells and enzymes. Cyclooxygenase (COX) family enzyme – COX-1 and COX-2 – is widely studied in inflammation disorder and critically responsible in the illness incident [15].

The inflammation, in general, is treated by nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs which are useful for reducing swelling and pain from inflammation. NSAIDs inhibit the COX enzyme which caused the conversion of arachidonic acid to prostaglandin become disrupted [16]. These drugs have a risk of gastrointestinal toxicity, cardiac toxicity, and others for prolonged use [17] so that traditional medicine or treatment from nature can be used as an alternative.

MATERIALS AND METHODS

Plant materials

T. crispa was collected from Padang, Indonesia. The plant specimens were properly identified and authenticated by Dr. Nurainas at the Department of Biology, Faculty of Science, Andalas University.

Instruments

UV lamp (Merck, Germany), pH meter, column chromatography (Pyrex), micropipette (Socorex), rotary evaporator (Bűchi), UV-visible spectrophotometer (Hitachi U-2910), Fourier-transform infrared spectroscopy (FTIR) Spectrum One (PerkinElmer), freeze dryer (Christ, Germany), water bath (Eyela SB-1000), centrifuge (Universal 32 R, Hettich Zentrifugen, USA), incubator (Thermo Scientific), microplate reader (BioRad × Mark™), Falcon tube, Eppendorf tube, 96-well plate (Iwaki), and EDTA vacuum tube were used.

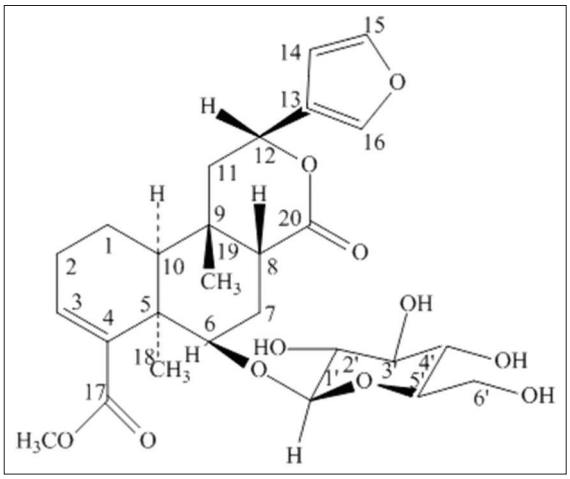

Fig. 1: Chemical structure of tinocrisposide [10]

Materials

T. crispa stem, dichloromethane (DCM), methanol pro analysis (Merck), ethyl acetate (Merck), hexane (Merck), acetic acid (Merck), silica gel 60 F_{254} (Merck, Germany), TLC, sodium dodecyl sulfate (Sigma-Aldrich), isotonic NaCl solution (PT Widatra Bhakti), dimethyl sulfoxide (Thermo Fisher Scientific, USA), distilled water, ibuprofen, and human blood were used.

Freeze-dried aqueous extract *Tinospora crispa* stems (FDAETPS) preparation

Freshly collected stems were cut, shade-dried for 2–3 days, and ground into powder. It was then gently boiled with distilled water (1:10) for 30 min, filtered, and allowed to cool at room temperature. The filtrate was then freeze-dried at −50°C and 0.1 atm for 9 h, and stored at 4°C before use.

Tinocrisposide isolation

The stems were cut, shade-dried, ground into powder, and macerated using methanol 3 times for 3 days each at room temperature. The extracts then evaporated under reduced pressure using a rotary evaporator to yield crude extracts. The extracts were dissolved in 5% acetic acid and allowed overnight, decanted, and then partitioned, sequentially, with hexane and DCM. Each fraction was evaporated and dried to yield n-hexane, DCM fractions. The DCM fractions then subjected into column chromatography over silica gel 60 and eluted using step gradient elution (3–8%) system with the mixture of DCM and methanol (3–8% methanol) which were combined on the basis of thin-layer chromatography (TLC) evaluation. TLC was carried out using the mixture of MeOH: DCM (1:9) as a solvent system and the fractions with same Rf values were combined and further purified by column chromatography to obtain tinocrisposide as white amorphous powder [16]. The powder then identified using TLC, ultraviolet spectrophotometry, and FTIR spectroscopy.

In vitro hemolytic test

Blood from the healthy human volunteer who had not taken any NSAIDs for 2 weeks before the experiment was collected using vacutainer tube EDTA, mixed with isotonic NaCl solution, and centrifuged at 3000 rpm for 5 min. The packed cells were washed with isosaline and a 5% HRBC suspension was made. Tinocrisposide (100, 200, 400, 600, 800, and 1000 µg/ml) and FDAETCS (100, 200, 400, 600, and 800 µg/ml) were prepared in a distilled water. 0.5 ml of sample was mixed with 0.5 ml HRBC suspension in Eppendorf tubes and incubated at 37°C for 1 h [18]. After incubation, the tubes were centrifuged at 1000 rpm for 5 min and the supernatant was transferred into 96-well plate. The absorbance of hemoglobin in the supernatant was measured at a wavelength, λ of 560 nm. As a positive

Table 1: Absorbance value of tinocrisposide and FDAETCS on hemolytic activity test

Sample	Concentration (µg/ml)	Absorbance			
		1	2	3	Mean±SD
Tinocrisposide	100	0.058	0.059	0.063	0.060±0.002
	200	0.061	0.064	0.058	0.061±0.003
	400	0.068	0.071	0.074	0.071±0.002
	600	0.068	0.079	0.069	0.072±0.007
	800	0.073	0.074	0.079	0.075±0.003
	1000	0.074	0.082	0.082	0.079±0.005
FDAETCS	100	0.063	0.062	0.065	0.063±0.002
	200	0.064	0.062	0.066	0.064±0.002
	400	0.066	0.070	0.063	0.066±0.004
	600	0.063	0.067	0.071	0.067±0.002
	800	0.079	0.077	0.076	0.077±0.002
Negative control (0.9% NaCl)		0.060	0.061	0.060	0.060±0.001
Positive control (5% SDS)		0.939	1.007	0.977	0.974±0.034

control, 5% SDS was employed and an isotonic NaCl solution was used as a negative control. Measurements were replicated 3 times for each concentration [19]. The hemolytic activity was calculated with the following equation:

$$\% \text{ Hemolytic} = \left[\frac{A_1 - A_2}{A_3 - A_2}\right] \times 100\%$$

Table 2: Hemolytic activity percentage of tinocrisposide and FDAETCS on HRBC

Sample	Concentration (µg/ml)	Hemolytic activity (%)
Tinocrisposide	100	0.032
	200	0.097
	400	1.203
	600	1.236
	800	1.641
	1000	2.079
FDAETCS	100	0.347
	200	0.473
	400	0.693
	600	0.992
	800	1.896
% SDS	5	100

HRBC: Human red blood cell

Where, A1=Absorbance of test sample
A2=Absorbance of negative control
A3=Absorbance of positive control

***In vitro* anti-inflammatory test**

This study was conducted using the HRBC membrane stabilization method. Blood from the healthy human volunteer who had not taken any NSAIDs for 2 weeks before the experiment was collected using vacutainer tube EDTA. Blood samples were mixed with isotonic NaCl solution and centrifuged at 3000 rpm for 5 min. The packed cells were washed with isosaline and a 10% HRBC suspension was prepared [20]. Tinocrisposide (100, 200, 400, 600, 800, and 1000 µg/ml) and freeze-dried aqueous extract (2000, 1000, 800, 600, 400, and 100 µg/ml) were dissolved in a distilled water. Reaction mixtures consisted of 1 ml test sample, 0.5 ml of HRBC suspension, and 1.5 ml distilled water. It was incubated at 37°C for 30 min and centrifuged at 3000 rpm. The hemoglobin content of the supernatant solution was measured spectrophotometrically at a wavelength (λ) of 560 nm. Measurements were replicated 3 times for each concentration. Ibuprofen that used as a standard and a control was made by substituting the test samples with isosaline [21]. The percentage of HRBC membrane stabilization was calculated using the following equation:

$$\% \text{ Stability} = 100 - \left[\frac{OD_{sample}}{OD_{control}}\right] \times 100\%$$

Table 3: Absorbance value of tinocrisposide and FDAETCS on HRBC membrane stabilization method

Tinocrisposide	Concentration (µg/ml)	Absorbance			
		1	2	3	Mean±SD
	100	0.817	0.819	0.817	0.818±0.001
	200	0.789	0.798	0.838	0.808±0.033
	400	0.777	0.820	0.798	0.798±0.022
	600	0.803	0.780	0.785	0.789±0.012
	800	0.741	0.788	0.791	0.773±0.028
	1000	0.734	0.752	0.796	0.761±0.032
FDAETCS	100	0.799	0.800	0.807	0.802±0.004
	200	0.781	0.792	0.808	0.794±0.014
	400	0.784	0.789	0.804	0.777±0.010
	600	0.724	0.795	0.855	0.791±0.066
	800	0.747	0.786	0.753	0.792±0.021
Ibuprofen	6.25	0.841	0.812	0.852	0.835±0.021
	12.5	0.836	0.870	0.792	0.832±0.039
	25	0.821	0.852	0.776	0.816±0.038
Control		0.815	0.901	0.879	0.865±0.045

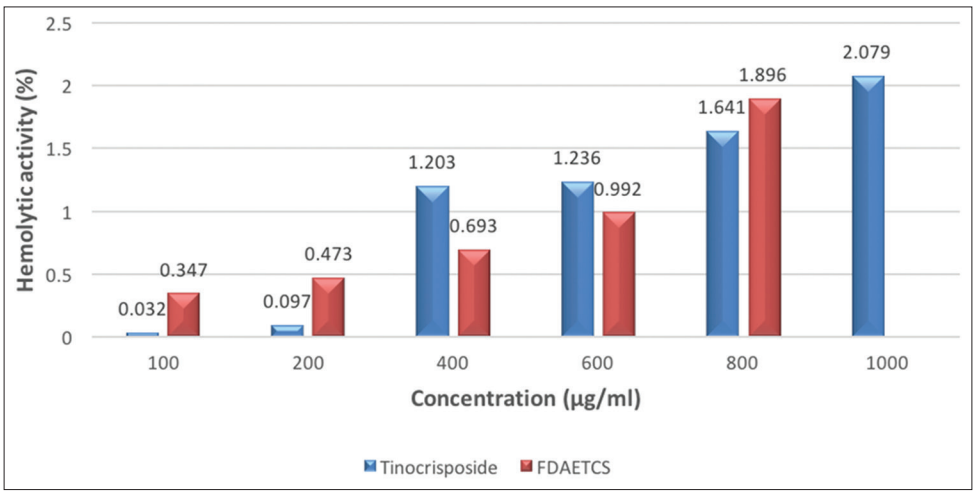

Fig. 2: Diagram of hemolytic activity percentage of tinocrisposide and FDAETCS on HRBC

Data analysis

All data were expressed as means ± standard deviation of result obtained from three independent experiments. Statistical analysis was performed using paired sample t-test. $P<0.05$ was considered statistically significant. The statistical package IBM SPSS Statistics Version 21 for Windows was used in the analysis.

RESULTS AND DISCUSSION

***In vitro* hemolytic test**

The hemoglobin in the HBRC supernatant that treated with tinocrisposide at concentration of 100, 200, 400, 600, 800, and 1000 µg/ml showed an absorbance of 0.060, 0.061, 0.071, 0.072, 0.075, and 0.0793, respectively. Meanwhile, the HBRC supernatant that treated with FDAETCS at concentration of 100, 200, 400, 600, 800, 1000, and 2000 µg/ml showed an absorbance of 0.063, 0.064, 0.066, 0.067, and 0.077, respectively (Table 1).

The percentage of calculated hemolytic activity of various concentrations of tinocrisposide and FDAETCS on HRBC is presented in Table 2.

Data in Table 2 showed that hemolytic activity percentage of tinocrisposide in the concentration of 100, 200, 400, 600, 800, and 1000 µg/ml on HRBC was 0.032, 0.097, 1.203, 1.236, 1.641, and 2.079%, respectively. We found a linear correlation between tinocrisposide concentration and hemolytic activity percentage, with regression equation, y=0.0023x−0.1312, and coefficient correlation, R=0.929. The same condition occurs in the FDAETCS that at the concentration of 100, 200, 400, 600, and 800 µg/ml showed the hemolytic percentage of 0.347, 0.473, 0.693, 0.992, and 1.896%, respectively. It also gave a linear correlation between FDAETCS concentration and hemolytic activity percentage, with regression equation, y=0.002x+0.0222, and coefficient correlation, R=0.895.

Table 4: Membrane stability percentage of various concentrations of tinocrisposide and FDAETCS

Sample	Concentrations (µg/ml)	Membrane stability (%)
Tinocrisposide	100	5.437
	200	6.533
	400	7.707
	600	8.748
	800	10.597
	1000	12.100
FDAETCS	100	7.283
	200	8.208
	400	10.944
	600	8.555
	800	8.401
Ibuprofen	25	5.620

Fig. 2 showed a clear comparison of hemolytic activity between tinocrisposide and FDAETCS in various concentrations. Hemolytic percentage with a value of <10% was interpreted as non-hemolytic, and a value of >25% was considered showing hemolytic effect. From the experiments, it is showed that tinocrisposide and FDAETCS have no hemolytic effect with a percentage value of <10% and no toxicity on HRBC so that this compound can later be used for drug formulations. Erythrocyte membrane is analogous to the lysosomal membrane, so compounds that caused a damage to erythrocyte membrane will also damage lysosomal membrane [22].

***In vitro* anti-inflammatory test**

Tinocrisposide and FDAETCS were tested if they are able to prevent water-induced lysis of red blood cells using membrane stabilization assay. The result of the HRBC membrane stabilization test is shown in Table 3.

Table 3 showed that by treating tinocrisposide in the concentration of 100, 200, 400, 800, and 100 µg/ml on HRBC membrane stabilization experiment will give the absorption of hemoglobin at wavelength, λ 560 nm by 0.818, 0.808, 0.798, 0.789, 0.773, and 0.761, respectively, with stability membrane by 5.437, 6.533, 7.707, 8.748, 10.597, and 12.100%, respectively. Meanwhile, the positive control ibuprofen 25 µg/ml only exerted the membrane stability of 5.620% (Table 4). We found a linear correlation between tinocrisposide concentration and membrane stability percentage, with the regression equation, y=0.0072x+4.8312, and the coefficient correlation, R=0.9932 (Fig. 4).

Data in Table 3 showed that by treating tinocrisposide in the concentration of 100, 200, 400, 800, and 1000 µg/ml on HRBC membrane stabilization experiment will give the absorbance of hemoglobin at wavelength (λ) 560 nm by 0.802, 794, 0.777, 0.791, and 0.792, respectively, with stability membrane by 7.283, 8.208, 10.944, 8.555, and 8.401%, respectively. It can be seen that the relationship between FDAETCS and its membrane stability percentage is parabolic, which gave a maximum at a concentration of the extract of 400 mg/ml with membrane stabilizing of 10.944%. This phenomenon is assumed because the extract also contains other compounds that can cause inflammatory or hemolysis of membrane erythrocytes. Tinocrisposide at the concentration of 100 µg/ml had HRBC membrane stability of 5.437% that close to membrane stability of the positive control, ibuprofen at the concentration of 25 µg/ml that revealed membrane stability of 5.620%. It means at those concentrations the efficacy of tinocrisposide was 96.74% of ibuprofen. If membrane stability of tinocrisposide compared to FDAETCS membrane stability, it can be seen an interesting phenomenon, namely, FDAETCS in the level concentration of 100–400 µg/ml showed higher efficacy then tinocrisposide, but in the higher concentration, tinocrisposide revealed evidently higher efficacy than FDAETCS did (Table 4 and Fig. 3).

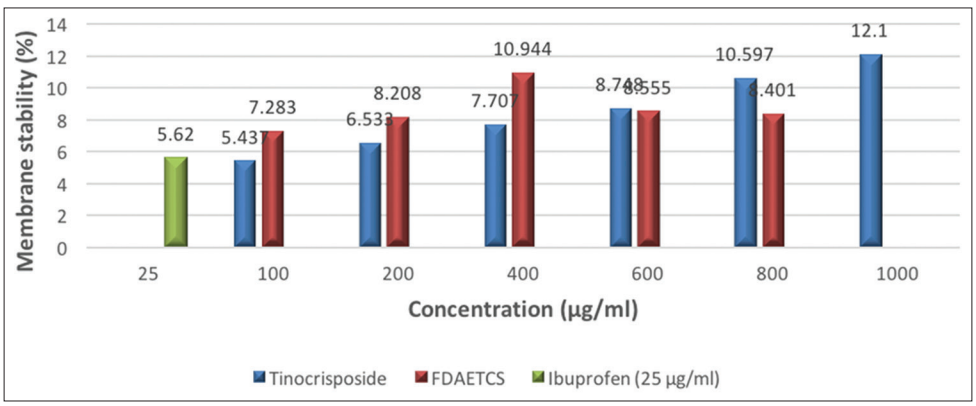

Fig. 3: Membrane stability percentage (%) of tinocrisposide, FDAETCS, and ibuprofen

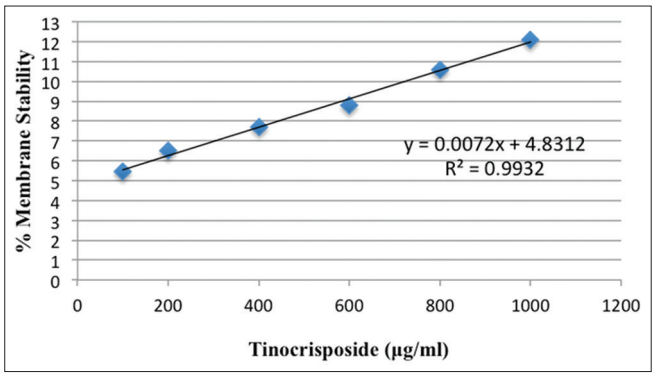

Fig. 4: Diagram between tinocrisposide concentrations versus membrane stability percentage

Anti-inflammatory agents control the biochemical processes involved during the inflammatory response by stabilizing the membranes of lysosomes [18]. The erythrocyte membrane is analogous to the lysosomal membrane, and its stabilization implies that tinocrisposide and FDAETCS may as well stabilize lysosomal membranes [23]. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which cause further inflammation and damage on extracellular release [24].

In this study, distilled water was used as an inductor that triggers hemolysis that can cause the release of inflammatory mediators. By adding 1.5 ml of distilled water into the well plate, it will decrease an osmotic pressure solution outside of the erythrocyte cell, while in the cell, it remains equivalent to 0.9% NaCl solution. Then, the water from outside of the cell will move into the cell, across the erythrocytes cell membrane which functions as a semipermeable membrane, this causes the swelling of the cell, and eventually rupture and hemolysis. In human body, an inflammatory mediator actually plays an important role in preventing the spread of infection and is also needed to repair tissue damage [18]. However, excessive inflammatory mediators can cause tissue damage and chronic inflammation [25].

CONCLUSION

It can be concluded that tinocrisposide and FDAETPS stem have an anti-inflammatory activity by increase the membrane stability of lysosome cell that has equal physiological properties with erythrocytes membrane cell and it has no hemolytic activity; therefore, tinocrisposide can be promoted and developed as a new anti-inflammatory drug candidate.

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AUTHORS' CONTRIBUTIONS

Conception and design of study: A.Z. Adnan, I.R. Sudji, D.I. Roesma, and M.D. Novida. Acquisition of data: M.D. Novida and H.A. Ali. Analysis and interpretation of data: A.Z. Adnan, M.D. Novida, D.I. Roesma, and A. Fauzana. Drafting the manuscript: F. Armin, M.D. Novida, and A. Fauzana. Revising the manuscript critically for important intellectual content: A.Z. Adnan and I.R. Sudji.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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