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Decreased Interleukin-4 Level of Type I Hypersensitive Mice Using Scopoloetin Isolated from Noni Fruit (*Morinda citrifolia* L.).

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ABSTRACT

An in vivo study of the activity of scopoletin isolated from noni fruit (*Morinda citrifolia* L.) on the level of interleukin-4 (IL-4) in type I hypersensitive male Swiss-Webster mice has been carried out. Scopoletin was isolated from dried noni powder by soxhletation method using dichloromethane, separated by column chromatography using silica gel as stationary phase and n-hexane-ethyl acetate (1:4) as mobile phase, then purified by column chromatography using Sephadex LH20 as stationary phase and methanol as mobile phase. Type I hypersensitive male mice were obtained by ovalbumin sensitization. Animal model were divided into 5 groups: negative control group, positive control group, and scopoletin-treated group (1; 3; and 10 mg/kg). The results showed that scopoletin at doses of 1, 3 and 10 mg/kg decreased the level of IL-4 of type I hypersensitive mice significantly ($p < 0.01$). The scopoletin at the dose of 10 mg/kg decreased the serum level of IL-4 to the normal level.

Keywords: noni fruit, morinda citrifolia, scopoletin, type I hypersensitivity, interleukin-4

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INTRODUCTION

Type I hypersensitivity reaction is an abnormal immunologic reaction to an antigen in those who had previously been sensitized to the antigen, and occurs when IgE antibody is produced in response to antigens or allergens [1]. Bronchial asthma, allergic rhinitis, and atopic dermatitis are diseases related to type I hypersensitivity reactions. Type I hypersensitivity reactions can be provoked by allergens such as pollen, food, cold, dry air, dust, smoke, animals, drugs, fungi, viruses, chemicals or industrial output and emotional stress [2,3].

The entry of allergens into the body causes an immune response with subsequent formation of IgE that binds to the surface of mast cells and basophils cells [4,5]. The exposure process begins with phagocytosis of allergen by macrophages. Macrophages destroy allergens into several peptide fragments and bind into MHC class II molecules and carried to the cell surface of macrophages and presented to the cells Tho (naive helper). Macrophages release several cytokines such as IL-1 (interleukin) and TNF-α (α- factor necrosis cancer). The Th cells that receive signals from macrophages undergo cell differentiation and proliferation into Th1 and Th2 cells. Allergens are also phagocyted by mastocyte and basophil cells, then these cells will release IL-4. Increased level of IL-4 causes the proliferation and differentiation of Th0 cells are limited to become Th2 cells only. The Th2 cells release several cytokines such as IL-4, IL-5, IL-10, and IL-13 [6]. IL-4 exhibits a direct effect on B lymphocytes that subsequently differentiate and proliferate into plasma, which in turn produces IgE [7,8]. One of the plants that has been used to treat allergies is noni fruit (*Morinda citrifolia* L.) [9]. Besides antiallergic, noni fruit has also been used as antibacterial, antiviral, anticancer, antihelmintic, analgesic, hypotensive, anti-inflammatory and immunostimulant, to reduce the growth of tumor cells, and anti-ulcer [10-12]. Nowadays, the use of herbs in medication is very common among the society. This should be supported by scientific research to prove the efficacy [13].

Previous studies showed that the extract of noni fruit could inhibit active cutaneous anaphylaxis reaction in mice induced by white albumin [14] and showed in-vitro inhibition of sensitized mastocyte degranulation induced by albumin [15]. Topical application of the extract could also suppress the inflammatory reaction [16]. The extract could also increase antibody titers in male mice induced by sheep red blood cells and could increase the number of lymphocytes, neutrophils and eusinofil stem cells [17].

One of the active compound in the ethanol extract of noni fruit is scopoletin. Dried powder noni fruit contains not less than 0.02% of scopoletin [18]. Hyung et. al. has reported that scopoletin can inhibit mastocyte degranulation in mice; inhibit the production of PGE_2 (prostaglandin E2), TNF-α, IL-1β, IL-6 and suppress COX-2 [19]. Scopoletin is also reported to decrease the level of IgE in type I hypersensitive mice [20]. The present study aimed to observe the effect of scopoletin isolated from noni fruit on the level of IL-4 in the type I hypersensitive male mice.

MATERIALS AND METHOD

Materials

Paper filter, soxhlet extraction apparatus, rotary evaporator, chromatography columns, vials, TLC plates and backings, desiccator, Pasteur pipette, syringe, measuring cups, animal scales, spatel, needle oral, analytical scales, containers (bottle), mortar and stamfer, surgical scissors, 365 nm UV lamp, centrifuge, centrifuge tubes, UV-Vis spectrophotometer 1601 (Pharmaspec 1700), IR spectrophotometer (Perkin Elmer 735) and BIO-RAD spectrophotometer.

The material used were: the meat of noni fruit that has been dried and ground, dichloromethane (DCM), hexane, ethyl acetate, methanol, distilled water, physiological saline, Na CMC, scopoletin comparator (Exrtasynthese, Lot 10,041,510), distilled water, HCl (hydrochloric acid) 12N, ovalbumin (No. Brands. Lot.20HO763 A-5253) and *mouse IL-4 kit Platinum ELISA* (eBioscience, No. BMS613. 887 944).

Isolation of scopoletin from noni fruit

The dried powder of noni fruit underwent soxhlet extraction by using dichloromethane as the solvent. The extract was evaporated in vacuo to obtain thick extract. The eluate was collected with vials and the

isolated scopoletin was monitored by thin-layer chromatography (TLC) with a light stain apparition ultra violet (UV) at a wavelength of 365 nm. The scopoletin was detected in the vial 6 through 12. The obtained spot was still tailing which indicated other compounds left. The scopoletin in those seven vials were combined and dried. To purify the obtained scopoletin, a column chromatography was employed using Sephadex LH20 as stationary phase and methanol as mobile phase. Scopoletin pure compound was analyzed by UV and IR spectroscopy.

Animals

Healthy male Swiss-Webster mice weighing 20-25 g were injected with ovalbumin 5 mg/20g intraperitoneally. Ovalbumin was injected again at the third day in the same dose subcutaneously. Animals were declared as sensitive if redness arisen at the site of injections in the seventh day.

The mice were divided into group I: normal animals; group II: type I hypersensitive mice given only physiological NaCl solution; group III, IV and V: type I hypersensitive mice given 1, 3, and 10 mg/kg of scolopeletin, respectively. The scopoletin was given immediately when redness arisen at the site of injection.

Determination of interleukin-4

After 24 hours of scopoletin administration, blood was taken by guillotine method. The blood was centrifuged 30 minutes later to obtain the serum. IL-4 level was determined by ELISA method.

RESULTS AND DISCUSSION

The scopoletin isolated from noni fruit was obtained 0.01%. According to Indonesian Herbal Pharmacopoeia I, 2008, noni fruit symplicia (dried powder) contains scopoletin compound not less than 0.02%. This shows that the present scopoletin extraction was not complete yet, hence the scopoletin compound obtained relatively fewer.

TLC examination of the scopoletin isolated from noni fruit and scopoletin comparator (Exrtasynthese, France) with n-hexane: ethyl acetate (1.5 : 3.5) as the eluent showed the same Rf value of 0.56 (Figure. 1).

Figure 1: Thin-layer chromatograms of scopoletin under the UV light 365 nm (1. Scopoletin isolated from noni fruit; 2. Scopoletin comparator)

The examination of the ultraviolet (UV) spectrum of the isolated scopoletin showed the same spectrum as compared to scopoletin comparator. Both spectra huddled together as shown in Figure 2. The comparison of the results of the wavelength and absorbance values of the two compounds are shown in Table 1.

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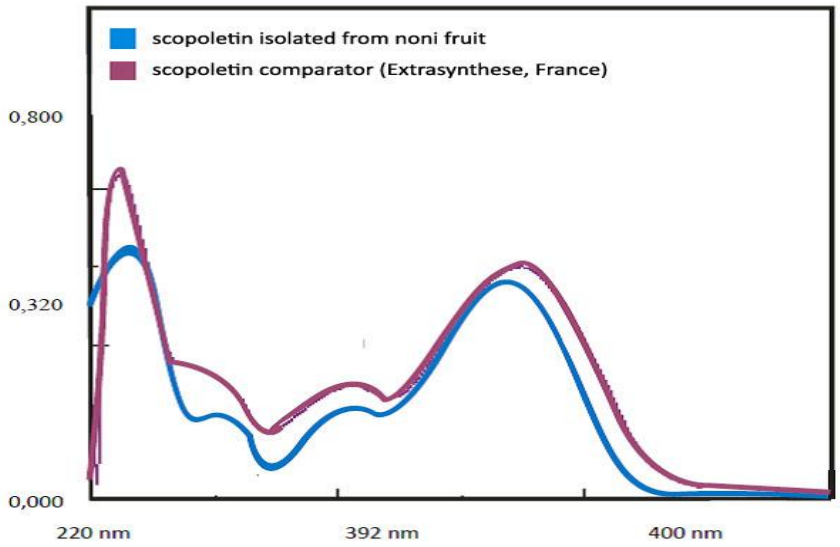

Figure 2: UV Spectrum of scopoletin isolated from noni fruit and scopoletin comparator

Table 1: Wavelength (λ) and the absorbance peak of scopoletin

No	Peak wavelength (nm)		Absorbance	
	Scopoletin isolated from noni fruit	Scopoletin Comparator	Scopoletin isolated from noni fruit	Scopoletin Comparator
1	345.50	345.2	0.411	0.481
2	297.0	296.0	0.151	0.237
3	253.0	254.0	0.119	0.271
4	228.4	228.6	0.423	0.671

The experiment started by sensitizing mice with ovalbumin 5 mg/20 g intraperitoneally. This route of administration was used to introduce the antigen to the immune system more quickly as the peritoneal cavity has many macrophage cells (MHC class II). Thus, the animals were expected to become sensitive to the antigen more rapidly. The next antigen exposure was given on the third day in the same dose subcutaneously where the type I hypersensitivity reaction did not appear. The type I hypersensitivity reactions appeared after the administration of ovalbumin subcutaneously on the seventh day characterized by redness around the site of injection. The redness is caused by the interaction between the ovalbumin antigen with IgE on the surface of mastocyte and basophil cells. When the second exposure of the same antigen happens, one molecule of the antigen binds two adjacent IgE molecules on the Fab fragment. This leads to enzymatic activity in the cell membrane and subsequently the release of chemical mediators stored in granules in the mast cells and basophil cells such as histamine, bradykinin, prostaglandins, leukotrienes and others. These mediators are responsible for the onset of type I hypersensitivity reaction. The histamines collectively increase capillary permeability and lead to vasodilatation of blood vessels in the skin of mice and caused the redness arise.

The level of IL-4 in type I hypersensitive mice following administration of antigen subcutaneously increased 388.17% after 24 hours as compared to normal group. This suggests that antigen exposure after the animals experienced type I hypersensitivity reaction increases the production of IL-4 cytokine which is responsible for type I hypersensitivity reactions.

Scopoletin was given at the doses of 1, 3, and 10 mg/kg orally to type I hypersensitive mice. After 1 day (24 hours) of administration, animals were sacrificed by guillotine, serum was separated and then the concentration of IL-4 was determined by ELISA method.

In the determination of IL-4 level, the standard curve was made by using the IL-4 standard at a wavelength of 450 nm. Regression equation of IL-4 standard was Y = 0.011 X with R^2 = 0.952.

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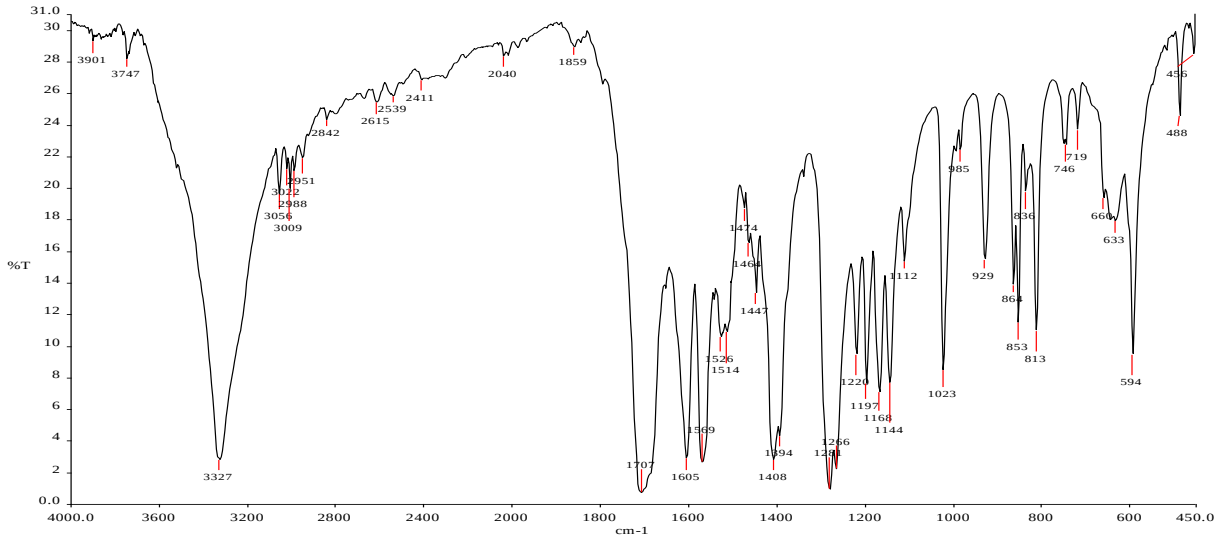

Figure 3: IR spectrum of scopoletin isolated from noni fruit

Table 2: Wavenumber (cm^{-1}) and functional groups of scopoletin

No	Wavenumber (cm^{-1})		Functional Group
	Scopoletin isolated from noni fruit	Scopoletin comparator	
1	3327 (3700-3100)	3339 (3700-3100)	Hidroxy
2	1707 (1900-1650)	1703 (1900-1650)	Ketones (carbonyl)
3	1605, 1569, 1514 (1600-1450)	1608, 1567, 1510 (1600-1450)	C=C
3	1605, 1569, 1514 (1600-1450)	1608, 1567, 1510 (1600-1450)	C=C
4	1447, 1408, 1394, (1465-1350)	1447, 1435 (1465-1350)	C-H
5	1220, 1197, 1168, 1144, 1112, 1023, 985 (1250-1000)	1220, 1190, 1161, 1141, 1100, 1019 (1250-1000)	C-O
6	864, 853, 813, 746, 719 (900-700)	862, 841, 821, 746, 714	Substituted sugar

The serum level of IL-4 in type I hypersensitive mice is presented in Table 3 and the comparison of serum levels of IL-4 among the groups of type I hypersensitive mice with various doses of scopoletin is presented in Figure 5. Scopoletin at the doses of 1, 3 and 10 mg/kg could decrease the serum level of IL-4 in type I hypersensitive mice significantly ($p < 0.01$). Decreased levels of IL-4 in type I hypersensitive mice causes a direct impact to Tho cells and plasma cells and decreases the production of IgE. Bonferroni test (Figure 4) revealed that the reduction of IL-4 levels by scopoletin at the doses of 1 and 3 mg/kg showed insignificant difference ($p > 0.05$), while the doses of 3 and 10 mg/kg showed significant difference ($p < 0.05$). Scopoletin at the dose of 10 mg/kg decreased the IL-4 level of type I hypersensitive mice to the normal level ($p > 0.05$).

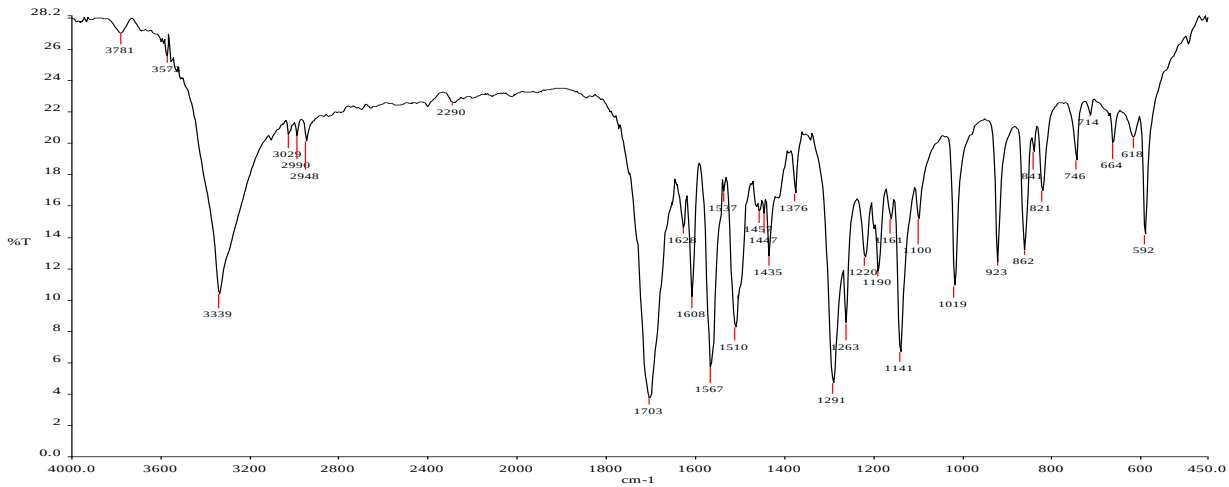

Figure 4: IR spectrum of scopoletin comparator

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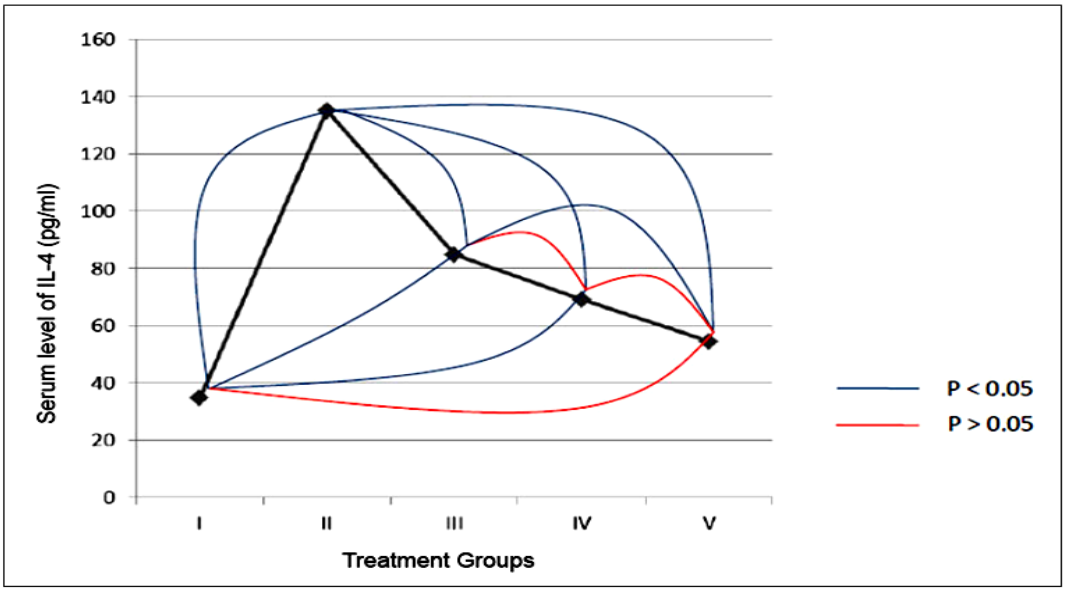

Figure 5: Relationship between doses of scopoletin isolated from noni fruit and serum levels of IL-4 in type I hypersensitive mice.

Table 3: Serum levels of IL-4 in the type I hypersensitive mice after administration of scopoletin isolated from noni fruit.

Treatment Group	Serum levels of IL-4 (pg/ml)					Averages (pg/ml)
	1	2	3	4	5	
I. Normal animals	47.27	34.73	28.36	35.27	28.55	34.84 ± 7.69
II. Control	139.64	156.18	123.45	146.91	110.00	135.24 ± 18.51
III. Scopoletin 1 mg/kg	84.91	86.91	86.18	76.73	89.64	84.87 ± 4.87
IV. Scopoletin 3 mg/kg	82.73	64.18	83.64	64.73	50.73	69.20 ± 13.95
V. Scopoletin 10 mg/kg	46.18	52.73	62.36	48.36	63.45	54.62 ± 7.93

The major function of IL-4 is in the regulation of the immune response mediated by IgE and mastocyte or eusinophil cells. The activity of IL-4 is not limited on B cells only, but also on T cells, macrophages, granulocytes, mastocytes, erythrocyte precursors and megakaryocytes. IL-4 acts as a stimulator of Th2 cells and inhibits the activity of macrophages, but this effect can be countered by the INFγ [6, 21]. Increased level of IL-4 in animals experiencing type I hypersensitivity reactions will affect the activity of CD4 T cells to undergo cell proliferation and differentiation toward Th2 cells and subsequently increase the production of IL-4. Furthermore, IL-4 has receptors on the plasma cells that will be activated to produce IgE. Thus, decreased level of IL-4 will also lead to decreased production of IgE [6, 22].

CONCLUSIONS

From the present study, we conclude that the administration of scopoletin at a doses of 1, 3, and 10 mg/kg can decrease serum levels of IL-4 type I hypersensitive mice ($p < 0.01$). Scopoletin at the dose of 10 mg/kg can decrease the serum level of IL-4 in type I hypersensitive mice to the normal level ($p > 0.05$).

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