Original Research Article

Ameliorative Efficacy of Combined Betel and Tea Tree Essential Oil-Incorporated Cream on DNCB-Induced Atopic Dermatitis-like Symptoms in BALB/c Mice

ABSTRACT

Aims: This study aimed to investigate the ameliorative efficacy of combined betel and tea tree essential oil-incorporated cream on DNCB-induced atopic dermatitis-like symptoms in BALB/c mice**.**

Methodology: cream formulation containing blended betel essential oil (1%) and tea tree essential oil (0.5%) was topically applied onto the dorsal skin of 2,4-dinitrochlorobenzene-sensitized BALB/c mice once a day during four weeks of assay. Phosphate-Buffered Saline (PBS) and protopic (tacrolimus 0.1%) were used as negative and positive control, respectively. All mice were subjected to the assessment of AD-like symptoms including the severity of skin lesions, frequency of scratching behavior and histological features.

Results: The blended essential oil-incorporated cream ~~was significantly effective in suppressing the~~ has significant effect against DNCB-induced eczematous skin lesions in BALB/c mice with the dermatitis score of 2.17 ± 0.75 (n = 6, $p < 0.05$) and the number of scratching behavior of 6.09 ± 1.28 (n = 6, $p < 0.05$) compared to ~~that of~~ negative control (7.00 ± 0.63 and 18.55 ± 1.23, n = 6, $p < 0.05$, respectively). ~~in week 4. Meanwhile, histological changes were markedly noted with the reduction of the clinical signs including hyperplasia, parakeratosis, spongiosis and acanthosis. Moreover, there was no~~

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~~significant difference in the thickness of epidermal/dermal between mice received blended essential oil-incorporated cream and protopic-treated mice.~~

Conclusion: These findings confirmed the ~~significantly~~ ameliorative efficacy of ~~combined~~ betel and tea tree essential oil-incorporated cream on DNCB-induced atopic dermatitis like symptoms in BALB/c mice. However, further studies are necessary to elucidate the possible synergistic effect of blended essential oils from betel and tea tree as well as the interplaying roles of major molecules present in essential oils~~, thereby ultimately exploiting the utilization of these valuable medicinal plants.~~

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Keywords: 2,4-dinitrochlorobenzene; Piper betle, Melaleuca alternifolia; essential oil; atopic dermatitis.

1. INTRODUCTION

1.1. Atopic dermatitis

Atopic dermatitis (AD) or atopic eczema is a chronic skin inflammatory disease that occurs commonly in young children, but it can also affect adults of all ages and genders with the hallmark of intense pruritus [1]. People with AD may typically experience periods where the disease gets worse and the symptoms become more severe (flare-ups), followed by periods where the skin condition improves and the symptoms are mild or even no signs of AD (remissions). The symptoms and frequency of AD flare-ups can be significantly variable from person to person, and from time to time depending on a person's age, phenotype and inflammatory response profile. Typical AD symptoms including erythematous eczematous skin lesions, xerosis, lichenification, cutaneous hyperactivity and hyperkeratosis in both intrinsic and extrinsic AD types manifest through acute, subacute and chronic AD cause serious disturbance for patients in daily life [2]. AD can develop any area of the body, but the most common parts to be affected are face, cheeks, scalp, hands, fingers, elbows, knees, arms, legs and feet. The pathogenesis of AD is not fully understood, but it is clear that AD may be likely attributed to a combination of various factors including genetics, skin condition (dry skin), immune-mediated responses, emotional triggers and provocations from environmental factors such as microorganisms, irritants, allergens, tobacco smoke, food or changes in climate [3]. It is noteworthy to know that filaggrin protein has been proven to be one of the most significant genetic risk factors for AD. Filaggrin is found in cells that make up the major structure of the stratum corneum of the epidermis. Filaggrin plays a vital role in maintaining the structural integrity of epidermis and building a strong skin barrier, thereby regulating skin moisture levels and protecting against pathogenic agents such as bacteria, allergens or irritants. Research indicated that people with AD might have a mutation of filaggrin gene (FLG) re-

sponsible for encoding the synthesis of filaggrin. Lack of expression of filaggrin protein may predispose to the weakening of skin barrier function. As a consequence, the skin becomes less effective in retaining proper moisture levels. Additionally, owing to the defective epidermis, bacteria or viruses can enter the outer layers of skin which in turn can exacerbate the severity of AD skin lesions. This is the reason why many people suffer from AD tend to have dry, scaly and infection-prone skin. Emollients, commonly known as specific ingredients of moisturizers, are effective in protecting damaged skin affected by AD. By softening the skin and improving skin hydration, emollients can enhance the skin barrier function and reduce skin dryness, thereby relieving itching, scaling, and protecting the skin from irritants. On the other hand, immunological responses, specifically different excessive immune responses of Th1 and Th2 cells in inflammatory regulation that leads to different hypersensitive reactions producing various mediators, are chiefly regarded as the central of AD pathogenesis. The treatments of AD, thus, aim to ameliorate the immune dysregulation caused by imbalance between Th1 and Th2, reduce pruritus intensity and recover functionality of the skin barrier, which is damaged by prolonged inflammatory response [4].

There is currently no cure for AD, but various therapies may help relieve symptoms or manage control triggering factors. Among various current therapies, topical remedies remain the mainstay of treatment for AD, owing to their proven effectiveness and generally less side effects than systemic medications. Common topical prescribed medications for AD include corticosteroids, calcineurin inhibitors, Janus kinase (JAK) inhibitors and phosphodiesterase 4 (PDE4) inhibitors. However, some existing AD treatments are bound to certain concerns that could negatively affect patients' adherence to the treatment: topical corticosteroids (TCS) are currently classified as the first-line treatment for AD flare-ups. Noticeably, the use of TCS as standard management associates with phobia of local side effects like skin atrophy, striae, steroid rosacea, senile/solar purpura, telangiectasia, hypertrichosis, pustular psoriasis or perioral dermatitis. Additionally, long-term use of TCS may be associated with a potentially serious side effect, namely topical steroid withdrawal (TSW) [5-8]. Topical calcineurin inhibitors (TCI) are corticosteroid-sparing immunomodulators used as second-line treatment for moderate to severe AD (tacrolimus) or mild to moderate (pimecrolimus). Notably, TCI might cause immunosuppression, local skin irritating effects (pruritus, erythema, burning), folliculitis, molluscum contagiosum, tinea incognito, facial acne, allergic contact dermatitis or rosacea-like dermatitis [9]. Indeed, the search for a novel and effective anti-AD agent deserves special attention considering its convoluted, frustrating nature and wide span of endemic areas. Accordingly, a serious demand for discovery and development of complementary approaches to AD has been imposed, promoting the practice of holistic methods and utilization of naturally derived materials to a great extent [10].

Generally, the alternative and complementary medications will be taken into consideration if AD does not respond to conventional therapies. Indeed, many people find natural remedies and naturally occurring ingredients can be beneficial to the treatment of AD. Practically, some essential oils have been proven potentially effective in ameliorating symptoms of AD over the years. Common essential oils used to combat AD include Roman chamomile (*Anthemis nobilis*), clove (*Syzygium aromaticum*), lavender (*Lavendula angustifolia*), rosemary (*Rosmarinus officinalis*), lemon balm (*Melissa officinalis*), tea tree (*Melaleuca alternifolia*), Australian sandalwood (*Santalum spicatum*), frankincense (*Boswellia carterii*, *Boswellia serrata* and *Boswellia ferreana*), peppermint (*Mentha arvensis*) and thyme (*Thymus vulgaris*). In fact, essential oils are not regulated by The Food and Drug Administration (FDA) to treat or prevent symptoms of AD; this is partly owing to lacking of many clinical trials or scientific evidence supporting the effectiveness of essential oils for treatment of AD. Nevertheless, essential oils are still regarded as one of the most alternative treatments of AD, owing to their significant pharmacological properties including antimicrobial, antioxidant and anti-inflammatory properties. Essential oils, thus, would not be recommended for replacing prescribed medications for AD. Instead, they could be a beneficial supplement as an adjunct to conventional therapy in the treatment and prevention of AD. In general, some essential oils can potentially ease symptoms of AD in various different ways, such as: (1) help repair skin damage, promote healing of wounds and skin cell regeneration; (2) significantly soothe and relieve pruritus thanks to anesthetic properties; (3) help reduce inflammation and protect against infections; (4) help relieve pain, swelling, redness and irritation; and (5) help rejuvenate dry skin and reduce scarring [11-15].

Piper betle L. (*P. betle*), commonly known as betel vine, belongs to genus Piper of the Piperaceae family. Betel leaf essential oil (BLO) is a complex chemical mixture consisting of approximately between 30 to 60 natural volatile compounds. These compounds mainly belong to classes of terpenes and phenols in various proportions depending on the botanical origin of the betel leaves. Major components of BLO include anethole, chavicol, chavibetol, eugenol, eugenol acetate, germacrene-D, isoeugenol, and safrole [2-9]. BLO possesses a variety of significant pharmacological properties including antiseptic, antioxidant, antimicrobial and radio-protective properties. Nevertheless, as evidenced from previous studies, the antiseptic property of BLO is attributed chiefly to the major constituents of BLO including chavicol, chavibetol and eugenol [16-19]. On the other hand, *Melaleuca alternifolia* (*M. alternifolia*), commonly known as tea tree, is a member of the family Myrtaceae native to Australia. Tea tree essential oil (TTO), a mixture of natural volatile compounds extracted mainly from tea tree leaves, has been widely used as an alternative and complementary therapy for a variety of skin conditions such as

acne, wounds or contact dermatitis. TTO possesses various significant biological activities including antimicrobial, antioxidant and anti-inflammatory properties. TTO is composed of approximately 100 various chemical compounds. Chemical analysis of TTO reveals the presence of terpinen-4-ol, *p*-cymene, γ-terpinene, α-terpineol, terpinolene, α-pinene, α-terpinene and 1,8-cineole [20-23]. As indicated from previous literature, the antibacterial and antifungal properties of TTO are attributed mainly to terpinen-4-ol and α-terpineol. In this regard, this study was primarily focused on investigating the ameliorative efficacy of combined betel and tea tree essential oil-incorporated cream on 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis-like symptoms in BALB/c mice.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,4-dinitrochlorobenzene (DNCB) was purchased from Sigma Chemical Co. (Singapore) and dissolved in the mixture of acetone/olive oil (1:4) to make 1% (w/v) and 0.5% (w/v) solutions. Protopic 0.1% (tacrolimus ointment) was obtained from a pharmacy. BLO and TTO were provided by the Laboratory of Applied Biochemistry, Department of Applied Biochemistry, School of Biotechnology, International University, Vietnam National University-Ho Chi Minh City, Vietnam with voucher No. HB-BIO-09-03-22. The data on the composition of both BLO and TTO were also available. The essential oil from *P. betle* contains 13 compounds representing approximately 95% of the total oil. The chemical composition of BLO which was determined by GC-MS revealed the presence of eugenol (39.21%) followed by other components: eugenol acetate (16.42%), 4-allyl-1,2-diacetoxybenzene (12.24%), terpinen-4-ol (6.58%), α-cadinol (6.13%), γ-terpinene (3.46%), and sabinene (2.14%). Meanwhile other compounds made contributions less than 2% each to the total composition of essential oil. On the other hand, the essential oil from *M. alternifolia* consists of 12 compounds accounting for almost 97% of the total oil. The major components of TTO were terpinen-4-ol (49.62%), followed by other components: γ-terpinene (18.08%), α-terpinene (9.16%), *p*-cymene (5.89%), α-terpineol (4.94%), terpinolene (3.47%), and α-pinene (2.02%). Meanwhile, other substances contributed lower than 2% each to the total composition of essential oil.

2.2. Animals

~~Female~~ BALB/c mice were procured from Pasteur Institute of Ho Chi Minh, Vietnam and allowed to acclimate in experimental conditions for 1 week. All mice were kept in controlled environment of 12 h of light and dark cycle, while a standard diet and water were provided *ad libitum*. All experiments on mice were in accordance with the *Guide for the Care and Use of Laboratory Animals* (8^{th} edition) [24] and *Principles of laboratory animal care* (NIH publication No. 85-23, revised 1985).

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3. Cream Preparation

Table 1. Formulation of the Topical Cream.

	Ingredient	Amount (%)
Oily phase	Cetearyl alcohol	2.50
	Ceteareth-20	2.50
	Ceteth-2	2.50
	Cetyl alcohol	1.00
	Isopropyl myristate	3.00
Aqueous phase	Propylene glycol	0.50
	Carbomer 940	0.30
	Phenoxyethanol	0.90
	Polysorbate 80	0.90
	BLO	1.00
	TTO	0.50
	Deionized water	Qs. 100%

The preparation of oily phase was conducted by melting together cetearyl alcohol, ceteareth-20, ceteth-2, cetyl alcohol and Isopropyl myristate in a beaker with continuous heating in a water bath at 70°C-75°C. Meanwhile, carbomer 940 was dispersed in warm deionized water (70°C-75°C) with agitation. Propylene glycol and preservative phenoxyethanol were then added to complete the formation of aqueous phase. When the oily and aqueous phase were at the same temperature of 50 ± 2°C, the aqueous phase was slowly added to the oily phase, with continuous stirring until the temperature had dropped to 40 ± 2°C. The essential oils were then emulsified by polysorbate 80 prior to being incorporated into the mixture. Finally, deionized water was added to make up 100 g of semisolid cream formulation. Homogenization of this combination was carried out by a homogenizer for 30 minutes until a smooth, clump-free cream with good integrity was obtained. pH Values of all prepared cream formulations were adjusted to 4.5-5.0 using 0.2% citric acid and 0.1% triethanolamine. The cream formulations were left to cool to room temperature prior to being filled into sterile air tight containers, sealed and kept in cool place. The composition of cream formulation is presented in Table 1.

3.1. Evaluation of cream formulations

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3.1.1. Physical observation

Physical characteristics including color, odor and feeling of application of prepared creams were ~~noted~~.

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3.1.2. Homogeneity

All the prepared cream formulations were subjected to visual and manual inspections, as the presence of any clump or disintegration was considered signs of heterogeneity.

3.1.3. Determination of pH

The pH values were determined using digital pH meter following the dissolution of 0.5 g of cream sample in 50.0 mL of deionized water. Prior to the pH examinations, the pH meter was calibrated using standard buffer solutions (pH 4, 7 and 10). The measurement of each cream was triplicated.

3.1.4. Stability study

The stability of cream formulations was assessed based on the changes in appearance, pH, odor, color and after-feel in a 2-month interval.

3.1.5. Skin Irritation

Ten human volunteers in the test for irritation received 1.0 g of each prepared cream for topical application on an area of 1 cm^2. The participants were asked to record and report any occurrence of swelling, redness or itchiness after 24 h.

3.2. Induction of AD and topical treatment on mice model

Induction of atopic dermatitis was carried out following a previously used protocol with slight modifications [25]. Briefly, dorsal skin of mice were shaved using depilatory cream before being sensitized with 100 µL of 1% DNCB on day 1, 3, 5 in first week. Animals whose dermatitis scores were not statistically different with similar clinical manifestations were then chosen and randomized in to 4 groups of 6. For the next 3 weeks, mice skin was challenged with 100 µL of 0.5% DNCB thrice a week, simultaneously with daily topical application of treatments. Group I: mice received 100 µL of PBS (normal mice). Group II: mice sensitized with DNCB received 100 µL of cream base (negative control). Group III: mice sensitized with DNCB received 100 µL of blended essential oil-incorporated cream. Group IV: mice sensitized with DNCB received treatment with protopic 0.1% (positive control). All mice were euthanized by inhalation of diethyl ether in week 4 for the purpose of skin histological assessment.

3.3. Scratching behavior observation

Scratching behavior of mice in terms of the number of scratching bouts was recorded following previously described method [26]. For each mouse, only back scratching with hind paws was counted in 20 minutes by visual observation once a week before the exposure to DNCB.

3.4. Evaluation of skin lesions severity

During the challenge and topical treatment of blended essential oil-incorporated cream, the development of AD was evaluated based on 4 clinical parameters including erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness that were marked on the scale of 0-3, as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) following Eczema Area and Severity Index (EASI)–lesion severity atlas [27]. Dermatitis score therefore ranged from 0 to 12 as a sum of severity points of these parameters and was assessed blindly by two individuals once every week.

3.5. Histological examination

Skin biopsies were collected on the last day of week 4 and immediately fixed in 10% neutral buffered formalin for 12 hours. The samples were then dehydrated by alcohol in graded concentration (50-100%), cleared in xylene and embedded in paraffin wax before being sectioned at a thickness of 4 µm for the purpose of H&E staining. Signs of acanthosis, spongiosis and parakeratosis were recorded. The thickness of epidermal and dermal layer was measured to assess the degrees of hyperplasia and hyperkeratosis. Procedures were performed under supervision and guidance of a doctor (Histopathology Department, University of Medicine, Ho Chi Minh City).

3.6. Statistical analysis

All the results were expressed in terms of Mean ± Standard deviation (SD). t-test, one-way and two-way analysis of variance (ANOVA) for comparisons between groups were conducted. Significance level was set to be less than 0.05. GraphPad Prism 6 was used for data analysis.

4. RESULTS

4.1. Evaluation of cream formulations

Table 2: Physical characteristics and skin irritation of cream formulations in the duration of 2 months.

Period	Color	Appearance	Homogeneity	pH	Skin irritation testing of creams		
					Edema	Erythema	Pruritus
Initial	White	Smooth, opaque	Good	4.82 ± 0.02	No case	No case	No case

After two months	White	Smooth, opaque	Good	4.70 ± 0.01	No case	No case	No case

All the prepared cream base formulations were opaque white, shining and easy to spread product with firm smoothness. Other blended essential oil-incorporated cream formulations were pearlescent white in color and had also listed characteristics along with a typical odor of essential oil. All creams gave a cooling sensation and good adherence on skin, while they could be easily removed by tap water. The homogeneity, pH values, stability study and skin irritation tests of these cream formulations were summarized in Table 2.

3.2 Clinical observation

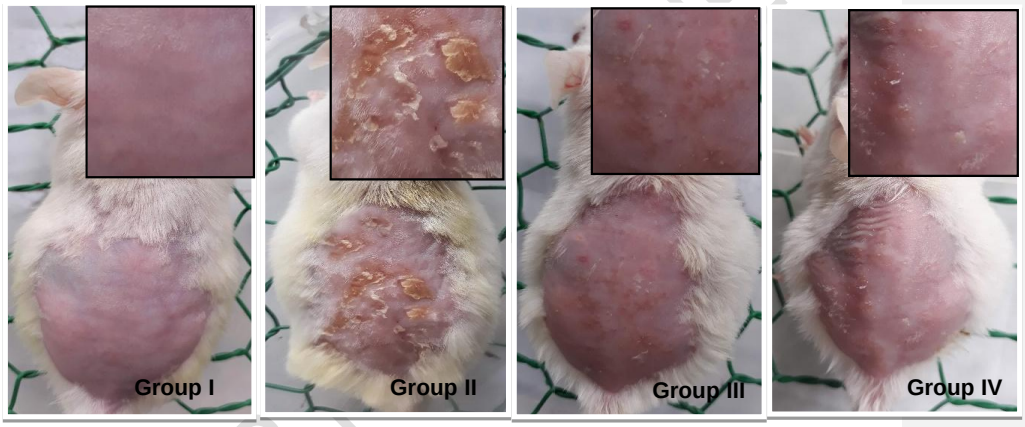

Figure 1: Effects of blended essential oil-loaded cream on DNCB-sensitized mice.
Clinical manifestations observed in mice in week 4. Group I: Normal mice; Group II: DNCB + cream base (negative control); Group III: DNCB + essential oil-incorporated cream; Group IV: DNCB + protopic 0.1% (positive control).

3.3 Evaluation of skin lesions severity

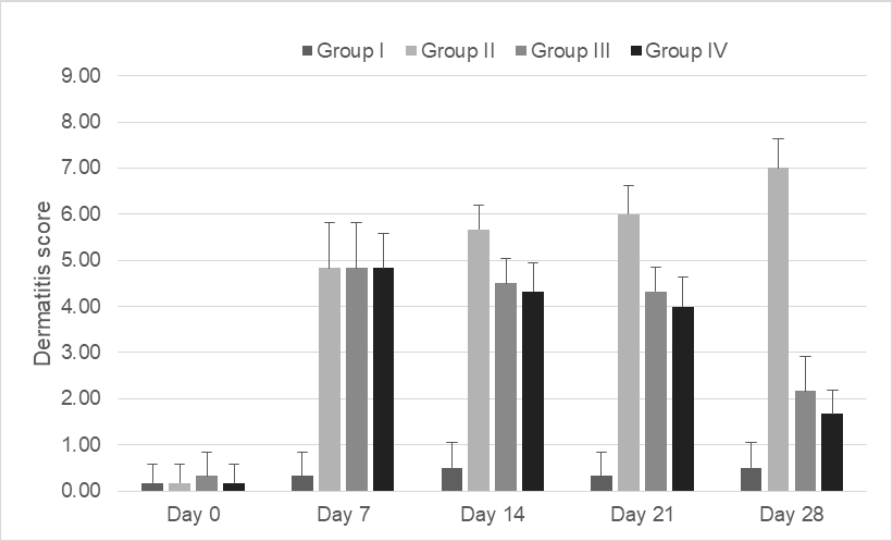

Figure 2: Dermatitis severity of experimental groups during treatment period. *Dermatitis score based on signs of erythema, edema, excoriation and lichenification of skin was evaluated once a week. Each bar represents the Mean ± SD (n = 6); p < 0.05 compared to negative control.*

3.4 Scratching behavior

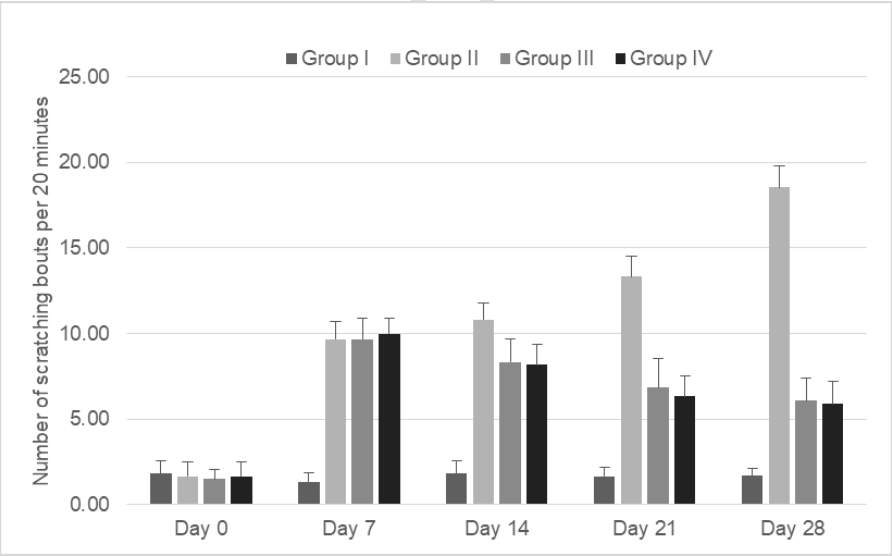

Figure 3: Scratching behavior during treatment period. *The number of scratching bouts by hind legs was recorded for 20 minutes weekly before sensitization of DNCB. Each bar represents the Mean ± SD (n = 6); p < 0.05 compared to negative control.*

In response to multiple repeated exposure to DNCB, the dorsal skins of challenged mice developed acute AD-like symptoms including the formation of pustules, typically with

consequent edema/papulation, erythema/hemorrhage, excoriation and scaling/dryness compared to control group received vehicle. Besides, erosion gradually aggravated skin condition. Mice from negative control exhibited the severity of skin lesions, characteristically a chronic symptom of lichenification. However, these features were well-illustrated in mice from negative control group and appeared to be less severe in others. Meanwhile, it can be clearly observed the skin conditions of blended essential oil-incorporated cream-treated mice were distinguishable from that of negative control, as crust formation, skin dryness and lesion areas were visibly ameliorated. For positive control mice which received topical application of protopic 0.1%, all acute and chronic AD-like symptoms were only mildly severe (Figure 1).

During four weeks of experiment, the development of AD was observed and evaluated by summing up the score given individually on 4 parameters including erythema, edema, excoriation and lichenification in the scale from 0 to 3 (0 (none), 1 (mild), 2 (moderate) and 3 (severe)). In general, there was no statistically significant difference among tested groups after one week of assay. However, it was clear to observe the significant difference from the day 14 onwards and extremely significant difference on the last day of treatment that the symptoms in group III mice were considered significantly different from that of negative control. The dermatitis score of group III was recorded 2.17 ± 0.75 ($n = 6$, $p < 0.05$) compared to that of group II (7.00 ± 0.63, $n = 6$, $p < 0.05$) after 28 days of experiment, denoting the effective therapeutic potency of blended essential oil-incorporated cream in reducing AD-like symptoms in BALB/c mice. Generally, there was no statistically significant difference between group III and IV throughout the experiment as protopic 0.1% notably suppressed AD-like symptoms from the second week. However, it was observable that the severity of skin lesions in group IV was milder than that of other investigated groups. Accordingly, the dermatitis score of group IV was 1.67 ± 0.52 ($n = 6$, $p < 0.05$) on day 28 (Figure 2).

The frequency of scratching bouts by hind legs in 20 minutes of each mouse was recorded weekly before DNCB challenge (Figure 3). Pruritus, as exhibited through scratching behavior, intensified in correspondence with severity of AD skin lesions in all experimental groups of mice. There was statistically significant difference in the number of scratching behavior (NSB) between group I (normal mice) and other groups (II, III and IV) after seven days of experiment, confirming the effect of DNCB in inducing AD-like symptoms in BALB/c mice; however, mice from group II, III and IV whose NSB were not significantly different to each other. Accordingly, NSB of group II, III and IV was recorded 9.67 ± 1.03, 9.67 ± 1.21 and 10.00 ± 0.82 ($n = 6$, $p < 0.05$), respectively. The NSB, thus, did not exhibit distinguishable clinical manifestations after seven days of assay. However, the NSB of mice in group II was higher than that of group III and IV on day 14 and

significantly greater than other groups after three weeks, and continued drastically increase until the end of the experimental period. Indeed, the cream base was truly not effective in suppressing itchiness as high scratching frequency was noted throughout the experimental period with the NSB of 10.83 ± 0.98, 13.33 ± 1.21 and 18.55 ± 1.23 (n = 6, $p < 0.05$) on day 14, 21 and 28, respectively. In contrast, it could be observed the effectiveness of both blended essential oil-incorporated cream and protopic 0.1% in reducing the NSB, notably changing on day 14 and continued showing the powerful efficacy until the last day of experiment. Noticeably, there was no statistically significant difference in NSB between group III and IV throughout the experimental period. The NSB of group III on day 14, 21 and 28 was recorded 8.33 ± 1.37, 6.83 ± 1.72 and 6.09 ± 1.28 (n = 6, $p < 0.05$), respectively, whereas the NSB of group IV was 8.17 ± 1.17, 6.33 ± 1.21 and 5.89 ± 1.33 (n = 6, $p < 0.05$), respectively.

3.5. Histological examination

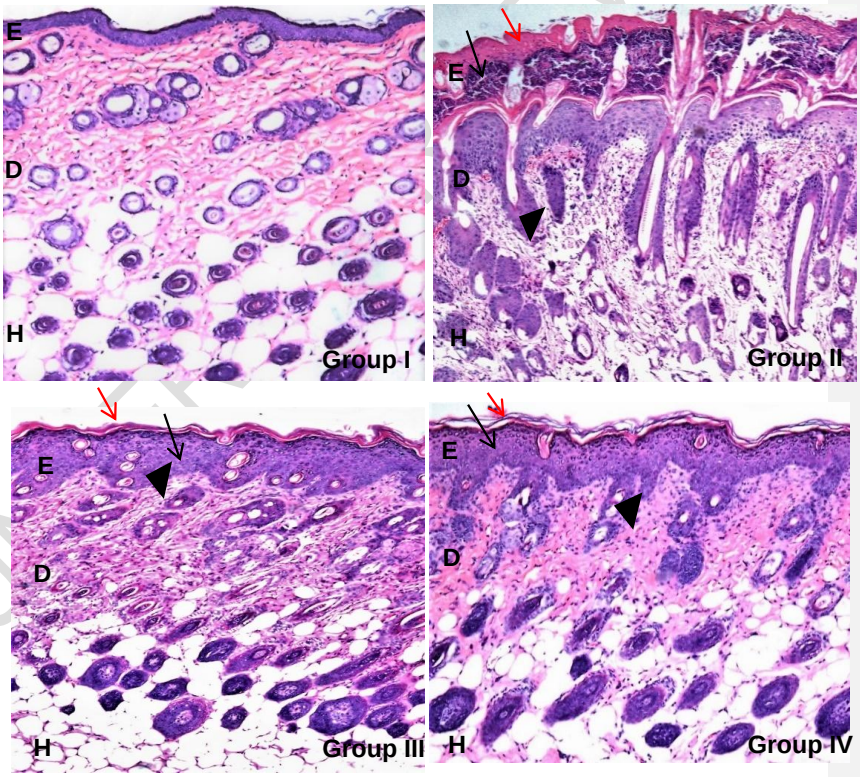

Figure 4: Effects of blended essential oil-incorporated cream on mice skin histology. Histological findings by H&E staining**.** *(Magnification: 10x); Epidermis, dermis and hypodermis layer were denoted by E, D and H, respectively; Signs of spongiosis (→), parakeratosis (→), pustule and elongation of rete ridges (◂) were presented***.**

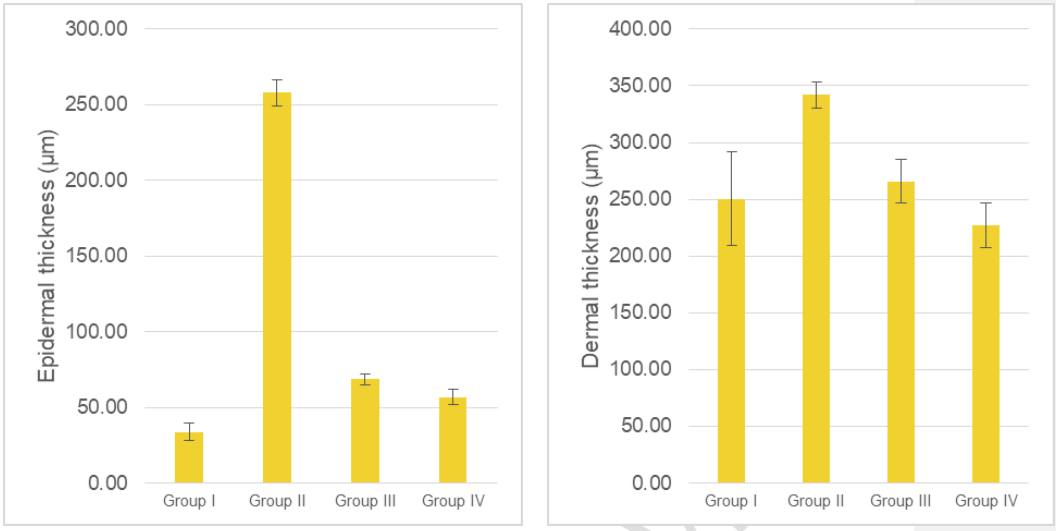

Figure 5: **Effects of blended essential oils-incorporated cream formulations on mice skin histology**. ***(Left)*** *Epidermal and* ***(Right)*** *dermal thickness of mice dorsal skin. Each bar represents the Mean ± SD (n = 6); p < 0.05 compared to negative control.*

As shown in Figure 4, mice dorsal skin was subjected to staining with H&E dye for the examination of histological features. ~~Pustules in the stratum corneum, hyperplasia, parakeratosis, spongiosis and acanthosis with elongation of rete ridges of moderate severity were clearly visible in negative control mice; such alterations resulted in epidermal and dermal thickening, as well as the appearance of abnormal-shaped hair follicles and sebaceous glands in these skin layers.~~ Exhibited features on the dorsal skin including weepiness, crusting, scaling, thickening and abrasion reflect the intensity of AD-like symptoms on DNCB-sensitized mice. However, those histopathological findings were found to be subdued partly in blended essential oil-incorporated cream-treated mice and significantly in positive control mice. ~~In fact~~, the thickness of epidermis layer in mice treated with blended essential oil-incorporated cream and positive control mice was significantly lower than that of negative control mice. Meanwhile, only the dermal thickness of mice treated with blended essential oil-incorporated cream and protopic was markedly reduced as compared to PBS-receiving mice (Figure 5).

Comment [A8]: Please, verify. This statement is misleading. A negative control group is not exposed to any treatment that is expected to have an effect.

3. DISCUSSION

AD is a chronic and complicated relapsing dermatological disease with high prevalence of 15–30% and 2–10% in children and adults, respectively. The disease is clinically characterized by intense pruritus, appearance of dry, eczematous skin lesions, particularly inflammatory responses causing edema, erythema, irritation and pain. It is noteworthy to

know that the inflammatory responses are commonly associated with an abnormal secretion of T helper (Th1) and T helper (Th2) cells which has been proven to be the culprits of the imbalance between Th1 and Th2 cells owing to different immune function. Th1 immune response is related to pathogenic infection, whereas Th2 immune response is involved in stimulating agents. Accordingly, the hypersensitivity of Th2 cells play a key role in the production of cytokine leading to the overproduction of serum immunoglobulin E (IgE), infiltration of mast cells and eosinophils, especially the release of mediators like histamine into inflamed skin tissue. These events, particularly the Th1/Th2 imbalance, could be considered to be specific biochemical parameters which can be used as indicators for clinical assessment of the AD pathogenesis. This refractory disease, thus, imposes an enormous financial burden, sleep disturbance and depression on patients in every daily life [28]. Chiefly responsible for AD development can be classified as intrinsic and extrinsic factors. Intrinsic factors are widely known as inherited downregulation of FLG, endocrine changes, or even psychological stress, whereas extrinsic factors commonly include food, climate, exposure to drugs, chemicals and allergens. These risk factors may promote a series of inflammatory reactions that exterminates intruding pathogenic agents, yet consequently perturbs the balance of immunological system and exacerbates severity of AD symptoms if repeated and prolonged. As a result, treatments with immunomodulatory action, especially those that target on mediating inflammatory responses, are required for the treatment of AD.

As reported from previous literature, essential oils have been traditionally used for hundreds of years for both aromatherapy and topical application, especially for the treatment and prevention of skin disorders. ~~In fact~~, the practice of blending essential oils is primarily exploited in the synergistic interactions which is well known as the therapeutic philosophy of herbal medicine where the combination of key constituents from medicinal plants is believed to be more effective than single isolated components. Nonetheless, the therapeutic effect of blended essential oils from BLO and TTO against AD has not been scientifically investigated. Suffice to say, this study is not expected to provide a cure for AD. Instead, this essential oil-based topical preparation may hopefully contribute as an adjunct therapy in the conventional treatment plan of AD. Both BLO and TTO have been intensively studied in recent years, basically elucidating their chemical composition and biological activities. Thanks to their natural intrinsic antimicrobial properties, both essential oils were shown effective in preventing the growth of skin bacteria, virus or fungi. On the other hand, both essential oils possess significant anti-inflammatory and anti-ulcer activities, and thus might be capable of interfering in immunologic responses of eczema pathogenesis. The major active ingredients of BLO are eugenol, chavibetol, chavicol, hydroxychavibetol and safrole whereas terpinen-4-ol, *p*-cymene, γ-terpinene, α-terpineol, terpinolene, and α-terpinene are accounted for a large proportion of TTO. The unique

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combination of these molecules present in essential oils may likely result in the synergistic effect in ameliorating AD-like symptoms. This study, thus, aims to investigate the anti-AD effect of combined BLO and TTO and contribute to the utilization of herbal medicines as skin diseases treatment, especially when current standard managements such as corticosteroids, immunosuppressive agents or phototherapy are bound to certain concerns for cost and safety. In this study, essential oils were loaded into a cream base at a concentration of 1% (BLO) and 0.5% (TTO) and topically applied on BALB/c mice with DNCB-induced AD. ~~In fact~~, the concentration of BLO and TTO used in the cream formulation was a result of an optimization process using checkerboard method. During the optimization process, two fold serial dilutions of essential oil (both BLO and TTO) were prepared by dilution with solvent to achieve a series of decreasing concentration (8%, 4%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0313% and 0.0156%). However, it is noteworthy to know that pure essential oil is lipophilic and thus cannot be dissolved in water. Additionally, owing to consisting of volatile compounds, essential oil is easy to vaporize when directly applying onto the skin surface, resulting in low bioavailability. Thus, essential oil should be necessarily formulated in cream, thereby enhancing the penetration of various components present in essential oil and possibly maximizing the therapeutic effectiveness of essential oil. Indeed, various non-active ingredients present in cream formulations not only played a key role in providing the rich resource of carrier for essential oils but also made a contribution to achieve the ideal homogeneity, spreadability and applicability which are known as typical characteristics of a cream. All prepared cream formulations had pH values ranging from 4.5 to 5.0, an acceptable pH span for topical preparation since it is close to that of human skin [29], and were safe to use as no adverse events were reported during skin irritation assay. However, the cream base had no ~~power~~ of alleviating AD-like symptoms including itchiness (Figure 4), and reducing epidermal/dermal thickness (Figure 5), since no significant improvements compared to PBS treatment was made.

Comment [A14]: Please,is this your result? If not, provide the reference.

Comment [A15]: Please, insert reference.

Comment [A16]: Please, replace power with ''effect''

The development of AD symptoms is attributed primarily to the inflammation responses, typically resulting in clinical symptoms such as swelling and redness that are accompanied by formation of blisters and eventually proceeds into dry, scaly and lichenified patches [30]. In this study, the BALB/c mice model were successfully induced by DNCB as can be seen from Figure 1-5 which showed the significant difference between normal mice and mice sensitized by DNCB with regards to clinical signs and pathogenic parameters including dermatitis score, number of scratching behavior, and the thickness of epidermis/dermis. To assess the severity and progress of DNCB-induced AD-like symptoms in this experiment, dermatitis score (0-12) based on 4 parameters including erythema/hemorrhage, edema, excoriation/erosion and lichenification was taken into account. These signs were significantly inhibited in mice which received the blended es-

sential oil-incorporated cream with the dermatitis score of 2.17 ± 0.75 compared to those of negative control (7.00 ± 0.63, n = 6, $p < 0.05$), as indicated by their scores in week 4 of assay. These findings clearly demonstrate the potential of the blended essential oil-incorporated cream in alleviating AD-like symptoms that are heavily influenced by pro-inflammatory responses.

Moreover, pruritus is a hallmark of AD in both acute and chronic phases. Pruritus-evoked scratching behavior, best known as a central symptom of AD, is typically regarded as a helpful indicator for assessment of AD, thereby assessing the skin's inflammatory response. The action of scratching may temporarily ease itching, but could induce skin damage, and trigger inflammatory response. As a consequence, the pruritus-scratching cycle could exacerbate the skin lesions, thereby further aggravating the severity of AD symptoms, negatively affecting the quality of both physical and mental life. Therefore, controlling pruritus should be chiefly regarded as one of crucial strategies in the AD treatment pathway. To evaluate the anti-pruritic effect of the blended essential oil-incorporated cream, the number of scratching bouts by hind paws was only recorded weekly in 20 minutes before the challenge of DNCB. As expected, the frequency of scratching behavior was notably suppressed in mice receiving the blended essential oil-incorporated cream with the NSB of 6.09 ± 1.28 compared to that of negative control with NSB of 18.55 ± 1.23 in the week 4 (n = 6, $p < 0.05$), firmly reflecting the significant effectiveness of the blended essential oil-incorporated cream in inhibiting pruritus during AD development.

Comment [A17]: Please, provide reference.

On the last day of the experiment, histopathological features of negative control mice have clearly shown the dorsal skin lesions accompanied with accumulation of inflammatory cells as compared with normal mice. It could be observed the oozing fluids in reddened, weeping skin lesions, possibly resulting from high scratching frequency. Additionally, hollow pustules in the stratum corneum were also noted, partly reflecting the pathological signs of an AD development. Meanwhile, hyperplasia, spongiosis and acanthosis were confirmed as the main causes of skin thickness, erosion and lichenification in chronic phase. As a consequence, defections of skin structures including parakeratosis, epidermal elongated rete ridges, hair follicles and sebaceous glands of unusual shapes and sizes in the dermis were visibly observed. These histological features, nevertheless, were found less severe in mice of group III and IV. In fact, the epidermal/dermal thickening of mice from group V and VI was significantly suppressed as compared to that of negative control. Notably, there was no significant difference in epidermal/dermal thickness between mice of group III and IV, denoting the ~~significantly ameliorate~~ efficacy of blended essential oil-incorporated cream in suppressing pathological alterations of AD.

Comment [A18]: Please, is this your result? If not, provide reference.

Comment [A19]: Please, delete.

4. CONCLUSION

Overall, this present research demonstrates the ~~significantly ameliorate~~ efficacy of combined BLO and TTO-incorporated cream on DNCB-induced AD-like symptoms in BALB/c mice ~~including pruritus, clinical manifestations and histopathological features. Although it is still unclear which mode of action has been accounted for the hindrance of this skin condition, high content of constituents present in essential oils, among which were eugenol, terpinen-4-ol, γ terpinene, α terpinene and α terpineol might be attributable. Moreover, since AD is typically characterized by the imbalance between Th1 and Th2 cells, there should be a comprehensive investigation on the inhibitory effects of blended essential oil-incorporated cream on relevant pro-inflammatory cytokines, chemokines or interleukins that mast cells and leukocytes release to control T cells derivation and functionality. Furthermore,~~ these findings would hopefully encourage more studies to elucidate the mechanism behind the possible synergistic interaction between natural intrinsic components of BLO and TTO, for the purpose of exploiting the potential of these valuable essential oils for the topical treatment against eczema-like skin lesions.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Both authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

Comment [A20]: ***What about the 10 persons used. ‘’***Ten human volunteers in the test for irritation received 1.0 g of each prepared cream for topical application on an area of 1 cm^2. The participants were asked to record and report any occurrence of swelling, redness or itchiness after 24 h”.

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