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Original Article

ANTI-INFLAMMATION EFFECTS OF BIOACTIVE FRACTION DLBS0533 CONTAINING PHALERIA MACROCARPA AND NIGELLA SATIVA ON ANIMAL MODEL

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ABSTRACT

Objective: DLBS0533 extract is a bioactive fraction obtained from combination of *Phaleria macrocarpa* and *Nigella sativa*. This present study aims to observe its potentially anti-inflammatory activities using carragenaan-induced paw edema in mice as animal model.

Methods: Mice were divided into control, positive control and dose groups. Diclofenac potassium was used as a positive control. Treatment DLBS0533 was given at dose 39, 78 and 156 mg/kg body weight (b. w.). Edema thickness was examined for 6 hours.

Results: Reduction in edema is shown in 27.26%; 30.71% and 32.72% at dose of 39, 78 and 156 mg/kg b. w., respectively. Comparation between dose groups and positive control group show that dose 156 mg/kg b. w. did not give significantly different. Therefore, dose 39 and 78 mg/kg b. w. also gave anti-inflammatory activities proved by reduction in edema.

Conclusions: Taken together, DLBS0533 potentially have anti-inflammatory activities.

Keywords: DLBS0533, Anti-inflammation, Mice, Edema, Carragenan.

INTRODUCTION

Inflammation is the body response from injury of cells and tissues due to different insulting factor, such as infection, chemical, thermal and mechanical factors [1]. Joint pain and swelling, related to arthritis or other diseases are disorders commonly associated with inflammation. Inflammation drugs can be grouped into steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). The most widely used medicine between the two groups for the treatment of inflammation-related disorders is NSAIDs. It acts primarily by inhibition cyclooxygenase (COX) pathway. Specifically it inhibits arachidonic acid metabolism into prostaglandins. Diclofenac potassium is one of NSAIDs which works non-selectively by inhibiting COX-1 and COX-2 [2].

Bioactive fraction DLBS0533 is fractionated from *Phaleria macrocarpa* and *Nigella sativa*. *P. macrocarpa*, commonly known in Indonesia as "mahkota dewa", is a plant originated from Papua Island, Indonesia and grows in many Indonesian areas. Traditionally, this plant has been used as anti-microbial, anti-fungal, anti-diabetic, anti-inflammatory and many more [3]. In addition, extract of *P. macrocarpa* are also reported for a number of pharmacological activities, including anti-tumor, anti-oxidant, anti-viral and vasodilator [4]. *Nigella sativa* is a plant originated in the Mediterranean region, but it has been cultivated in other area, such as Asia. Indonesia is a potential area for its growth due to the suitable tropical climate. *N. sativa* has been traditionally used as analgesic, anti-pyretic, anti-inflammatory and anti-microbial [5].

Study by Hendra, et al. resulted potent antioxidant and anti-inflammatory activities of *P. macrocarpa* fruit extract through inhibition of nitric oxide (NO). A study held by Seif show potent anti-inflammatory activities of *N. sativa* against osteoporosis via inhibition of COX activity [6]. Previous study result by Tjandrawinata et al. (2010) show anti-inflammatory activities of another *Phaleria macrocarpa* fraction, DLBS1425. It is an fraction of *P. macrocarpa* fruits which contains 20.26% phalerin. This fraction confers its anti-inflammatory effects by inhibiting COX-2 mRNA. Thereby causing a decreased in Prostaglandin (PGE) synthesis [7]. Another previous study confers its anti-cancer agent which targets genes involved in both cell survival and apoptosis in MDA-MB-231 breast cancer cells [8].

In this present study, DLBS0533 which is combination of *Phaleria macrocarpa* and *Nigella sativa* was interesting to observe for potentially its anti-inflammatory activities. Carrageenan-induced paw edema in mice was chosen as the model study for inflammation.

MATERIALS AND METHODS

Test and control articles

DLBS0533 was prepared by Dexa Laboratories of Biomolecular Sciences (Cikarang, Indonesia). DLBS0533 is extracted from a combination of two herbs, namely *Nigella sativa* seed and *Phaleria macrocarpa* fruits. *Nigella sativa* was purchased from Kulon Progo (Yogyakarta, Indonesia), while *Phaleria macrocarpa* was provided from Bantul (Yogyakarta, Indonesia). *Nigella sativa* seed and *Phaleria macrocarpa* fruits (1:3) were percolated using water. Extract was dried using evaporator at 45ºC. Dried fraction is named as DLBS0533 and analyzed by Thin Layer Chromatography (TLC), using Silica gel 60 F_{254} (Merck, USA) with mixed solvent ethyl acetate/acetone/formic acid/water (8:2:1:1). TLC resulted in two spot with Rf ±0.25 and Rf ±0.70 was examined under $UV_{366\ nm}$*.

DLBS0533 was given at doses of 39, 78 and 156 mg/kg body weight (b. w.), which were equivalent to 300, 600 and 1200 mg/70 kg in human dose, respectively. Diclofenac potassium at dose of 9.1 mg/kg b. w. was used as positive control in this study. Carrageenan 0.5% (w/v) was used to induce edema in mice. Distilled water was used as solvent and given as negative control.

Test animals and housing

Two untill three-month-old male Swiss mice (weighing 25-35 g) were obtained from Biological Laboratories, Faculty of Pharmacy, Sanata Dharma University, Jogjakarta. Animals were treated similarly with respect to the food, cage and drinking water. They were fed with standard rodent food and *ad libitum* drinking water. The room temperature was maintained at $22^0C \pm 3^0C$ with relative humidity of 30% to 70%. The animals were exposed to 12 h-12 h light dark cycle. All experimental precedures for animal use have been approved with approval number KE/FK/613/EC by Medical and Health Research Ethics Committees, ministry of national education, Faculty of Medicine Gadjah Mada University. All

experimental animals were acclimatized for ± 2 weeks prior to the commencement of study.

Study design

Mice were randomly divided into positive control group, negative control and dose groups, consisting of 10 mice in each Mice were fasted for 24 hours before administration of DLBS0533, only drinking water was given *ad libitum*. Carrageenan-induced paw edema in mice was used as animal models. 0.5% Carrageenan was given via subplantar route to induce edema. Then, each group was treated orally with distilled water, diclofenac potassium and treatment dose of 39, 78 and 156 mg/kg b. w. DLBS0533, respectively. Measurement of edema was performed using calipers post-carrageenan induction and treatment. Edema thickness was examined every 30 minutes for 6 hours post-carrageenan induction edema (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 minutes). The calculation of the edema volume was conducted using formula as follows:

$$Tu = Tt - To \quad (1)$$

Tu: Thickness of mice foot edema in particular time

Tt: Thickness of mice foot after 0.5% carrageenan induction

To: Thickness of mice foot before 0.5% carrageenan induction

Area Under Curve (AUC) was calculated for each minute within 0-6 hours using trapezoid method formula, as below:

$$AUC_{tn-1} = \frac{t_n \, T_{tn-1} + T_{tn}}{(t_n - t_{n-1})} \quad (2)$$

T_{tn-1}: Average edema volume on t_{n-1}

T_{tn}: Average edema volume on t_n

Percentage inhibition of inflammation was calculated according to the formula:

$$Inhibition\ of\ Inflammation = \frac{(AUC_{0-x})_0 - (AUC_{0-x})_n}{(AUC_{0-x})_0} \ x\ 100\% \quad (3)$$

$(AUC_{0-x})_0$ = average AUC_{0-x} for negative control group, $(AUC_{0-x})_n$ = AUC_{0-x} for each animal given test drug at dose of n.

Statistical analyses

Area Under Curve (AUC) was used to calculate percentage inhibition of inflammation (% reduction in edema). Statistical analyses was conducted between each treatment group to negative and positive control group. It was significantly different if $p<0.05$. The results were analyzed using *Kolmogorov-Smirnov* test, followed by ANOVA with 95% confidence interval. *Scheffe* test was used for significantly different results. *GLM repeated measures* test was performed to know the differences of edema thickness graph between each group.

RESULTS

Examination of edema thickness, AUC and percentage inhibition of inflammation were shown in table 1, 2 and 3 and also Fig. 1. Table 1. shows edema thickness during 6 hours of observation.

Minute 0 represented paw edema thickness as a baseline after carrageenan induction and treatment. Fig. 1 is a graph of mean edema thickness during 6 hours of observation.

Table 2 shows the calculation of AUC from edema thickness observed. Therefore, Table 3 shows the percentage inhibition of edema. This present study resulted in reduction of edema 27.26%, 30.71% and 32.72% at dose of 39, 78 and 156 mg/kg b. w., of DLBS0533 respectively.

The positive control group shows reduction 48.65% in edema. Statistical analyses between treatment dose groups to negative and positive control show that all dose groups gave significantly different with negative groups. Then, only dose of 156 mg/kg b. w. of DLBS0533 is not significantly different compared to diclofenac potassium at dose 9.1 mg/kg b. w. (Table 2 and table 3).

Table 1: Mean edema thickness (mm) to time (minute) in anti-inflammatory effect using 0.5% carrageenan-induced mice animal model

Treatment	Average Edema Thickness (mm) on Minute												
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**	**330**	**360**
Negative control (Distilled water)	2.24	1.92	1.90	1.69	1.93	1.80	1.68	1.92	1.59	1.60	1.29	1.07	1.24
Positive control (Diclofenac potassium 9.1 mg/kg b. w.)	0.39	0.64	0.67	0.83	1.10	1.49	0.89	1.23	0. 87	0.76	0.74	0.69	0.45
DLBS0533 39 mg/kg b. w.	1.27	1.25	1.11	1.30	1.45	1.29	1.44	1.49	1.27	1.21	0.80	1.02	0.79
DLBS0533 78 mg/kg b. w.	1.51	1.21	0.99	1.54	1.12	1.31	1.17	1.14	1.20	1.29	0.87	0.77	0.65
DLBS0533 156 mg/kg b. w.	0.95	0.74	0.78	1.22	1.53	1.56	1.29	1.71	1.14	1.08	1.02	0.73	0.56

Data depicted on both table 1 and Fig. 1 suggested that treatment of DLBS0533 in all dose group lead to reductions in paw edema thickness, thus signifying DLBS0533 as anti-inflammatory agent. This effect is pronouncedly depicted on Fig. 1 in which the downward trends of the line represented reduction in carrageenan-induced paw edema thickness in mice.

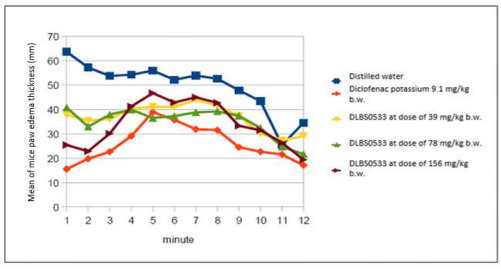

Fig. 1: AUC Graph in Minute 0-360 in association between measurement time (minute) to mice paw thickness (mm)

DISCUSSION

This present study was conducted to assess the anti-inflammatory effect of DLBS0533 using carrageenan-induced mice. Carrageenan-induced paw edema is a widely used test to determine anti-inflammatory effect. It is a simple and routine animal model for evaluation in site of inflammation, without any injury or damaged to the paw edema [9]. Carrageenan also has been known to have sensitive response for inflammation [10].

Carrageenan, as irritant substances, induced cells injury through releases of mediators which cause inflammation. The development of carrageenan-induced edema is a biphasic event. The initial phase (after an hour) is associated to the release of serotonin, histamine

and bradykinin. The late phase (after an hour) is mainly due to the neutrophil infiltration into the inflammatory site and production of large amounts of pro-inflammatory mediators, such as prostaglandins (PGE) and various cytokines. The inflammatory edema reached its maximum level at the third hour and after that it started declining [11]. A previous study by Posadas et al. indicated that injection of carrageenan in the mouse paw caused a biphasic response: an early inflammatory response that lasts for 6 hours and a second late response that peaks at 72 hours, declining at 96 hours [12].

Previous study by Alemi et al. resulted that alcoholic extract of *N. sativa* seeds has an anti-inflammatory effects to rat's neuronal cells [13]. Crude fix oil of *N. sativa* also shows inhibitory effect to COX and 5-lipooxygenase (5-LO) pathways of arachidonate metabolism in rat peritoneal leukocytes. It also shows dose-dependent inhibition of thromboxane and leukotriene (LT) [14].

The most abundant and active component of *N. sativa* is thymoquinone (TQ). TQ is believed to exert anti-inflammatory effect by inhibiting 5-LO and LT synthesis in a dose-dependent manner [15]. *N. sativa* is also reported to contain phenolic. This compound has diverse physiological properties, including analgesic and anti-inflammatory activities [16]. Beside that previous study by Hendra et al. shows NO inhibitory effect of *P. macrocarpa* extract in a dose-dependent manner. The highest dose of the extract shows the highest inhibition percentage of NO [3]. NO is recognized as a mediator and regulator of inflammatory responses, and it is involved in several inflammatory disorders [17, 18]. Antioxidant and anti-inflammatory activities of *P. macrocarpa* was due to the presence of flavonoids and phenolic compounds [3,19]. Flavonoid has been identified for its potential in inhibiting COX, thus it inhibits the formation of PGE [20]. Therefore, combination of *P. macrocarpa and N. sativa* need to study for its anti-inflammatory activities.

Table 2: Mean AUC to time (minute) in anti-inflammatory effect in 0.5% carrageenan-induced mice animal model

Treatment	Mean AUC on Minute												AUC
	0-30	30-60	60-90	90-120	120-150	150-180	180-210	210-240	240-270	270-300	300-330	330-360	
Negative control (Distilled water)	63.68	57.21	53.78	54.26	55.92	52.17	53.91	52.59	47.87	43.38	35.36	34.53	604.64
Positive control (Diclofenac potassium 9.1 mg/kg b. w.)	15.51	19.71	22.61	29.06	38.88	35.72	31.85	31.49	24.48	22.62	21.48	17.07	310.46
DLBS0533 39 mg/kg b. w.	37.77	35.39	36.11	41.28	41.10	40.88	43.98	41.54	37.29	30.14	27.26	27.09	439.80
DLBS0533 78 mg/kg b. w.	40.76	32.91	37.91	39.90	36.44	37.28	38.81	39.18	37.37	32.37	24.63	21.45	418.98
DLBS0533 156 mg/kg b. w.	25.35	22.80	30.09	41.25	46.38	42.86	44.99	42.71	33.27	31.46	26.28	19.37	406.79

Table 3: Percentage inhibition of inflammation in 0.5% carrageenan-induced mice animal model

Group	AUC of foot edema in mice on minute 0-360	Inhibition of inflammation (%)	Difference to negative control	Difference to positive control
Negative control (distilled water)	604.64	0	-	S
Positive control (diclofenac potassium 9.1 mg/kg b. w.)	310.46	48.65	S	-
DLBS0533 at dose of 39 mg/kg b. w.	439.80	27.26	S	S
DLBS0533 at dose of 78 mg/kg b. w.	418.98	30.71	S	S
DLBS0533 at dose of 156 mg/kg b. w.	406.79	32.72	S	NS

S: Significantly different ($p<0.05$), NS: Not significantly different ($p>0.05$)

Result of this study shows DLBS0533 has potentially inhibition of the inflammation activity (as anti-inflammatory). Since DLBS0533 is a bioactive fraction of combination between *P. macrocarpa and N. sativa,* it potentially contains thymoquinone, flavonoids and phenolic compound. Those combination compounds may have strong anti-inflammatory activities.

It resulted DLBS0533 at dose of 156 mg/kg b. w. gave the most potent anti-inflammatory effect and was not significantly different to positive control (diclofenac potassium) at dose of 9.1 mg/kg b. w. DLBS0533 at dose of 156 mg/kg b. w. where equivalent to 1200 mg/70 kg human weight have a same effect in reduction in edema with diclofenac potassium 9.1 mg/kg b. w. where equivalent to 70 mg/70 kg human weight. Therefore, DLBS0533 at dose 39 and 78 mg/kg b. w. also gave anti-inflammatory activities that was shown in the percent inhibition of edema percentage inhibition of edema. This present study show DLBS0533 as anti-inflammatory agent. Further studies are required to establish the safety of DLBS0533 in animal and human models, followed by clinical trials to elucidate its effect in human patients.

CONCLUSION

In conclusion, result of the present study suggested that DLBS0533 has anti-inflammatory effect. DLBS0533 at dose 156 mg/kg b. w. (equivalent to human dose 1200 mg/70 kg b. w. human dose), may provide strong anti-inflammatory effect same as diclofenac potassium 9.1 mg/kg b. w. (equivalent to human dose 70 mg/70 kg b. w. human).

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CONFLICT OF INTERESTS

The author(s) declared no conflicts of interest with respect to the authorship and/or publication.

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ABBREVIATIONS

NSAIDS - Non-steroidal anti-inflammatory drugs, COX-Cyclooxygenase, NO-Nitric oxide, PGE-Prostaglandin, b.w.-Body weight LO-Lipooxygenase, LT-Leukotriene, TQ-Thymoquinone

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