Topical anti-inflammatory activity of *n*-hexane extract of *santalum album* linn leaves on rat ear oedema induced by croton oil

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Abstract. *Santalum album* Linn (*S.album*) has been traditionally used to treat inflammation. This research aimed to evaluate topical anti-inflammatory activity of *n*-hexane extract of *S.album* leaves on croton-oil induced rat ear oedema. The anti-inflammatory activity test was done using 25 Wistar rats which divided randomly into five groups. Group 1 (negative control, croton oil 35% in acetone only), group 2 (positive control, hydrocortisone drug 2.5% and croton oil), and groups 3, 4, and 5 treated with 10, 20, and 40 mg/ear of *n*-hexane extracts respectively along with the croton oil. The rat ear-oedema thickness was measured manually using micrometre after 6 hours of observation. The topical application of *n*-hexane extract of *S.album* leaf at doses 10 and 20 mg/ear, and hydrocortisone significantly reduced croton-oil induced rat ear oedema with percentage inhibition of 36, 52, and 73 % respectively. However, at a higher dose of 40 mg/ear, it did not significantly inhibit inflammation as compare to negative control. Histology analysis result also revealed that *n*-hexane extract at doses 10 and 20 mg/ear, and hydrocortisone decreased significantly infiltration of leukocytes. Based on this result, it can be concluded that *n*-hexane extract of *Santalum album* Linn leaf showed topical anti-inflammatory activity in a dose dependent manner.

1. Introduction

Human skin is an outer layer that cover the entirely of the body surface giving protection of an internal organs. Therefore, it is highly risk to get injury and invasion of pathogen which may lead to inflammation [1]. The reponse of inflammation aims to alleviate pathogen and repair tissue damaged. The inflammation process is due to the release of mediator from tissues and migrating cell and associated with prostaglandin, leucotriene, bradykinin, histamin, platelet-activating factor, and interleukin-1 (IL-1) [1]. A variety of stimuli such as cytokine, UV radiation and irritant 12-O-tetradecanoyl-13-phorbol acetate (TPA) when exposed to the skin can cause various inflammatory skin diseases [2]. Currently, commercially available antiinflammatory drugs for skin diseases are mostly ineffective and produce side effect [3]. Therefore the search for an alternative drugs which are safer, inexpensive, and more tolerable for the treatment of skin diseases is necessary [3].

Many plants have been traditionally used in ancient times to treat inflammation. *Santalum album* Linn (*S.album*), locally known as cendana, is the indigenous tree of East Nusa Tenggara province of Indonesia. The wood has been used for furniture, wood carving, statue of god, incense stick, funeral pyres, and religious practices [4]. The extracted oil from cendana is widely used in perfume industry, cosmetic, food flavouring, and aromatherapy [5]. In Indonesian traditional medicines, cendana has
been used to treat urinary tract infection, gastric irritability, gonorrhea, chest pain, skin diseases, nausea, dysentery, and inflammation [4,6]. Pharmacological activities of the essential oil, wood, and root of *S. album* have been studied extensively worldwide [7]. They have been reported to have anti-ulcer, antibacterial, antifungal, antiviral, antioxidant, antipyretic, anti-inflammatory, anticancer, anti-hyperglycemic, and anti-hyperlipidemic activities, metabolic effect, genitourinary system effect, central nervous system effect, genotoxicity effect, cardioprotective, insecticidal, and aromatherapy [7].

Pharmacological activities and phytochemical constituents of leaves part of *S. album* have also been studied extensively [8,9,10]. However, there is only few research has been found especially in Indonesia investigating pharmacology and biological active constituents of the leaves part of *S. album*. Our previous study demonstrated that *n*-hexane extract of *S. album* leaf exerted a potent oral anti-inflammatory activity in a dose dependent manner on hind paw oedema induced by carrageenan [11]. This research aimed to evaluate topical anti-inflammatory activity of *n*-hexane extract of *S. album* leaf using skin inflammation model induced by croton oil.

### 2. Material and Methods

#### 2.1. Plant material

The leaves of *S. album* used in this study were collected from Camplong Conservation Forest, Kupang-East Nusa Tenggara-Indonesia.

#### 2.2. Chemical

Analytical grade of methanol, *n*-hexane, acetone, paraffin wax, and formalin were purchased from local supplier Brataco. While croton oil, xylene, phosphate buffered saline, haematoxyline-eosin were purchased from Sigma Aldrich, and hydrocortisone cream was purchased from local pharmacy.

#### 2.3. Animal

25 Male Wistar rats (180-200 g) housed at 25 ± 2°C and with received standard food and water ad libitum were used in these experiment that were performed during the light phase of the cycle. The animals were acclimated to experimentation condition for 7 days prior testing.

#### 2.4. Extract preparation

The fresh leaves of *S. album* were washed, air dried and then blended to yield powders. The powder leaves (750 g) were extracted with methanol at room temperature (3x 24 hours). The extract was then filtered and concentrated in vacuo to yield crude methanol extract. The crude methanol extract was fractionated into *n*-hexane extract.

#### 2.5. Ear Oedema measurement

Oedema was expressed as the increase of ear thickness due to application of croton oil. Ear thickness was measured manually using a micrometer secrupe before and after treatment of croton oil.

#### 2.6. Croton Oil Induced Ear Oedema

The method used to promote oedema was based on method described by Lodonkar, with modification [12]. A solution of 20 μL croton oil 35% in acetone was topically applied to groups of wistar rats on the right ear and left ear was remained untreated. The *n*-hexane fraction (10, 20, and 40 mg/ear in acetone) and 10 mg of cream hydrocortisone 2.5% /ear in acetone were applied topically 30 minutes after the application of croton oil. The thickness of the ear was measured 6 hr after induction of inflammation. Percentage of inhibition inflammation was calculated according to the equation described by Abedi [13]:

\[
\% \text{ inhibition} = \left[1 - \frac{\Delta P_t}{\Delta P_c}\right] \times 100\% \quad \text{(1)}
\]
where: $\Delta P_t = \text{the difference in ear thickness of sample}$
$\Delta P_c = \text{the difference in ear thickness of control (croton oil)}$

2.7. Histology Analysis
Histological analysis was carried based on method described by Mescher with modification [14]. The animals were anesthetized at the end of the experiment by injecting with ketamine. The ears plug are biopsied 5 mm in diameter and fixed in PBS- formalin 10% for 24 hr. Subsequently, each sample was dehydrated with increasing concentration of alcohol (50, 70, 90, 96, and 100%) respectively for 2 hr, each treatment. After dehydrating, each sample was subjected to clearance process by immersing in xylene for 24 hr until transparent. Finally, each sample was embedded in paraffin wax for 1 hr, sectioned at 5 µm using microtome and stained with haematoxylin-eosin. A representative area was selected for qualitative light microscopic analysis for inflammatory cellular response with a 40x objective. In this experiment, only 3 of each group samples were analysed and data were analysed using student T-Test and presented descriptively.

2.8. Statistical Analysis
The results are presented as mean ± SEM. The statistical significance between the groups was assessed by one-way analysis of variance (ANOVA) followed by a post hoc Tukey multiple comparison except for data histology analysis. The accepted level of significance

3. Results and Discussion
3.1. The effect of n-hexane Extract of S.album leaves on Croton Oil Induced Ear Oedema
The S.album (cendana) plants have been traditionally used for treatment of many diseases including inflammation [4,6]. Phytochemical study revealed that methanol extract of S.album leaf contained a numerous class of secondary metabolites including alkaloid, flavonoid, phenolic, terpenoid, tanin and cardiac glycosides [15] which are potential to exert anti-inflammatory activity [16]. Previous study has proved the antiinflammatory activity of n-hexane extract of S.album leaves administered orally on rat hind paw oedema induced by carragenan [11]. This study was done to evidence the topical anti-inflammatory activity of the n-hexane extract of S.album leaves on rat ear oedema provoked by croton oil.

Croton oil is a constituent of Croton tiglium L plant containing phorbol ester with TPA predominantly as active compounds. Induction of acute inflammation by croton oil occurs by activating phospholipase $A_2$ (PLA$_2$) which releases arachidonic acid (AA) from the cell membrane [3] which in turn metabolism of arachidonic acid to prostaglandin by cyclooxygenase (COX-2) and to leukotriene by 5-lypoxygenase (LOX) [17]. When croton oil applied topically on rat ear, it enters epidermis and dermis causing cell damage. Subsequently, histamine mediators are released due to cell damage which result in vasodilation (dilation of arteri, venule) and expansion of capillaries in the dermis layer. This promotes an increase in blood flow. The contraction of endothelial cell occurs rapidly after binding to histamine, bradykinin, prostaglandin and leukotriene within 15 to 30 minutes. This leads to protein leakage and promotes oedema. The oedema can develop within 4 to 6 hours which triggered by inflammatory mediator such as cytokine, TNF-$\alpha$ and IL-1. Furthermore, leukocytes emigrates from central blood flow to the endothelial surface [18]. Hence, inflammatory parameters such as erythema (redness) and thickened of ears or oedema and infiltration of leukocytes can be observed.

Treatment of croton oil topically to rats ear promoted an oedema as signed by the increase in right ear thickness compared to left. The effect of topical application of n-hexane extract on croton oil induced ear oedema was summarised in Table 1. As can be seen from Table 1, the n-hexane extract, at doses 10, 20 mg/ear significantly reduced croton oil induced-rats ear oedema by inflammation inhibition of 36 and 52 % respectively. The effect of n-hexane extract at dose 20 mg/ear in inhibiting inflammation was comparable to positive control drug hydrocortisone (72%). However, at a higher dose of 40 mg/ear, it
did not elicit significant effect on reduction of ear thickness and only inhibit inflammation of 18% which statistically was not differed from negative control (croton oil).

Table 1. The effect of \( n \)-hexane extract on thickness of croton oil induced ear oedema

| Treatment                        | 6 hours After application of croton oil | Inhibition of Inflammation (%) |
|----------------------------------|----------------------------------------|--------------------------------|
|                                  | Ear thickness (mm) (n=5)                |                                 |
| Croton oil 35%                   | 0.2560±0.02074\(^a\)                  | -                              |
| Hydrocortisone cream 2.5%, 10 mg/ear | 0.0700±0.04950\(^b\)                  | 72                             |
| \( n \)-hexane, 10 mg/ear        | 0.1640±0.04775\(^c\)                  | 36                             |
| \( n \)-hexane, 20 mg/ear        | 0.1240±0.03162\(^b\)                  | 52                             |
| \( n \)-hexane, 40 mg/ear        | 0.2100±0.00837\(^a\)                  | 18                             |

Means within the same column followed by different letter are significantly different (P<0.05) based on the ANOVA one-way analysis followed by Tukey’s multiple comparison.

3.2. Histological Analysis

Histological analysis was performed to confirm the anti-inflammatory effect of \( n \)-hexane extract of \( S.\) \( album \) on cell infiltration. Leukocyte emigration from central blood flow to the endothelial surface was observed in histological analysis. The results of the histological analysis of the croton oil (negative control), \( n \)-hexane extract and positive control hydrocortisone was depicted in Figure 1.

The histological result (Fig.1) found that infiltration of lymphocytes apparently instead of neutrophil. In acute inflammation, infiltration of neutrophil was occured in early stage and lymphocytes was in a late stage. Hence, the appearance of lymphocytes suggested that the acute inflammation has proceed in a latest stage of inflammatory process or it could lead into a chronic inflamation [18]. Application of \( n \)-hexane extracts topically at doses 10, 20 mg/ear and hydrocortisone produced significant reduction in infiltration of lymphocytes as compared to negative control (croton oil) but at dose of 40 mg/ear, the infiltration of lymphocytes did not decrease significantly in comparison with negative control. This result confirmed the topical antiinflammatory activity of \( n \)-hexane extract of \( S.\) \( album \) leaves on croton oil induced ear oedema.

The topical anti-inflammatory activity elicited by the \( n \)-hexane extract of \( S.\) \( album \) leaves may due to the presence of methyl palmitic, methyl linoleic, oleic acid, and asarone which identified in one fraction of the \( n \)-hexane extract in our previous study. They were known to posses antiinflammatory activity by interfering the metabolism of AA to prostaglandin [11]. Additional study demonstrated the identification of lupeol, nerolidol in the \( n \)-hexane fraction which are active as antibacterial activity towards \( Staphylococcus aureus \) [19]. Lupeol and nerolidol have been reported to have anti-inflammatory activity [20,21]. Therefore, the presence of this compounds in the \( n \)-hexane extract of \( S.\) \( album \) leaves produced an interesting topical anti-inflammatory activity which are able to interfere with oedema and leukocytes infiltration.
Figure 1. Histological analysis of $n$-hexane extract effect on oedema and leukocytes infiltration in ear oedema induced by croton oil. Picture illustrative of rats ear transverse section stained with hematoxylin-eosin (magnify of 400 x) collected 6 hr. A. Healthy cell (0 lymphocytes). B. Negative control (croton oil, 29 lymphocytes). C. $n$-Hexane extract 10 mg/ear (19 lymphocytes). D. $n$-hexane extract 20 mg/ear (15 lymphocytes). E. $n$-hexane extract 40 mg/ear (25 lymphocytes). F. Hydrocortisones cream 2.5% 10 mg/ear. Green circle indicated the presence of lymphocytes.

4. Conclusion
The $n$-hexane extract of *santalum album* Linn leaves exhibited a potent topical antiinflammatory activity on croton oil induced rat ear oedema in a dose dependent manner.
Acknowledgments
Authors wishing to acknowledge financial support from The Faculty of Mathematics and Natural Sciences, Udayana University through research scheme of Penelitian Unggulan Program Studi year 2018.

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