RESEARCH ARTICLE

Arjunolic acid protects against DNCB-induced atopic dermatitis-like symptoms in mice by restoring a normal cytokine balance

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ABSTRACT. ***Purpose:*** **Atopic dermatitis (AD) is a chronically relapsing, pruritic, eczematous skin disorder accompanying allergic inflammation. AD is triggered by oxidative stress and immune imbalance. The effect of oral arjunolic acid (AA) on 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis in mice was investigated.** ***Methods:*** **Repeated epicutaneous application of DNCB to the ear and shaved dorsal skin of mice was performed to induce AD-like symptoms and skin lesions: 250mg/kg AA was given orally for three weeks to assess its anti-pruritic effects. Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6, IL-10, immunoglobulin (Ig)E and caspase-3 were assessed by ELISA.** ***Results:*** **We found that AA alleviated DNCB-induced AD-like symptoms as quantified by skin lesions, dermatitis score, ear thickness and scratching behavior. Levels of reactive oxygen species in the AA group were significantly inhibited compared with those in the DNCB group. In parallel, AA blocked a DNCB-induced reduction in serum levels of IL-4 and IL-10 associated with an attenuation of DNCB-induced increases in serum TNF-α, IL-6, IgE and caspase-3.** ***Conclusions:*** **The results indicate that AA suppresses DNCB-induced AD in mice via redox balance and immune modulation, and could be a safe clinical treatment for AD.**

Key words: caspase-3, IgE, IL-4/6/10, tumor necrosis factor (TNF)-**α**

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is increasingly common in infants and children [1]. The incidence of AD has increased dramatically, especially in industrialized countries, affecting approximately 3% to 5% of the adult population in the western world, and 30% of the worldwide pediatric population [2]. It is characterized by dryness, hyperkeratosis and fissures due to elevated serum immunoglobulin E (IgE) levels, leading to defective skin barriers and infiltration of inflammatory cells, such as lymphocytes, macrophages, eosinophils, and mast cells [3]. For many years, therapeutic strategies for AD have been dominated by local or systemic corticosteroids [4] However, they have many undesirable effects [5]. The search for new agents active against dermatitis is thus of great interest: naturally occurring products provide an important source of a wide range of potential new agents.

The plant *Terminalia arjuna* (Combretaceae) has been studied primarily for its medicinal uses. One of its constituents, present in the bark, is arjunolic acid, which is a natural, pentacyclic triterpenoid saponin that has potent antioxidant activity [6] Arjunolic acid has been shown to possess protective activities in many organ pathophysiologies such as sodium nitrite-induced cardiac toxicity [7], type 1 diabetes-induced vascular inflammation [8], and cisplatin-induced testicular toxicity [9]

Although, arjunolic acid is known to have protective effects against several inflammatory diseases, no previous work has studied its effect in AD. We used a 1-chloro-2,4-dinitrobenzene (DNCB) model of AD in mice to investigate the effects of arjunolic acid in AD, as well as its effect on oxidative stress, cytokine balance and caspase-3.

METHODS & MATERIALS

Animals and treatment outlines

The animal protocol was approved by the local ethics committee. Four-week-old BALB/c mice were housed under specific pathogen-free conditions at 22 ± 2°C with a 12-h light-dark cycle. AD-like skin lesions were induced in mice using DNCB. Briefly, the dorsal hair was removed. After 24 hours, 100 μL of 1% DNCB solution (acetone:olive oil, 3:1) was applied to the back skin, and 10 μL were applied to each of the face and the back of both ears (day4) for sensitization. Five days after dorsal hair removal, 0.2% DNCB was applied to challenge the dorsal skin (150 μl), face, and the back of both ears (10 μL each), three times a week for three weeks. Mice were allocated to the following groups each of which comprised 10 mice:

– control group : mice received the standard diet, and received no treatment.

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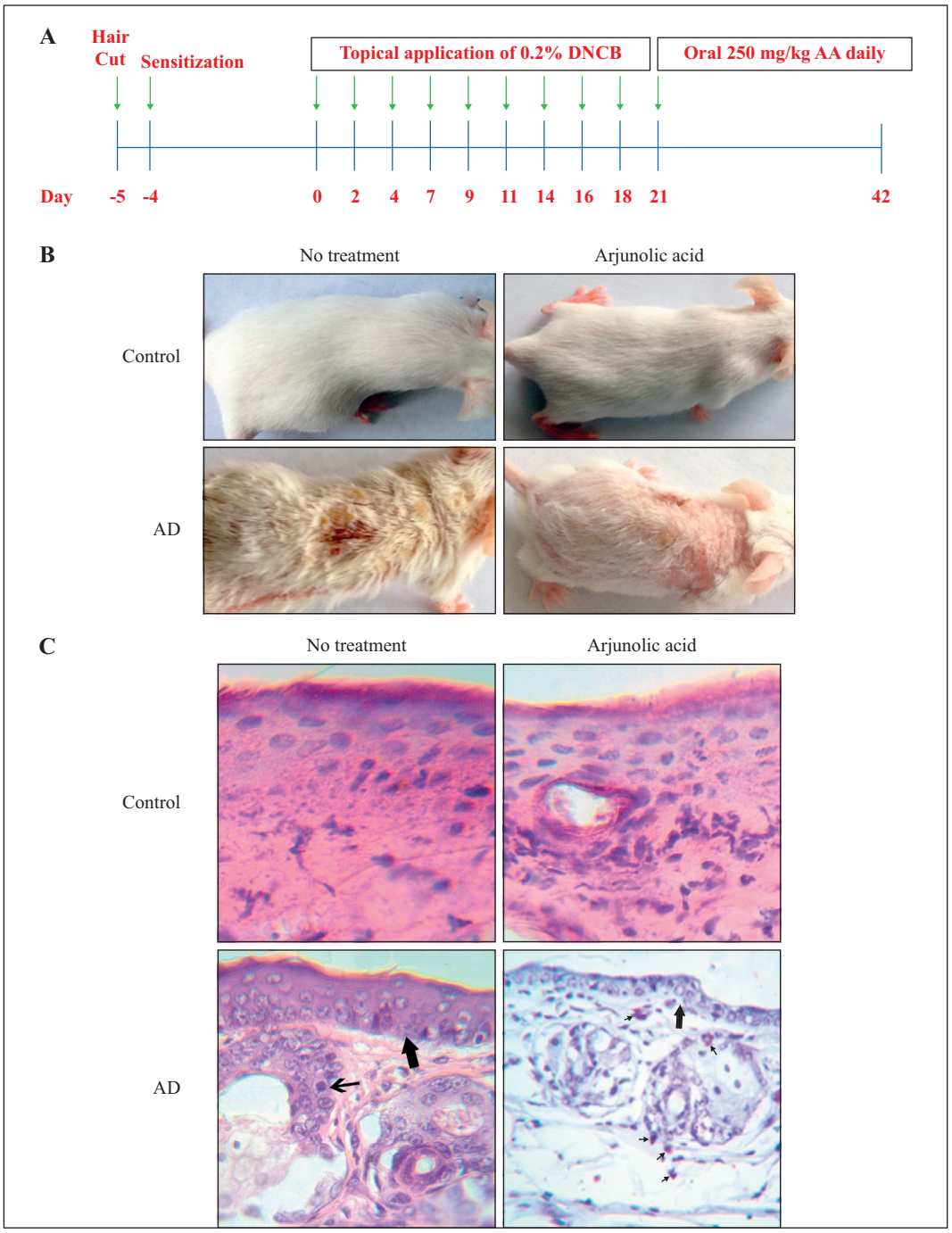

Figure 1

Effect of arjunolic acid on DNCB-induced atopic dermatitis (AD) in mice. **(A)** Experimental design. **(B)** Images of skin lesions from the different groups of mice were taken on the last day of the experiment. **(C)** Paraffin-embedded sections of mice skin stained with H&E stain. Histological examination showed that there was a pronounced acanthosis of the epidermis (broad arrows) and infiltration by mast cells (thin arrows) in the AD group. A variable decrease was noted in the group treated with arjunolic acid.

– arjunolic acid-treated control group (250 mg/kg): mice were supplemented with 250mg/kg arjunolic acid (Hangzhou Dayangchem, Zhejiang, China), dissolved in phosphate-buffered saline (pH 7.4), given by oral gavage, daily for three weeks.

– DNCB-induced AD group: mice were treated with DNCB as described above.

– arjunolic acid-treated group: mice were treated with DNCB for three weeks, followed by oral administration of 250 mg/kg of arjunolic acid for another three weeks.

The experiment design is summarized in *figure 1A*.

Measurement of scratching frequency

Mice were placed into cages and the number of scratching frequency was counted over a 10-minute period. This measurement was repeated five times during the last two days.

Skin lesions, dermatitis score and ear thickness

To compare any improvements of the condition of the skin following treatment with arjunolic acid, mice were anesthetized and photographs were taken on the last day of the study, just before sacrifice. The dermatitis score was measured once a week. Scores of 0 (none), 1 (mild), 2

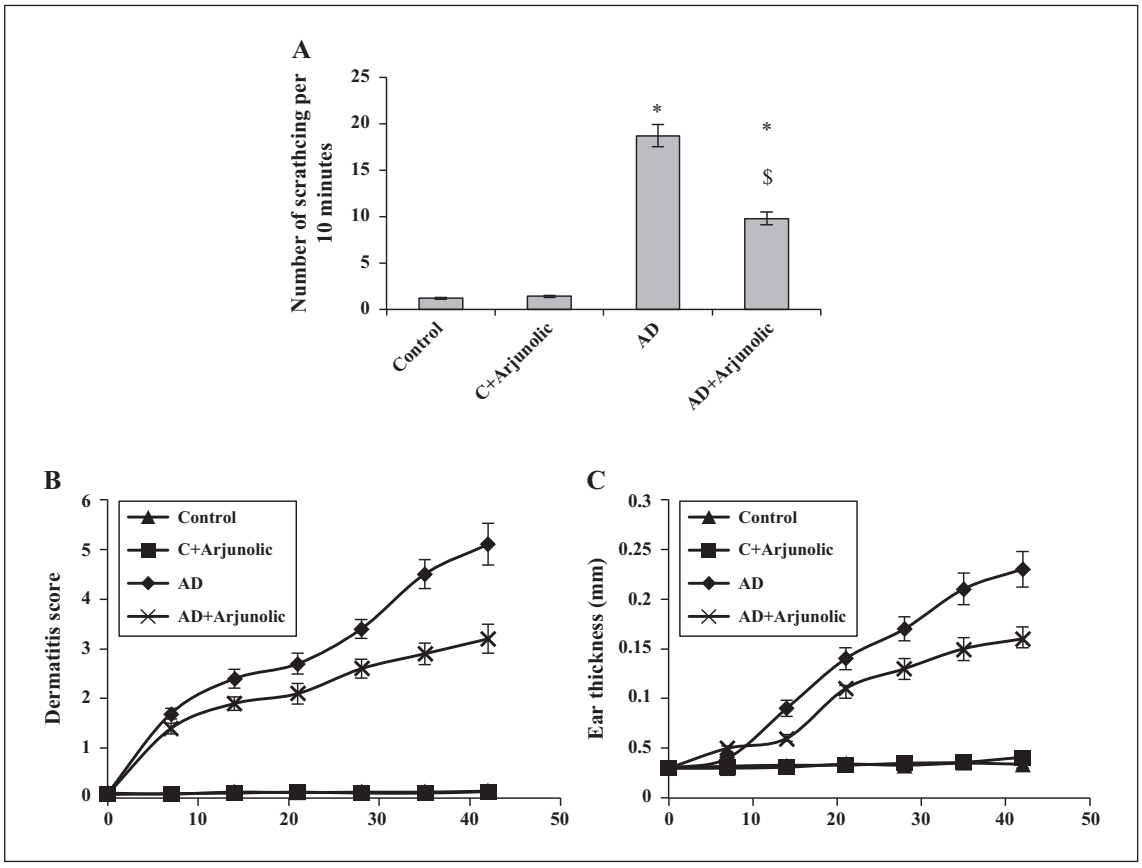

Figure 2
Effect of atopic dermatitis (AD) alone and in combination with arjunolic acid (250 mg/kg/day) on scratching frequency per 10 minutes **(A)**, dermatitis scores **(B)** and ear thickness **(C)**. *: significant difference compared with the control groups at $p<0.05$. #: significant difference compared with the AD group at $p<0.05$.

(moderate), and 3 (severe) were given for each of the four symptoms: (i) erythema/hemorrhage, (ii) edema, (iii) excoriation/erosion, and (iv) scaling/dryness. A total dermatitis score, indicating clinical severity, was defined as the sum of all scores (maximum score: 15). Mouse ear thickness was also measured and recorded once a week using a caliper.

Animal sacrifice and collection of samples

The animals were sacrificed by decapitation. Trunk blood was collected and centrifuged at 3000 rpm for five minutes. Serum samples were separated and stored at -80°C.

Morphological analysis of skin tissue

The skin samples were cut, fixed in 10% buffered formalin and embedded in paraffin. Five micrometer thickness sections were cut and stained with Mayer's hematoxylin and eosin (H&E) for examination using a Nikon Digital Camera (Japan).

Assessment of oxidative stress

Serum malondialdhyde (MDA) concentrations were measured as described previously [10, 11]. Briefly, after precipitation of the proteins by trichloroacetic acid, thiobarbituric acid was reacted with MDA to form thiobarbituric acid-reactive substances, which were measured at 532 nm.

Blood reduced glutathione (GSH) concentrations were measured as described previously [12] This assay depends on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by glutathione, which forms a yellow anion that can be measured at 412 nm.

ELISA

The serum levels of the biochemical parameters were measured by ELISA using commercially available tumor necrosis factor (TNF)-α, interleukin (IL)-6 and caspase-3 ELISA kits (eBioscience Inc., San Diego, CA, USA).

Estimation of IgE concentration

IgE was determined on Vidas instruments (bioMérieux, Inc., Durham, NC, USA).

Statistical analysis

For descriptive statistics of quantitative variables, the mean ± standard error was used. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov–Smirnov (K–S) test. One-way analysis of variance (ANOVA) was used to compare the means between groups. Once differences between the means were found, *post hoc* Bonferroni correction tests were calculated. Statistical computations were performed using Microsoft Excel 2007. Statistical significance was predefined as $P\leq0.05$.

Table 1
Differential WBC counts for different groups studied (mean ± SE):

	Control	Control + arjunolic acid	Atopic dermatitis	Atopic dermatitis + arjunolic acid
WBCs	5.36 ± 0.48	5.14 ± 0.42	13.1 ± 0.29*	9.74 ± 0.63*#
Neutrophils	1.46 ± 0.11	1.42 ± 0.12	4.29 ± 0.32*	1.93 ± 0.12#
Lymphocytes	3.46 ± 0.39	3.49 ± 0.31	5.47 ± 0.69*	6.27 ± 0.43*
Monocytes	0.36 ± 0.03	0.37 ± 0.03	1.56 ± 0.48*	0.81 ± 0.05*#
Eosinophils	0.063 ± 0.019	0.059 ± 0.027	0.83 ± 0.12*	0.445 ± 0.015*#
Basophils	0.013 ± 0.003	0.013 ± 0.002	0.97 ± 0.075*	0.3 ± 0.03*#

*: significant difference compared with the control groups at $p<0.05$.
#: significant difference compared with atopic dermatitis group at $p<0.05$.

RESULTS

Arjunolic acid attenuated DNCB-induced AD-like symptoms

On last day, the ear and dorsal skin of the AD mice showed severe erythema, erosion and dryness that had been ameliorated by treatment with arjunolic acid (*figure 1B*). In addition, DNCB treatment resulted in significant increase in the number of scratches (18.7 ± 1.2 scratches/10min) as compared with the control groups (*figure 2A*). The dermatitis score was significantly higher in DNCB-treated mice (5.1 ± 0.42) compared with control animals (0.4 ± 0.03) (*figure 2B*). Ear thickness had gradually increased in AD mice (0.23 ± 0.018 mm) compared with control mice (0.034 ± 0.004 mm) (*figure 2C*). In parallel, microscopic examination of skin sections stained with H/E showed pronounced acanthosis of the epidermis and infiltration by mast cells in the AD group (1c). However, treatment with arjunolic acid markedly attenuated the DNCB-induced AD-like symptoms in the DNCB group, without affecting the control group.

Effect of arjunolic acid on differential white blood cell (WBC) count

As shown in *table 1*, AD resulted in a significant increase in total and differential WBC counts as compared with the control group. The arjunolic acid-treated group showed significant decreases in all WBC counts as compared with the AD group, without affecting the control group.

Arjunolic acid blocked AD-induced enhancement in oxidative stress

AD caused significant increases in the serum levels of MDA (9.13 ± 0.11 nmol/mL), as well as significant decreases in the reduced glutathione concentrations (0.13 ± 0.002 mmol/L) compared with the control group (3.98 ± 0.07nmol/mL and 0.45 ± 0.028 mmol/L, respectively). Arjunolic acid-treated mice showed a significant reduction in MDA levels (5.18 ± 0.12 nmol/mL) and significant increases in concentrations of reduced glutathione (0.24 ± .029 mmol/L) compared with the AD group, without affecting the control group (3.84 ± 0.09 nmol/mL and 0.46 ± 0.031 mmol/L, respectively) (*figure 3*).

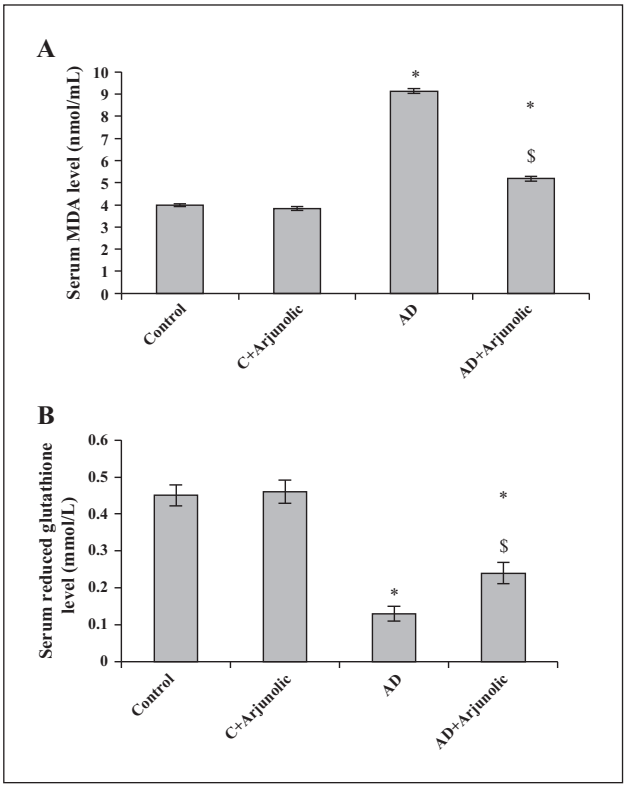

Figure 3
Effect of atopic dermatitis (AD) alone and in combination with arjunolic acid (250 mg/kg/day) on serum levels of malondialdehyde (MDA, **A**) and reduced glutathione **(B)**. *: significant difference compared with the control groups at $p<0.05$. #: significant difference compared with the AD group at $p<0.05$.

Arjunolic acid inhibited AD-induced increases in IgE

As shown in *figure 4A*, AD significantly increased the serum level of IgE (36.1 ± 0.55 U/mL) as compared with the control mice (13.63 ± 1.2 U/mL). Treatment of mice with arjunolic acid significantly reduced the elevated serum IgE in the AD group (25.8 ± 0.81 U/mL), without affecting the control mice (13.98 ± 1.14 U/mL).

Arjunolic acid reversed the AD-induced increase in serum pro-inflammatory cytokines

As regards proinflammatory cytokines, as demonstrated in *figures 4B, C*, we found a significant increase in serum concentrations of TNF-α (262.82 ± 20.57 pg/mL) and IL-

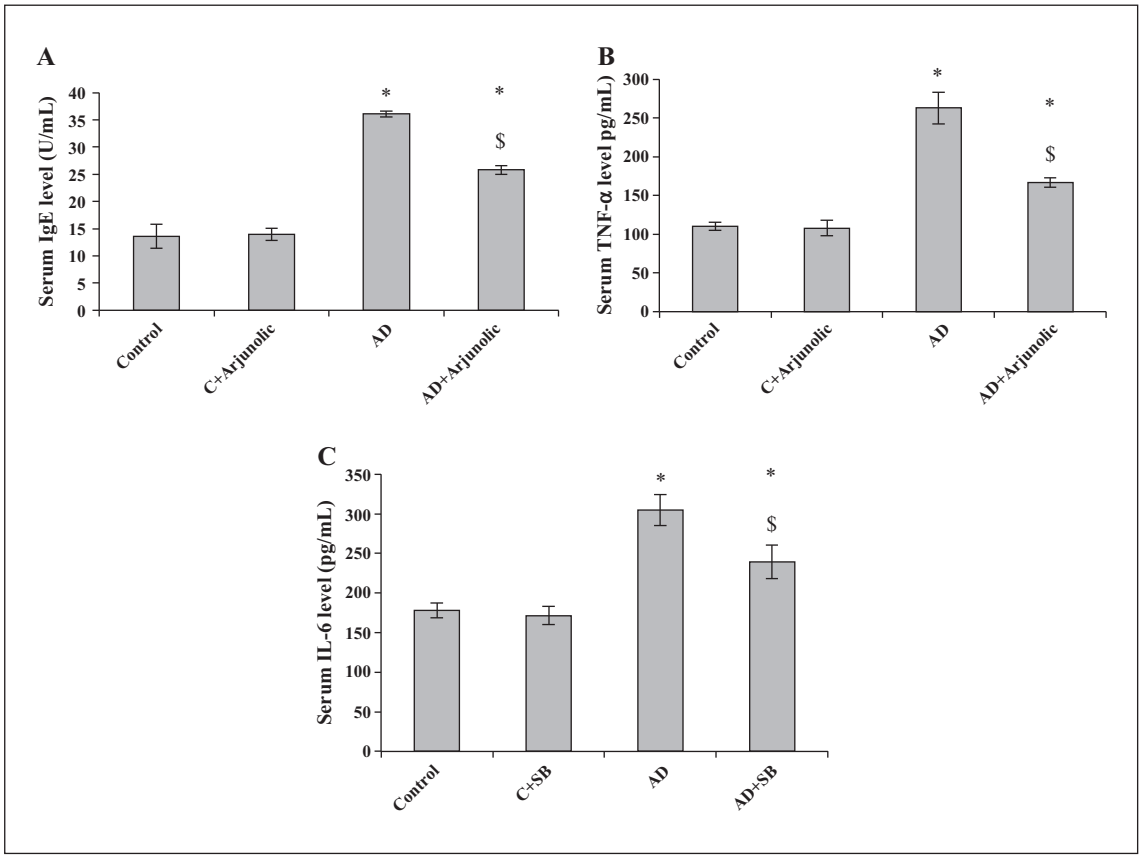

Figure 4

Effect of atopic dermatitis (AD) alone and in combination with arjunolic acid (250 mg/kg/day) on serum levels of immunoglobulin (Ig)E **(A)**, tumor necrosis factor (TNF)-α **(B)** and interleukin (IL)-6 **(C)** in mice. *: significant difference compared with the control groups at $p<0.05$. #: significant difference compared with the AD group at $p<0.05$.

6 (304.7 ± 19.9 pg/mL) in the AD group compared with the control group (110.26 ± 5.52 and 178.2 ± 9.5 pg/mL, respectively). The treatment of AD mice with arjunolic acid resulted in significant reductions in serum TNF-α (166.92 ± 6.27 pg/mL) and IL-6 (239.4 ± 21.2 pg/mL), compared with the AD group, although levels were still significantly higher than in the control group: arjunolic acid did not affect the control group (107.7 ± 9.98 and 171.4 ± 11.4 pg/mL, respectively).

Arjunolic acid reversed the AD-induced increase in serum anti-inflammatory cytokines

We measured the effect of both AD and arjunolic acid on the serum levels of anti-inflammatory ctyokines. We found a significant reduction in serum concentrations of IL-4 (76.8 ± 5.4 pg/mL) and IL-10 (59.59 ± 2.38 pg/mL) in the AD group compared with the control group (149.7 ± 11.8 and 292.83 ± 6.46 pg/mL, respectively). The treatment of AD mice with arjunolic acid resulted in significant increases in serum IL-4 (116.8 ± 9.5 pg/mL) and IL-10 (151.52 ± 8.4 pg/mL) compared with the AD group, and it did not affect the control group (152.4 ± 8.7 and 301.1 ± 10.5 pg/mL, respectively). However, the serum levels of anti-inflammatory cytokines in AD mice treated with arjunolic acid were still significantly lower than those in the control group (*figure 5*).

Arjunolic acid inhibited AD-induced activation of the apoptotic pathway

Next, we investigated the effect of both AD and arjunolic acid on serum caspase-3 (*figure 6*). AD caused significant increases in serum levels of cleaved caspase-3 (12.57 ± 0.44 U/mL) when compared with the control group (8.05 ± 0.53 U/mL). Treatment with arjunolic acid resulted in a significant reduction in the serum activity of caspase-3 (10.41 ± 0.21U/mL), but it did not affect the control group (7.97 ± 0.48 U/mL).

DISCUSSION

A defective skin barrier that allows increased allergen and pathogen penetration characterizes AD, an important, common, chronic or relapsing inflammatory disease of the skin that often precedes asthma and allergic disorders [13] Application of DNCB onto mice skin results in AD-like symptoms that include significant increases in ear thickness, dermatitis score and scratching frequency, when compared with the control group. DNCB and other haptens provide a useful model for epicutaneous allergen exposure through intact or disturbed skin.

A major problem with AD is that it is a common inflammatory skin disorder and the agents used as treatment such as steroids and antihistaminic drugs can have severe side effects, which reduce the length of time they can be used, and limit their clinical application. Therefore, a

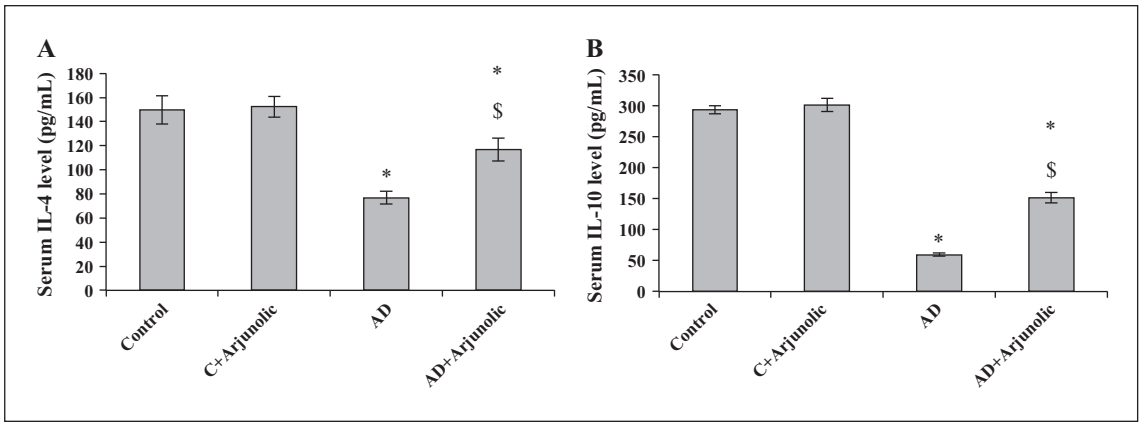

Figure 5
Effect of atopic dermatitis (AD) alone and in combination with arjunolic acid (250 mg/kg/day) on serum levels of interleukin (IL)-4 **(A)** and IL-10 **(B)** in mice. *: significant difference compared with the control groups at $p<0.05$. #: significant difference compared with the AD group at $p<0.05$.

good deal of research is being done to find new treatments to reduce the inflammatory response. Various experimental and clinical investigations have reported that natural immune modulators from herbal extracts or derivatives may have therapeutic effects in AD [1]. In this study, we used arjunolic acid, which has been shown to have an anti-inflammatory action in different body organs [7, 8, 14]. In addition, treatment of AD mice with arjunolic acid has been shown to significantly alleviate DNCB-induced increases in ear thickness, dermatitis score and scratching frequency. Although, arjunolic acid has been reported to affecting arachidonic acid metabolism through inhibition of cyclooxygenase [15], this is the first study that illustrates the protective role of arjunolic acid in DNCB-induced AD.

Free radicals mediate lipid peroxidation, which is considered to be the main mechanism of cell membrane destruction [16]. Such lipid hydroperoxides decompose under physiological conditions to generate highly cytotoxic aldehydes, in particular MDA. However, we have found significant increases in MDA and significant decreases in reduced glutathione levels in AD mice indicating that AD is an oxidative stress-mediated disease. Treatment with the antioxidant, arjunolic acid, significantly reduces AD-induced oxidative stress that is associated with inhibition of peak AD symptom severity. The antioxidant effects of arjunolic acid are thought to be mainly due to its ability to scavenge oxygen-derived free radicals and to chelate metal ions [15].

Two hypotheses concerning the causes AD have been proposed; the first hypothesis suggests an immunological disturbance that leads to IgE-mediated sensitization, with epithelial barrier dysfunction [16]. The second implicates an intrinsic defect in the epithelial cells leading to barrier dysfunction [17]. The immunological features are considered to be an epiphenomenon. IgE expression is known to cause both acute and chronic phase skin inflammation. In addition, clinical observations suggest a correlation between serum IgE and the extent of disease [18]. Patients suffering from AD are known to exhibit elevated serum levels of total IgE [19]. In addition, TNF-α is essential mediator of AD [20, 21]. Therefore, the upregulation of total serum IgE and TNF-α levels are hallmarks of AD [22]. In the present study, the concentrations of serum IgE and TNF-α fell following treatment with arjunolic acid as compared to the DNCB group. Arjunolic acid has been reported

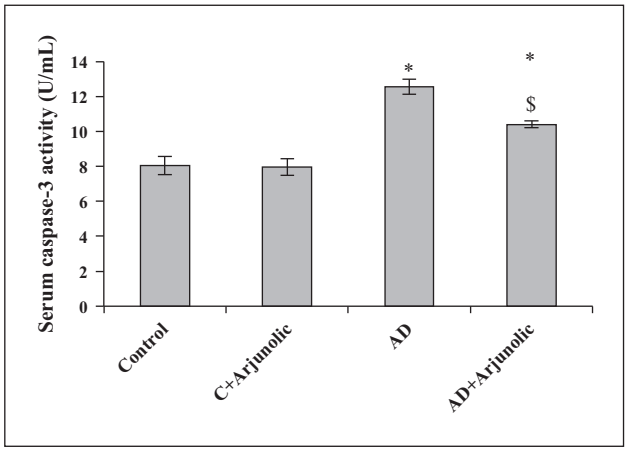

Figure 6
Effect of atopic dermatitis (AD) alone and in combination with arjunolic acid (250 mg/kg/day) on serum levels of caspase-3 in mice. *: significant difference compared with the control groups at $p<0.05$. #: significant difference compared with the AD group at $p<0.05$.

to affect arachidonic acid metabolism through inhibition of cyclooxygenase [15]. We found that arjunolic acid significantly reversed the DNCB-induced reduction of serum levels of anti-inflammatory cytokines, IL-4 and IL-10, in mice.

Activation of the MAPK signaling pathway ultimately results in direct or indirect phosphorylation and/or activation of NF-κB, as well as alterations in gene expression [23]. We found that treatment with arjunolic acid significantly reduced cleaved caspase-3 levels in the DNCB group. Arjunolic acid has been reported to block both extrinsic and intrinsic cell death in sodium nitrite-induced cardiac damage [7], cadmium-induced hepatic damage [24] and acetaminophen-induced renal injury [25].

CONCLUSION

We have shown for the first time that arjunolic acid can ameliorate DNCB-induced AD-like symptoms via multiple mechanisms, such as: (1) reducing AD-induced oxidative stress; (2) blocking AD-induced expression of IgE; (3) attenuating AD-induced increases in serum pro-inflammatory cytokines such as TNF-α and IL-6; (4) attenuating AD-induced reductions in serum anti-

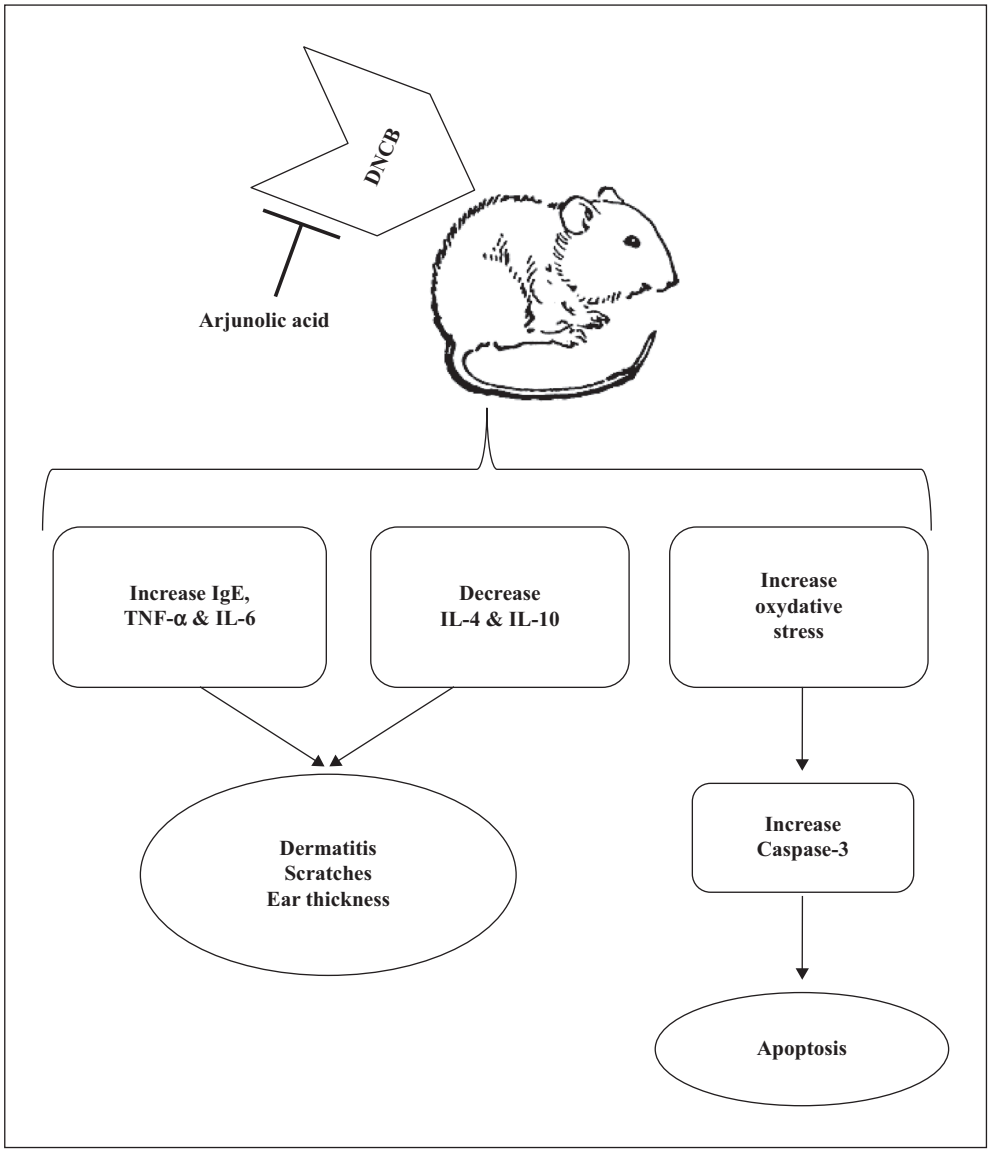

Figure 7
Schematic representation of the mechanism of the protective action of arjunolic acid against 2,4-dinitrochlorobenzene-induced atopic dermatitis-like symptoms.

inflammatory cytokines such as IL-4 and IL-10; and (5) blocking AD-induced expression of the apoptotic marker caspase-3. The mechanisms of action of arjunolic acid in DNCB-induced AD-like symptoms are summarized in *figure 7*. Clinical trials of arjunolic acid in human patients will be needed. We believe that our experimental results can be readily translated to clinical use, as arjunolic acid is a natural food supplement and could be easily added to patient protocols.

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