Original Research Article

Effects of Lemon balm essential oil application on atopic dermatitis-like immune alterations in mice

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A b s t r a c t

Lemon balm oil has been used as anti-inflammation agent, but there has been no scientific study to date on the possible effects of Lemon balm oil on alleviating atopic dermatitis.

BALB/c mice were sensitized with 2, 4-dinitrochlorobenzene for atopic dermatitis induction. Thereafter, Lemon balm oil was applied on the dorsal skin for 4 weeks. Scratching behavior was measured. Blood and spleen were collected for measuring IgE and cytokine, respectively.

Serum IgE levels were significantly lowered in 3% Lemon balm oil application group after 3-week application. The Lemon balm oil application showed a significantly lower spleenocyte interleukin-10 level and an increasing tendency in Interferon- level compared with that of saline application. Scratching frequency of the Lemon balm oil application group had a lower tendency than the control groups.

This study may demonstrate Lemon balm oil's immunoregulatory potential for alleviating atopic dermatitis through influencing of Th2 / Th1 cell activation.

Keywords: atopic dermatitis, cytokine, Lemon balm oil, IgE, mice.

Introduction

Atopic dermatitis is an immunologic disease induced from an imbalance favoring type-2 helper T (Th2) cell. Activated Th2 cells produce cytokines such as interleukin (IL)-4 and IL-10 which are important in switching antibody production from B cells to predominantly IgE production against the allergen. Hyper IgE production is a hallmark of atopic dermatitis in humans, and well documented in transgenic mice or NC/Nga mice suffering from atopic dermatitis-like skin lesions [1, 2]. Interferon (INF) - is secreted from type-1 helper T (Th1) cells, natural killer cells or other immune cells. Therefore, analysis of the spleenocyte IL-10 and IFN- levels could reflect the alteration of homeostasis between type-1 and type-2 immune responses. Atopic dermatitis is a chronic skin disorder and has been medically treated using steroids, antihistamines, immunosuppressive agents, and other medications. But many studies have reported that long-term use or abuse of these agents may cause various side effects, therefore relevant recent studies have focused upon complementary therapies based on alternative medicine [3, 4]. Aroma oils are natural essential oils consisting of volatile organic compounds containing original scents and therapeutic substances. It is well known for positive effects in insomnia, relief from anxiety and stress, recovery from fatigue, muscular relaxation, anti-allergic actions [5, 6].

Lemon balm (LB) is also named as *Melissa officials*. Infusions prepared with the aerial part of LB are used in folk medicine for the treatment of fevers and colds, asthma, indigestion, depression, mild insomnia, epilepsy, and skin diseases [7, 8, 9, 10]. The scientific reported uses include: antioxidant [7], sedative [11, 12], anti-inflammatory, hepatoprotective, digestive [13, 14], anti-bacterial, antifungal, antiviral, anti-histaminic [7, 15, 16, 17], immunostimulating activity [18], antiproliferative [19], antinociception [20].

LB has traditionally been used also include skin disorder such as eczema and skin inflammation [10, 21]. And recent research has begun to confirm these traditional uses. Ippoushi et al [22] demonstrated that methanol extract of lemon balm leaves shows potent in vitro inhibitory effect on hyaluronidase, an enzyme which cleaves the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue, is well known to be involved in allergic reaction [23, 24]. However, there has been no scientific in vivo study to date on the possible effects of LB oil on alleviating atopic dermatitis. Therefore, this study tested the application of LB oil on BALB/c mice demonstrating atopic dermatitis-like immune alteration and skin lesions.

## Materials and Methods

Experimental design

Experimental schedule is as shown in Figure 1A. Briefly, at first mouse paws were implanted with magnets. After atopic dermatitis-like disorders were induced on the dorsal skin, LB oil or saline was painted. In a 4-week period of the oil treatment, the blood samples

were collected and the scratch behavior were measured for 2 times. In the end mouse blood and spleen were collected for IgE and cytokine analysis, respectively.

A.
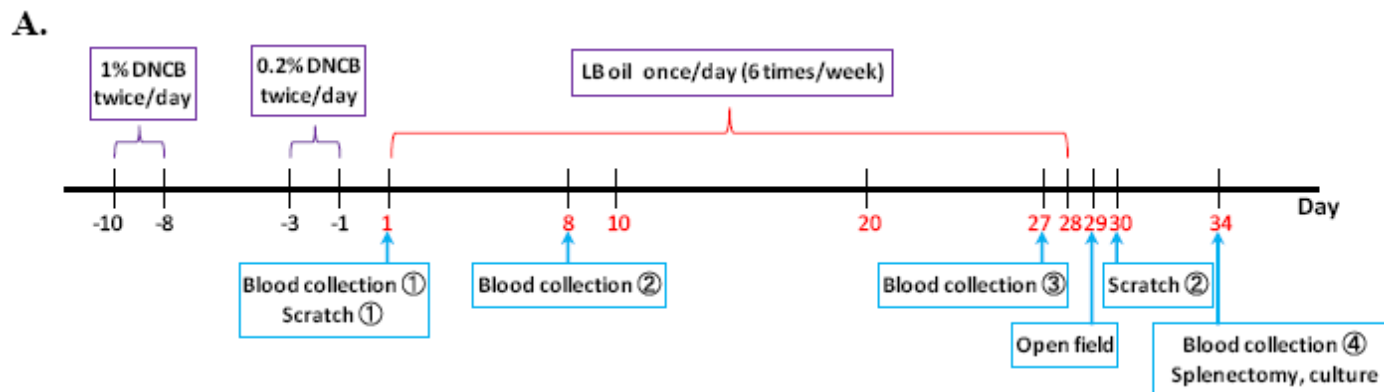
B.
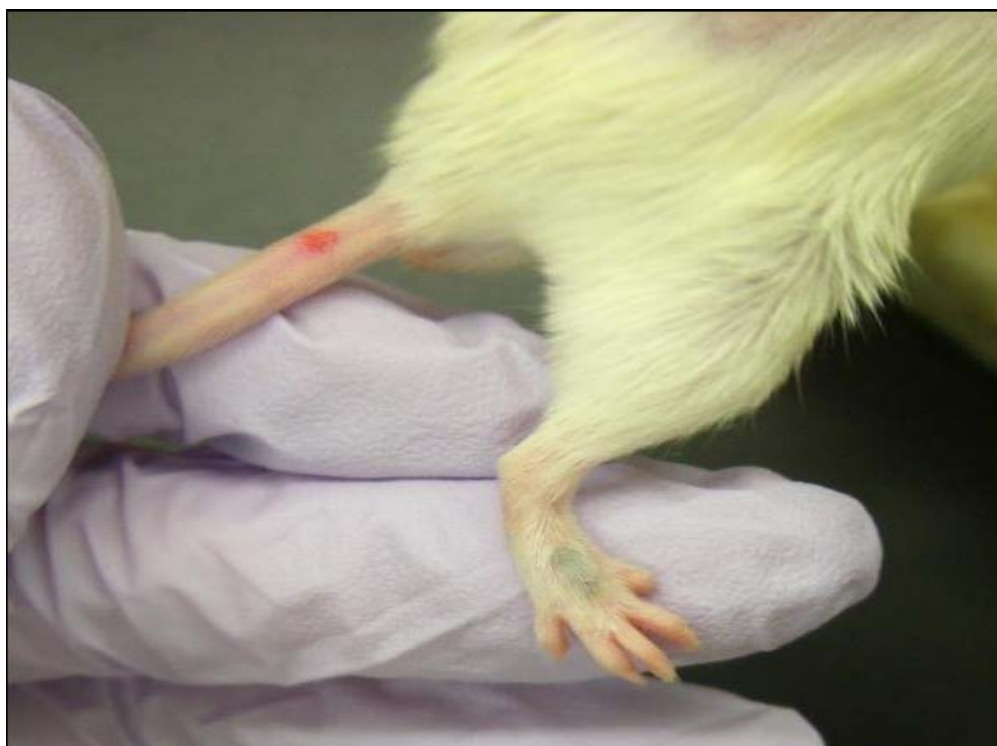
Figure 1. Experiment design (A). Macroscopic photograph of magnet into the hind paw of mouse (B).

Composition of LB oil

For the experiment, this study used LB oil (Primavera, German) as 100% pure and natural essential oil tested by the Pharmacognosy Laboratory of Sojo University. The compositions of Primavera LB oil were previously reported by Schnitzler et al [15].

Animals

Male BALB/c mice (SPF grade), 7 weeks old, were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). Mice were housed under the following controlled conditions: temperature (24 ± 2 °C), humidity (50 ± 10%) and a 12-hour light/dark cycle (7:00 a.m. to 7:00 p.m.). Food and water were available *ad libitum*. There was a one-week adaptation period, after which the mice were divided into four groups. The normal group was applied with saline throughout atopic dermatitis induction stage and oil treatment period. The control group was applied with saline following induction of atopic

dermatitis. The experimental groups were applied with 1% and 3% LB oil respectively, following atopic dermatitis induction. Three % aroma oil is usually selected in the clinical application. All experiments were conducted in strict accordance with the Guidelines of the Japanese

Pharmacological Society for the Care and Use of Laboratory Animals. The study was submitted to, and approved by the Ethics Committee of Sojo University.

Induction of atopic dermatitis-like immunologic and skin disorders

For induction of atopic dermatitis-like immunologic and skin disorders, DNCB was applied onto mice skin as described by Lee et al. [25]. Briefly, after complete removal of dorsal hairs in area of approximately 8 cm2, 100 μl of 1% DNCB was applied on their dorsal skin twice every 3 days for sensitization. In the next week, 100 μl of 0.2% DNCB was applied on their dorsal skin twice every 3 days for challenge. DNCB was dissolved in a 4:1 mixture of acetone and olive oil. As soon as the challenge was completed, 1% and 3% LB oil were applied once a day (70 μl, 6 times per week) on the dorsal skin for 4 weeks. Saline was applied onto skin of the control mice.

Measurement of Serum IgE levels

100 μl of blood was sampled at the completion of second challenge and 1- and 2-week after initiating oil application via periorbital sinus. After the 4-week oil application period, under ether anesthesia these mice were sacrificed, their bloods were collected from heart. The blood was allowed to stand for 2 hours at room temperature, then centrifuged at $12,000 \times$ g for 15 minutes at 4℃ to separate the serum. Serum IgE levels were determined using a sandwich ELISA method, Mouse IgE Quantitation Kit (Bethly, Montgomery TX, USA) were used as outlined by the manufacturer. Optical densities were determined using a micro-plate reader (Immunomini NJ-2300, Biotech, Tochigi, Japan) at 450 nm.

Measurement of cytokine in splenic cell culture solution

Immediately after the blood collection, their spleens were removed. The spleens were minced, and a single-cell suspension was obtained in a minimum essential medium (MEM). Splenic cells (5 105 cells) were stimulated with immobilized anti-CD3 mAb (5 μg) for 48 hours in a CO2 incubator. The levels of IL-10/IFN- in culture supernatants were determined using a sandwich ELISA method, Mouse IL-10/IFN- ELISA Kit (eBioscience, San Diego CA, USA) were used as outlined by the manufacturer. Optical densities were determined using a micro-plate reader at 450 nm.

Measurement of toxicity

The effect of LB oil on cell viability was examined by MTT assay. Briefly, splenocytes from normal BALB/c mice were seeded into 96-well plates at a density of $3 \times 10$6 cells and then incubated at 37 ℃ for 24 hours. The cells were then treated with 10, 30, 100, 300, 1000 μg/ml LB oil for 48 hours in a CO2 incubator. The absorbance was recorded using a micro-plate reader at 570 nm.

Evaluation of scratching behavior

The scratching behavior from the hind toes of mice was detected and evaluated using Microact® (Neuroscience, Tokyo, Japan). The use of the MicroAct® device has been previously validated [26, 27]. Briefly, under ether anesthesia a small Teflon-coated magnet (1 mm in diameter, 3 mm long) was implanted subcutaneously into the dorsal side of both hind paws of the mice the day before the first recording of scratching behavior (Figure 1B). The magnet remained in situ throughout the whole experimental period. At least 30 min before the recording was started, the mice were placed in the plastic chambers to calm the animals, in order to reduce the stress level during the initial phase of the recording. The

mouse with magnets was placed in the observation chamber (11 cm in diameter, 18 cm high), surrounded by a round coil. Movement of hind paws with the implanted magnets induced an electric current in the coil, which was amplified and recorded by the MicroAct® software. The extent of the recording time was 30 minutes, and the assessment was performed on the first day and on the 34th day after LB oil application at the same time of day every time. Under the present experimental condition, the MicroAct® analysis program used the following settings to register scratch events: Threshold (V) 0.1, Event Gap (sec) 0.2, Max Freq (Hz) 20.0, Min Freq (Hz) 2.0, Min Duration (sec) 1.5. To avoid a possible effect of central nervous inhibition, an open-field test is also valued.

Statistical Analysis

Each value represents the mean ± S.E.M. for 5~6 mice. Comparisons between the two groups were carried out using a Student's t-test. Multiple comparisons were performed using Dunnet-test. Probability (p) values less than 0.05 were considered to be statistically significant.

Results

Effect of LB oil on cytotoxicity

To determine the effect of LB oil on cell viability, An MTT assay was performed to assess toxicity in splenocytes. Cells were treated with 10, 30, 100, 300, 1000 μg/ml LB oil, and the cell viability was unaffected by LB oil treatment at all concentration (Figure 2). These results suggest that LB oil is non-cytotoxicity to cell.
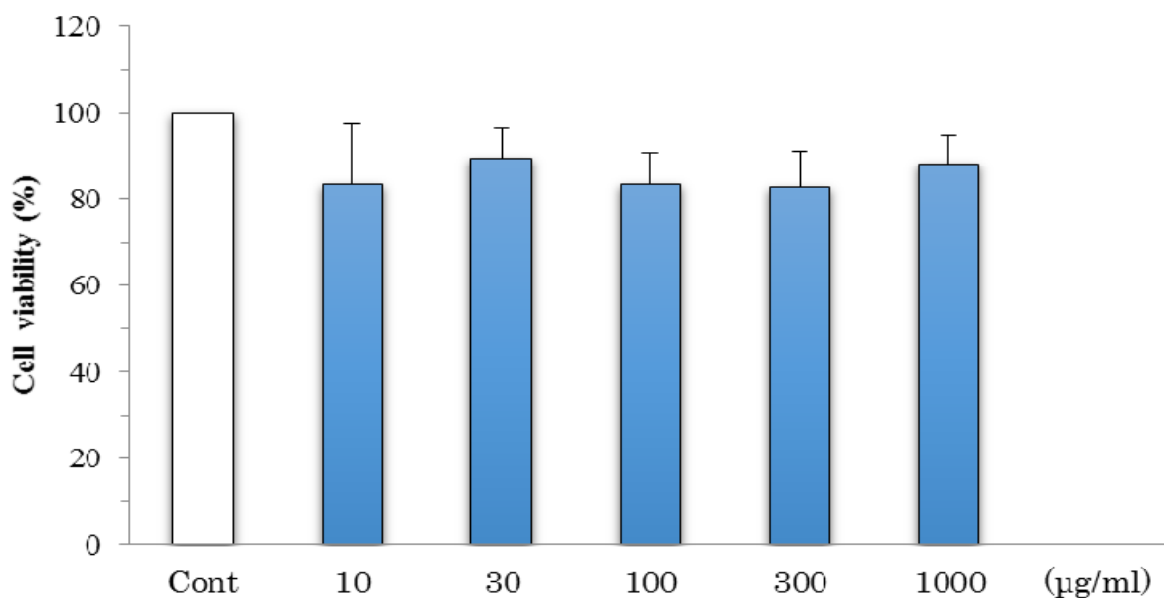
Figure 2. Cytoxicity of LB oil on BALB/c splenocytes. Cells were treated with LB oil (10, 30, 100, 300, 1000 g/ml) for 48 hours, after which the cell viability was assesses by MTT assay. Data shown represent the mean ± S.E.M. (n=3).

Observation of skin change

The DNCB skin application model was adopted to induce atopic dermatitis-like phenomena in mice. Mice at the completion of 0.2% DNCB second challenge are observed cutaneous inflammation compared with that before challenge (Figure 3B and Figure 3A). After 4-week application with 1% and 3% LB oil, the amelioration of cutaneous inflammation (wound area) were better than that of control mice as shown in Figure 3D, 3E and Figure 3C.

(B)

(A)

(C)

(D)

(E)
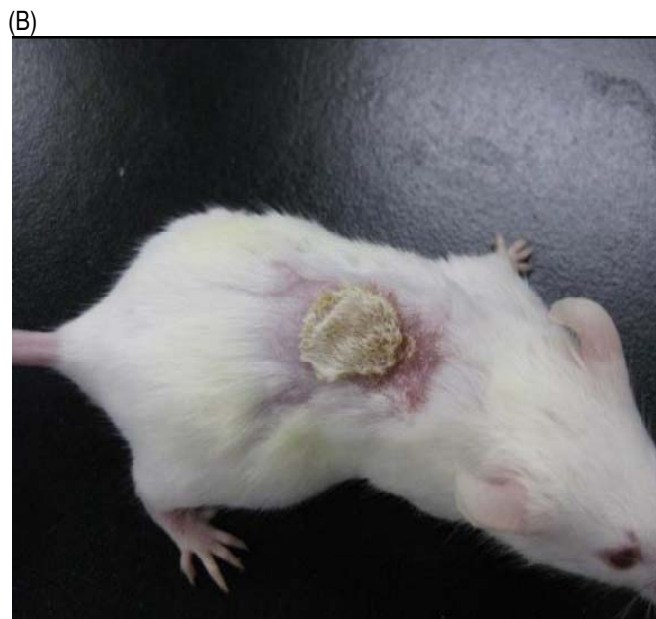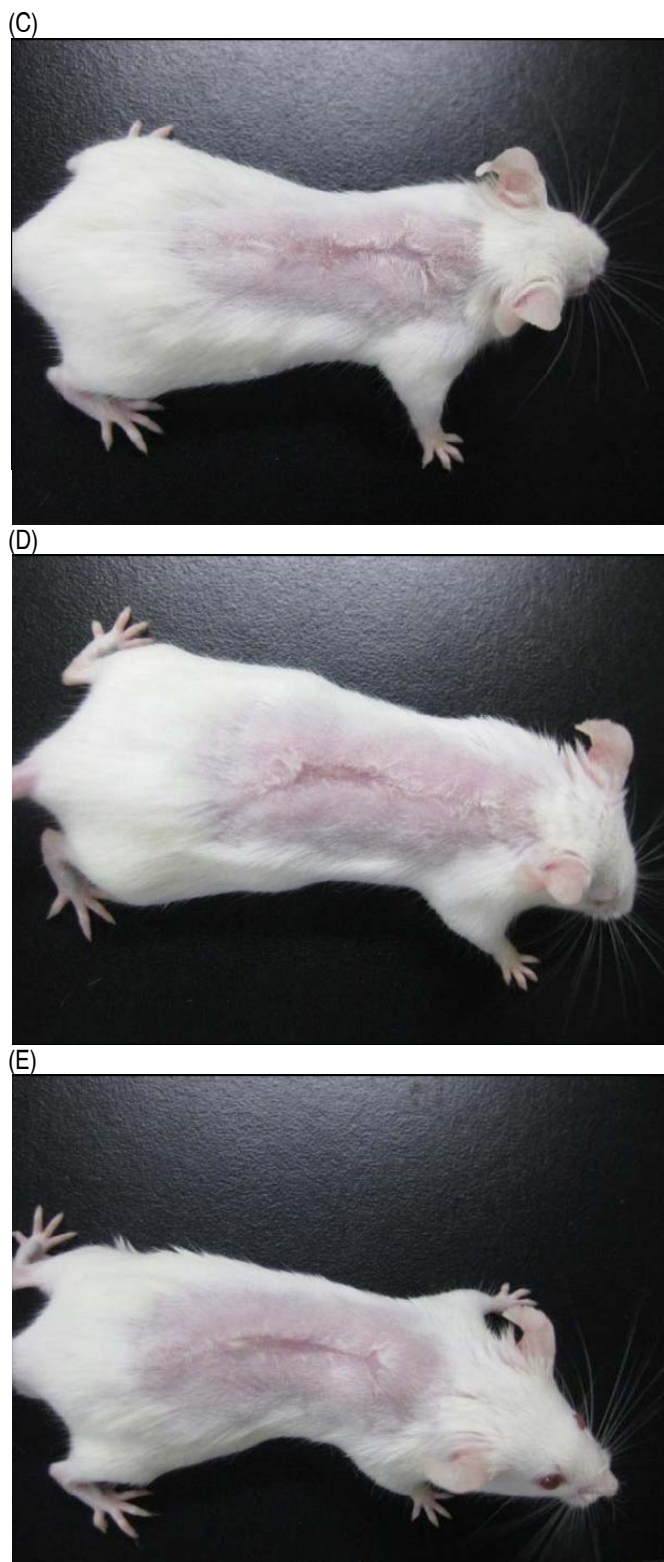
Figure 3. Macroscopic photograph by various applications. Saline application as the normal (A). Atopic dermatitis mouse skin just after induction by the application of 2,4-

dinitrochlorobenzene (DNCB) (B). Saline application after atopic dermatitis induction as the control (C), and 1% and 3% Lemon balm (LB) oil application after atopic dermatitis induction as the experiments respectively (D, E). C, D and E are macroscopic photographs at 4-week after various applications.

LB oil-mediated suppression of IgE hyperproduction

Mice at the completion of 0.2% DNCB second challenge demonstrated significantly higher levels of serum IgE (approximately 6.1 times) than the normal group (Figure 4), implying a successful induction of atopic dermatitis-like immune alteration. Serum IgE levels were significantly downregulated in the experimental 3% LB oil group after application for 27 days, while the saline-applied control group still demonstrated significantly upregulated IgE levels compared to the normal mice. And 27 days of 1% LB oil application resulted in downregulation of IgE levels with no statistical significance compared to the saline treated control mice.
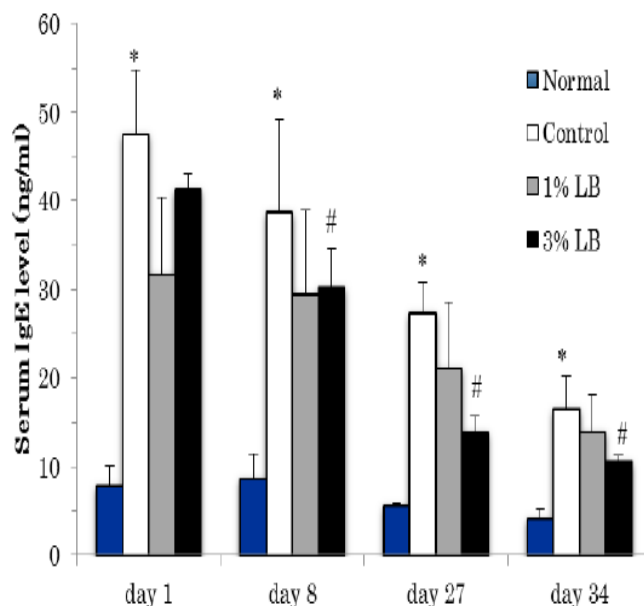

Figure 4. LB oil-mediated suppression of IgE hyperproduction. Serum IgE levels were measured at the completion of second DNCB challenge, 2 and 4 weeks after initiating dermal application of test compounds. The results are expressed as means ± S.E.M. (n=3~6). *: $P < 0.05$, significantly different from the Normal. #: $P < 0.05$, significantly different from the Control.

Effect of LB oil application on production of cytokine from splenic T cells

Four weeks of 1% and 3% LB oil applications resulted in significant lower IL-10 production concentration-dependently (291.42 and 213.25 pg/ml, respectively) compared to the saline treated control mice (484.88 pg/ml) (Figure 5), while 3% LB oil application resulted in a little higher (155.15 pg/ml, approximately 1.44%) IFN-

production with no statistical significance compared to the control mice (107.95 pg/ml).

A.

B.
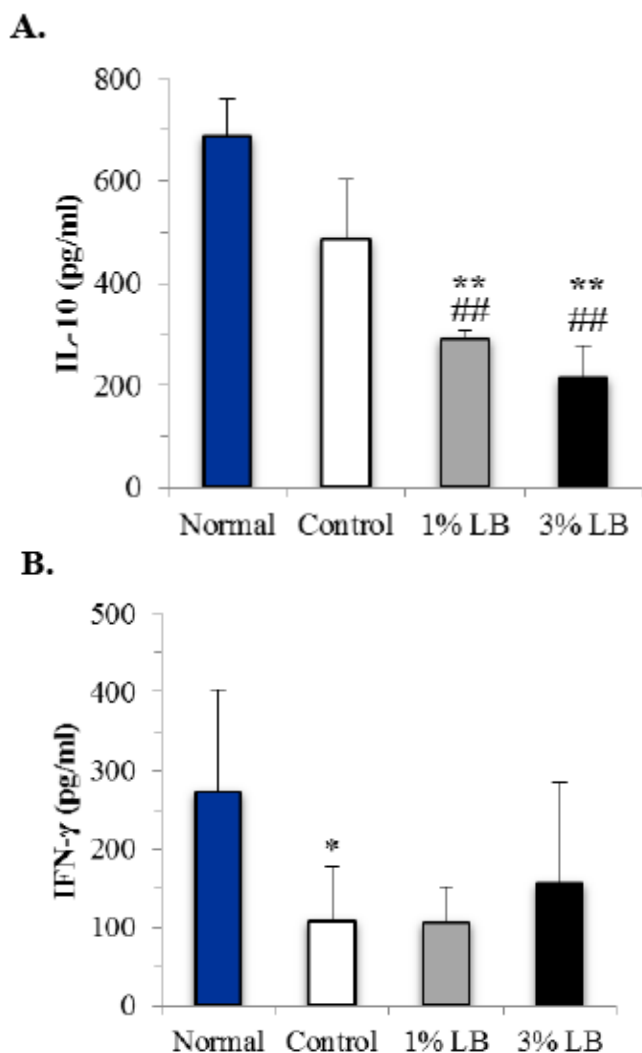
Figure 5. Effects of LB oil application on production of cytokine from splenic T cells. Splenocytes were stimulated with immobilized anti-CD3 mAb for 48 hours. Culture supernatants were collected for measurement of IFN- and IL-10. The results are expressed as means ± S.E.M. (n=3~6). *: $P < 0.05$, **: $P < 0.01$, significantly different from the Normal. #: $P < 0.05$, ##: $P < 0.01$, significantly different from the Control.
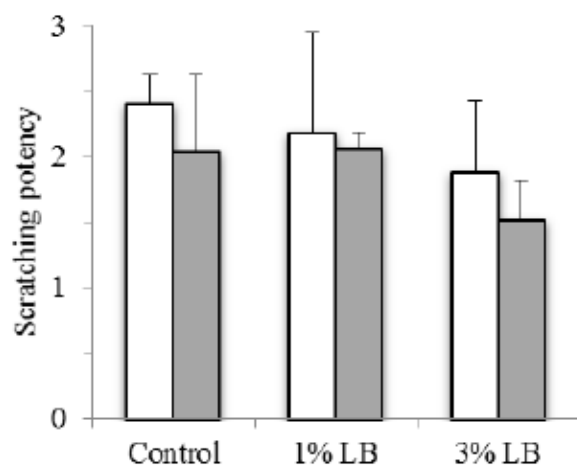
Decreased tendency of scratching frequency in LB oil-applied mice

The scratching behavior on back skin was observed for 30-min on one day after the second DNCB challenge (day 1) and on the day 30 after initiating LB oil application (day 30). It was found that 1% and 3% LB oil group (275 and 217 times, respectively) showed a lower (approximately 91% and 72%, respectively) tendency of scratching frequency than the saline treated control group (300

times) at day 30 (Figure 6). The total scratching duration of the LB oil groups (17.43 and 13.62 seconds, respectively) were a little shorter (approximately 92% and 72%, respectively) than the control group (19 seconds). The scratching potency of 3% LB oil groups (1.52) were weaker (approximately 74%) than the control group (2.03). The scratching speed was not changed among various groups. The scratching beats from the normal mice in 30 min duration are similar with previous report [27].

A.

B.
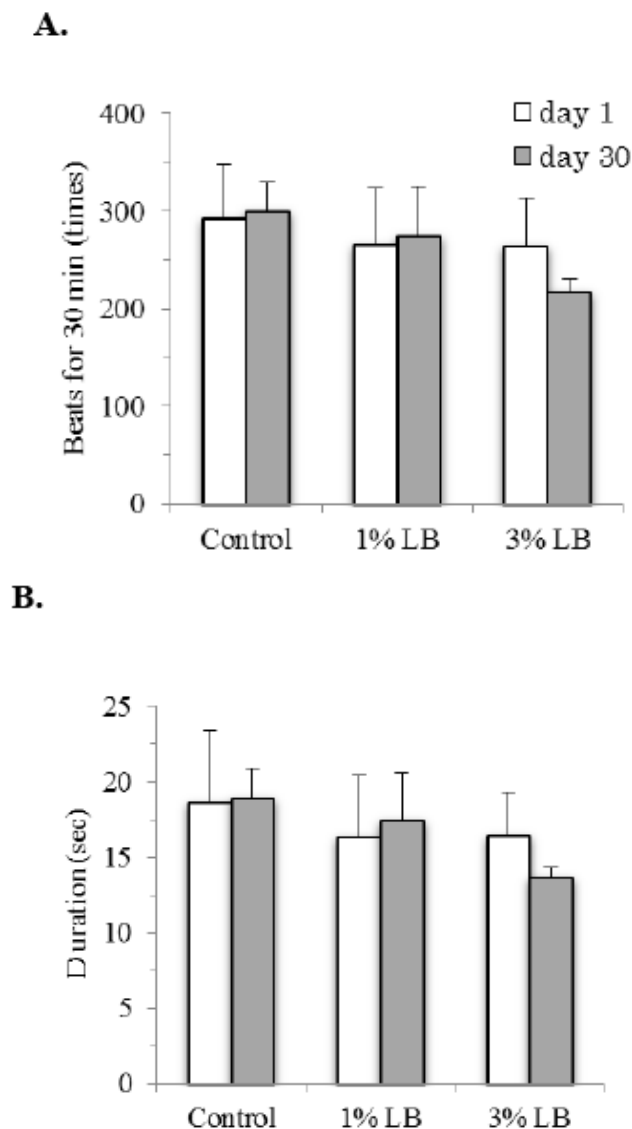
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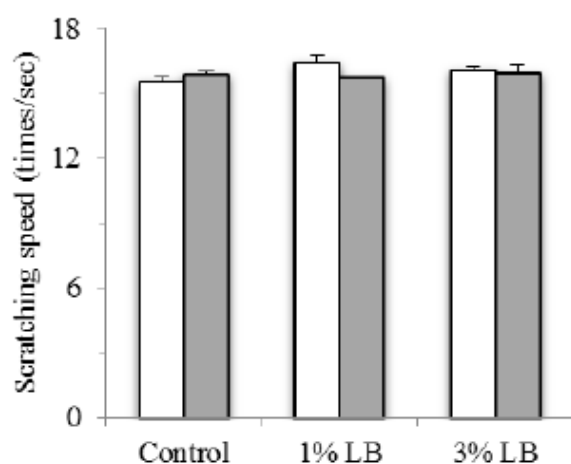
Figure 6. Decreased tendency on scratching frequency in LB oil-treated mice (A). Frequency of scratching on back skin was measured for 30 min one day after the second DNCB challenge (day 1) and the 30th day after initiating LB oil application (day 30). Total scratching time within 30 minutes (B). Scratching

Discussion

The present study was undertaken to evaluate the efficacy of LB oil alleviating atopic dermatitis-like immune alterations in mice. Even though LB has been used for the folk remedy of eczema by placing the fresh leaf on insect bites and wounds, and LB oil-contained formulation for the pruritic skin disorder [28, 29], there has been no scientific study investigating LB oil on the treatment or prevention of atopic dermatitis. Our study is the first to demonstrate LB oil's immunoregulatory potential for alleviating atopic dermatitis through influencing of Th2 / Th1 cell activation. Overall, we found that LB oil

possessed an ability to influence Th2 / Th1 cell activation because the dermal application of LB oil resulted in the suppression of IgE over-production, the downregulation of IL-10 production from Th2 cells, and upregulation tendency of IFN- production from Th1 cells. In addition, frequent scratching due to itchy sensations was a little alleviated following LB oil application onto the damaged skin involved with the pathogenesis of atopic dermatitis, further there isn't central nervous inhibition by the open-field test after 4-week application of LB oil (data not shown). Pruritus is a symptom found in a variety of skin disorders like atopic dermatitis and contact allergic dermatitis. For atopic dermatitis cases, it has been reported that scratching due to itchy sensations causes skin damage, promotes inflammation and thereby further aggravates pruritus [30, 31, 32]. Therefore, it is important to reduce itchy sensations and scratching frequency to prevent aggravation of skin lesions due to pruritic disorders and improve quality of life. LB oil has been reported to exhibit an *in vitro* antibacterial activity against Gram-positive strains, and a high activity against Candida albicans [33]. And it also has been used for the treatment of Herpes simplex [16, 17]. It contains several major constituents (citral, citronellal, trans-caryophyllene and rosmarinic acid). Citral and citronellal are well known for their antimicrobial effects [33], and the anti-inflammatory mechanism of citral is via the NF-kappaB pathway [34]. Furthermore, the hyaluronidase inhibitor in the methanol extract of leaves of lemon balm was identified with rosmarinic acid, a component which is demonstrated the possible clinical use as a therapeutic agent for atomic dermatitis [22, 35]. The most important aspect to be discussed is LB oil's systemic effect on the modulation of the *in vivo* type-2 response. The type-2 response becomes predominant when differentiation or activation of Th2 cells is preferred, but development or stimulation of Th1 cells is suppressed. Considering that the skewedness toward type-2 responses is a background mechanism for the occurrence of atopic dermatitis, dermal application of LB oil could systematically block the pathogenesis of atopic dermatitis through correcting the immune homeostasis skewed in favor of Th2. Considering our experimental results, it could be concluded that the application of LB oil on the skin of atopic dermatitis cases could alter production of IL-10 from Th2 cells, IFN- from Th1 cells, also alter IgE production and may alter histamine production, performing a series of immunoregulatory functions to alleviate the occurrence or progression of atopic dermatitis.

Conclusions

Hence, it is believed that the application of LB oil on atopic dermatitis cases will be significantly meaningful for public health and alternative medicine. However, it is not clear which constituent(s) of LB oil contribute to the LB oil-mediated alleviation of atopic dermatitis-like immunologic and skin alterations in mice at the moment. Future studies will be directed towards identifying which components of LB oil are responsible for its immunomodulatory effects.

Author contributions

Acknowledgements

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