Effect of Aescin in Psoriatic-Induced Animal Model: Immunohistochemical and Pathological Study

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Abstract:

Background: Aescin is a mixture of the triterpene saponins extracted from the seeds of the horse chestnut tree Aesculus hippocastanum. Aescin has a venotonic, anti-inflammatory, antioxidant and anti-edematous characteristics that are mostly connected to the agent molecular mechanism.

Objective: The present study aims to investigate the potential effects of Aescin on psoriasis induced by Imiquimod in male rats, ncluding its effect on the level of tumor necrosis factor alpha, Ki-67 and the histopathologic features of the psoriatic skin.

Methods: Thirty-six albino male rats were divided into six groups each group containing 6 animals, psoriasis was induced by Imiquimod to five of the groups, while for the last group vasaline was applied and the group served as a control group. The animals were then treated with topical Aescin, topical clobetasol, combination of topical Aescin and clobetasol and oral Aescin, finally all animals were sacrificed and the dorsal back skin was taken to perform histopathological and immunohistochemical analysis.

Results: regarding the level of Ki-67, Strong expression of Ki-67 was seen in the group who received Imiquimod only, where the scoring of Ki-67 was notably lowered among the other groups. However, the lowest expression was noticed in the group that were treated with the combination of topical Aescin and clobetasol. While the number of TNF-α positive cells and the intensity of immunostaining were higher in the induction group who received Imiquimod only and the lowest among the group who received the combination of topical Aescin and Clobetasol. Lastly the histopathologic analysis shows that the histopathologic features of psoriasis was markedly affected by the anti-inflammatory effect of Aescin and clobetasol, which was noticed through inhibition of proinflammatory markers, and the decrease in capillary permeability.

Conclusion: Topical Aescin alone or in combination with clobetasol reduced Ki-67 expression successfully; furthermore, the combination of topical Aescin and Clobetasol decreased TNF- score and had the strongest anti-inflammatory activity more than the other groups. Lastly Aescin was able to alter the histopathologic features of the psoriatic skin through its anti-inflammatory, venotonic and anti-edematous activity.

Keywords: Psoriasis, Imiquimod, Aescin, Clobetasol, Ki-67, TNF-α.

تأثير الأياسين على الصدفية المستحثة في نموذج حيوان: دراسة كيميائية مناعية ومرضية

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الخلاصة:

الخلفية: Aescin هو خليط من صابونين ترايتيربين المستخرج من بذور شجرة كستناء الحصان Aesculus hippocastanum. يحتوي Aescin على خصائص مقوية للأوردة ومضادة للالتهابات ومضادة للأكسدة ومضادة للوذمات ترتبط في الغالب بالآلية الجزيئية للعامل.

الهدف: تهدف الدراسة الحالية إلى دراسة التأثيرات المحتملة لـ Aescin على الصدفية التي يسببها Imiquimod في ذكور الجرذان، بما في ذلك تأثيره على مستوى عامل نخر الورم ألفا (TNF- α)، Ki-67 والسمات النسيجية للجلد الصدفي.
الطريقة: تم تقسيم ستة وثلاثين ذكور جرذان ألبينو إلى ست مجموعات تحتوي كل مجموعة على ٦ حيوانات، تم تحفيز الصدفية بواسطة Imiquimod إلى خمس مجموعات، بينما تم تطبيق vasaline للمجموعة الأخيرة وعملت المجموعة كمجموعة ضابطة. عولجت الحيوانات بعد ذلك باستخدام الأيسين الموضعي، والكلوبيتاسول الموضعي، ومزيج من الأيسين الموضعي وكلوبيتاسول والإيسين الفموي، وأخيراً تم التضحية بجميع الحيوانات وأخذ الجلد الخلفي الظهري لإجراء التحليل التشريحي المرضي والكيميائي المناعي.

النتائج: فيما يتعلق بمستوى Ki-67، شوهد تعبير قوي عن Ki-67 في المجموعة التي تلقت Imiquimod فقط ، حيث تم تخفيض درجة Ki-67 بشكل ملحوظ بين المجموعات الأخرى. ومع ذلك، لوحظ أقل تعبير في المجموعة التي عولجت بمزيج من Aescin الموضعي وكلوبيتاسول. بينما كان عدد الخلايا الإيجابية لـ TNF-α وشدة التلوين المناعي أعلى في المجموعة الحثية التي تلقت Imiquimod فقط والأقل بين المجموعة التي تلقت مزيج Aescin الموضعي وكلوبيتاسول. أخيرًا، أظهر التحليل التشريحي المرضي أن السمات النسيجية لمرض الصدفية قد تأثرت بشكل ملحوظ بالتأثير المضاد للالتهابات لـ Aescin وclobetasol، والذي لوحظ من خلال تثبيط العلامات الالتهابية، وانخفاض نفاذية الشعيرات الدموية.

الخلاصة: أيسين الموضعي بمفرده أو بالاشتراك مع كلوبيتاسول قلل من تعبير Ki-67 بنجاح؛ علاوة على ذلك، أدى الجمع بين Aescin وClobetasol الموضعي إلى خفض درجة TNF وكان له أقوى نشاط مضاد للالتهابات أكثر من المجموعات الأخرى. أخيرًا، كان إيسكين قادرًا على تغيير السمات التشريحية المرضية للجلد الصدفي من خلال نشاطه المضاد للالتهابات، والمضاد للأوردة، والمضاد للتورم.

الكلمات المفتاحية: الصدفية، Imiquimod، Aescin، Clobetasol، Ki-67، TNF-α.

Introduction:

Psoriasis is a typical chronic inflammatory condition causes red, scaly plaques on the skin, which can appear anywhere on the body but are most usually found on the scalp, lower back, elbows, and knees [(1)]. Psoriasis is not contiguous, and can occur at any age but it most frequently affects individuals between the ages of 15 and 30 [(2)]. Due to the disfiguring and incapacitating symptoms of psoriasis, those who are affected must deal with major social and psychological problems [(3)]. Activation of keratinocytes and immune cells causes the excessive proliferation of keratinocytes in this immune-mediated disease, which has both inherited and environmental roots [(4)]. Psoriasis was once believed to be a skin disorder that only impacted the skin, but it is now widely acknowledged that it is an inflammatory disease that affects the entire body [(5)]. The disorder frequently co-occurs with various comorbidities such diabetes mellitus, metabolic syndrome, cardiac problems, psoriatic arthritis, and others. Chronic renal and inflammatory bowel

disorders are more common in those with psoriasis [6]. It is characterized by an accelerated TNF- (tumor necrosis factor)/IL-23/IL-17 axis [7]. The three primary histologic features of psoriasis are epidermal hyperplasia, dilated, dilated blood vessels in the dermis, and an inflammatory infiltrate of leucocytes, predominantly into the dermis [8]. Aescin is a mixture of the triterpene saponins extracted from the seeds of the horse chestnut tree Aesculus hippocastanum [9]. Beta Aescin is the active ingredient of the mixture and is the chemical form present in most of the commercially available pharmaceutical drugs [10], Because of its wide range of pharmacological activity, Aescin has been the subject of many research over the past ten years [9]. It used to treat chronic venous insufficiency (CVI), edema, hemorrhoids, varicose veins, hematoma, venous congestion, post-thrombotic syndrome, and arthritis due to its potent anti-inflammatory, venotonic, and anti-odematous effects [11]. Treatment with aescin enhances venous tone and produces a "sealing effect" at the site of injury by raising calcium ion sensitivity, decreasing the permeability of small veins, and boosting venous contractile activity [12]. The anti-inflammatory effects of aescin have been demonstrated in animal models where aescin act by reducing leukocyte activity and adhesiveness and in result it reduces the production of inflammatory mediators [13]. In this study imiquimod which is a non-nucleoside heterocyclic amine that is a ligand for Toll like receptor7 and 8 [14] was used to induce psoriasis, it acts by boosting both the innate and adaptive immune systems [15]. Imiquimod rises IL-23, IL-17A, and IL-17F expression in the epidermis. The IL- 23/IL-17 axis plays a crucial role in the development of IMQ-induced dermatitis that is similar to psoriasis [16]. This study aims to investigate the potential effects of aescin on psoriasis induced by imiquimod in male rats.

Materials and Methods:

Chemicals

Aescin powder was purchased from arkure health care, India. Imiquimod cream was purchased from MEDA, Sweden. Clobetasol ointment was purchased from GSK, UK and Aescin gel was prepared at the Department of Pharmaceutical Chemistry, College of Pharmacy, Mustansiriyah University from Aescin powder, carbopol940, triehanolamine and propylene glycol. Carbopol940 was purchased from ASESCHEM, India. Triethanolamine was purchased from Charco, India and propylene glycol was purchased from Brouwland, Belgium. Ki-67 immunohistochemistry kit was purchased from cell signaling technology, USA and TNF-α immunohistochemistry kit was purchased from Ray Biotech, USA.

For the preparation of Aescin gel, the polymer (carbopol 940) had been gently dissolved into sufficient amount of water with constant stirring using a magnetic stirrer. Aescin powder, propylene glycol, and triethanolamine were added respectively to the gel. The weight of the gel was then brought to 120g by gently adding purified water, as shown in table 1.

Table 1: compositions of Aescin gel formation 0.6% W/W

Ingredients	Amount (g)
Aescin powder	0.72
Carbopol 940	0.278
Propylene glycol	1.64
triethanolamine	0.09
Purified water to	120

Animal groups

Thirty-six male albino rats were obtained from the National Center for Drug Control and Research/Ministry of Health. They were between 12 and 16 weeks old, and weighed (150-200 gm). The animals were given free access to food and water at a temperature of 25°C ±5° during natural cycles of light and darkness.

Rats were randomly divided into six groups (six rats in each group) and treated as follows: Group-I (healthy control): received vehicle ointment (Vaseline) topically as the negative group for 10 days, then the same vehicle used to prepare aescin gel were administer for the next 14 days.

Group-II (Induction group): received 120mg of 5% W/W imiquimod cream topically for 10 days then Vaseline ointment for 14 days.

Group-III (clobetasol group): received 120mg of 5% W/W imiquimod cream topically for 10 days, then 0.42mg/g clobetasol propionate ointment 0.05% for the next 14 days.

Group-IV (Aescin gel group): received 120mg of 5% W/W imiquimod cream topically for 10 days, then Aescin gel 0.04g/kg for the next 14 days.

Group-V (combination group): received 120mg of 5% W/W imiquimod cream topically for 10 days, then Aescin gel 0.04g/kg and 0.42mg/g clobetasol propionate 0.05% ginen at the same time for the next 14 days. Group-VI (oral Aescin group): received 120mg of 5% W/W imiquimod cream topically for 10 days, then Aescin suspension 10mg/kg orally by oral gavage for the next 14 days.

Immunohistochemical Analysis

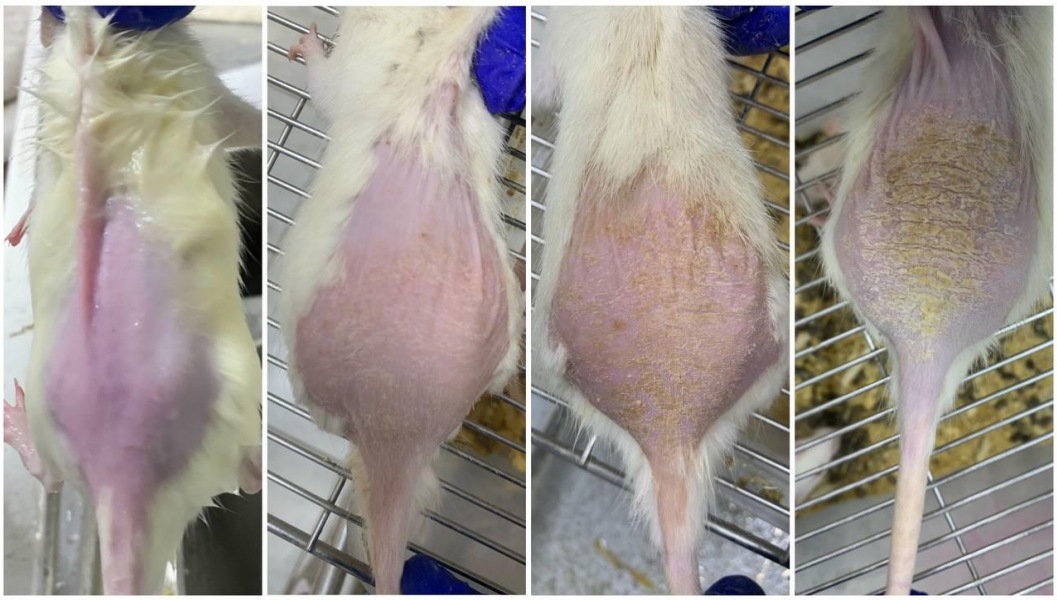
Immunohistochemical analysis was performed as following [17], [18]: Each paraffin block was divided into five-millimeter sections and then put on positively charged slides. All slides were placed in the PT-linker for automatic HIER (Heat-induced Epitope retrieval) with a preheated retrieval solution after which they were allowed to cool until the temperature was reduced to 65

°C. Each slide was prepared for automated immunostaining by being drained, plotted, and placed in its designated location in the Autostainer. Each slide had hydrogen peroxide applied to it, which was let to sit for 15 minutes before being washed twice with washing buffer. Ki-67 mAb was diluted at a concentration of 1: 200 while direct utilization of TNF- alpha was made. Each slide had EnVision FLEX/HRP added to it before being washed with washing buffer (Phosphate buffer saline (PBS) after 25 minutes of incubation. After waiting for ten minutes, DAB was added to the parts. Following two PBS washes on each slide, each tissue section received Mayer's Hematoxylin. All slides were then cleaned with distilled water, dehydrated with alcohol in descending concentrations, clarified with xylene, and mounted with DPX. The slides were reviewed, graded, and photographed. All pictures were taken using a single Light microscope and IHC

score was calculated by professional pathologist.

Histopathological Analysis:

The skin of sacrificed animals was surgically removed. The histopathologic analysis performed as following [19], [20]: In order to prevent autolysis, the tissue was fixed as soon as the skin was removed, and the samples were incubated in 10% formalin. Alcohol was employed to extract the water from the tissue. Before being embedded in paraffin, the dehydratant (alcohol) was removed with xylene then tissue was embedded in paraffin. The tissue was divided into 5-7 m slices using a rotary microtome, following which the sections were floated in a warm water bath. The sections were then picked up on microscopic slides, which were then heated for around 15 minutes to aid in the sections' adhesion to the slide. The paraffin wax was totally removed from the slides by dipping them in a xylene jar, which was followed by a series of descending alcohol concentrations (90%, 70%, 50%, and 30%), followed by a rinse with distilled water. After which hematoxylin stain was applied for 10 minutes. Following that, 1% Eosin stain was applied for 30 seconds. A cover slide was used to protect the slides and the slides were examined under a light microscope by an expert pathologist, the rats back skin are shown in figure 1.



Control	80mg	100mg	120mg

Figure 1: The dorsal backs of the rats after 10 days of Imiquimod application

Results:

Determination of Ki-67 expression

Different patterns, intensities, and percentages of positive nuclear Ki-67 expression were seen among the animal tissue samples. Group II shows high expression of Ki-67-stained DAB by IHC and counter-stained by Mayer's Hematoxylin in the dermal layer. While group I shows: Normal expression of Ki-67 counterstained with DAB in the basal layer of the epidermis (stratum basale) (Negative scoring), group III show immunostaining of low expression of Ki-67 limited to the epidermal layer, which is expressed in more than one row of cells, group IV show immunostaining of Ki-67 with DAB demonstrating intermediate expression (17%) after treatment with topical Aescin. Mostly only one row of the basal layer of stratum basale was expressing Ki-67, with less expression in the dermal layer, group V show positive expression limited to the basal layer of the stratum basale with the lowest percent (3%) among all groups, with few scattered cells in other layers of the stratum basale and no Ki-67 expression was seen in the dermal layer. and group VI show longitudinal section through rat skin with intermediate (23%) score of Ki-67 both in the epidermis (multiple layers) and dermis layers. Immunohistochemical staining with DAB and counterstained with Mayer's Hematoxylin in group VI show reduced proliferative activity of Ki-67 in stratum basale in the epidermis. As shown in figure (2).

Ki-67 score was calculated according to the following equation:

$$\text{Ki-67 percentage} = \frac{\text{Suprabasal Ki-67 positive cells}}{\textit{Total epidermal Ki-67 positive cells}} \times 100$$

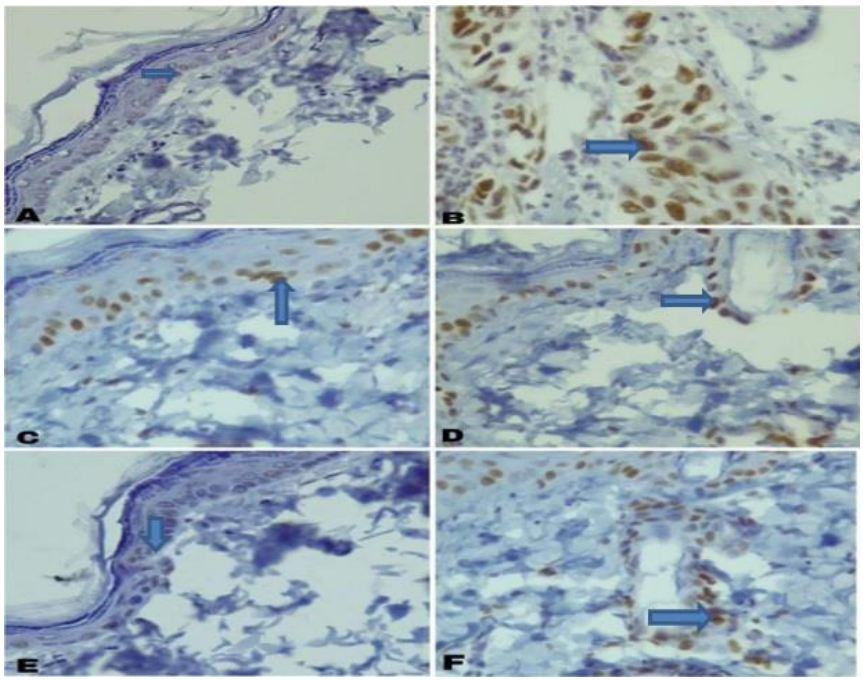


Figure 2: Expression of Ki-67 (pointed by blue arrows) in the six animal groups. A: Group I (control group), B: Group II (induction group), C: Group III (clobetasol group), D: Group IV (topical aescin group), E: Group V (combination of topical aescin and clobetasol group), F: Group VI (oral aescin group). X100

Table 2: The different patterns of immunoexpressions of Ki-67 (Ki-67 score).

Groups	Treatments	Ki-67 score
Group I	control group (Vaseline)	2%
Group II	Imiquimod	73%
Group III	Imiquimod + Clobetasol ointment	12%
Group IV	Imiquimod + Aescin gel	19%
Group V	Imiquimod + Aescin gel + clobetasol ointment	8%
Group VI	Imiquimod + oral Aescin	23%

Table 3: KI-67 mean values among the different animal groups.

Groups	KI-67 Mean ±SE
Control	1.75±0.23f
Induction	70.64±1.49a
Topical Aescin	18.38±0.67c
Topical clobetasol	12.55±0.44d
Combination	8.41±0.38e
Oral Aescin	23.95±0.93b
LSD	2.30

Means with a different letter are significantly different (P<0.05).

Determination of TNF-α expression

The amount of TNF-α positive cells and the degree of immunostaining were greater in group II (Immunostaining of TNF-α express score 5 of staining with score 3 of intensity total scoring 15), while they were lowest in the animals in group V (Almost normal expression was seen under low magnification). These cells were distributed differently in the other groups. In group I, negative expression of TNF-α with immunostaining with DAB. In group III expression of TNF-α was mostly in the epidermis layer, the expression was not only limited to the keratinocytes of stratum basale but also scattered positive expression in the dermal lymphocytes. While for the animals in group VI high score (4) of TNF-α immunostaining, but with grade 1 intensity was demonstrated, the expression was generally stronger at the base, with the tendency of decreasing in the upper layers. As shown in figure (3).

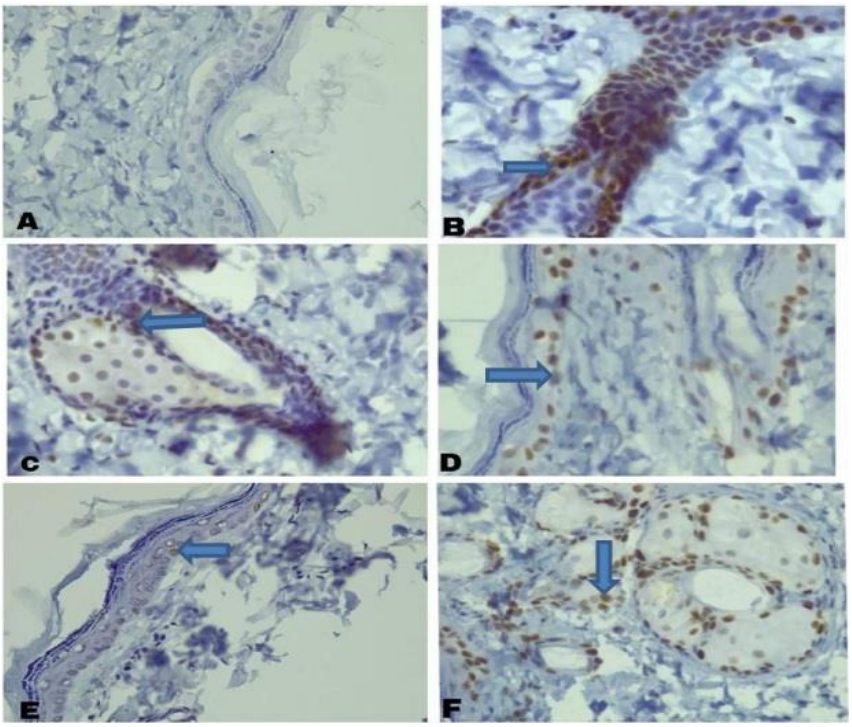


Figure 3: Expression of TNF-α (pointed by blue arrows) in the six animal groups (A: control group, B: induction group, C: clobetasol group, D: topical aescin group, E: combination of topical aescin and clobetasol group, F: oral aescin group). X100

Table 4: the different pattern of immunoexpression of TNF-α (TNF-α score).

Groups	Treatments	TNF-α
Group I	control group (Vaseline)	0
Group II	Imiquimod	15
Group III	Imiquimod + Clobetasol ointment	6
Group IV	Imiquimod + Aescin gel	6
Group V	Imiquimod + Aescin gel + clobetasol ointment	2
Group VI	Imiquimod + oral Aescin	8

Table 5: TNF-α mean values among different animal groups.

Groups	TNF Mean ±SE
Control	0.00±0.00b
Induction	14.50±1.22a
Topical Aescin	6.00±0.00b
Topical clobetasol	6.00±0.00b
Combination	1.67±0.51b
Oral Aescin	7.33±0.1.03a
LSD	2.30

Means with a different letter are significantly different (P<0.05)

Histopathology

Male rats in the group I displayed a typical histological skin picture. With appropriate levels of rete ridges, the stratum corneum seemed normal without excessive thickening as seen in (Figure 1). Sebaceous glands were also observed around the longitudinal striations of hair follicles. There were no indications of dilated blood vessels, inflammation, or proinflammatory cell infiltration in the connective tissue of the dermis layer. Group II show Deep rete ridges of the epidermis with deeply stained stratum corneum and epidermis due to infiltration of neutrophils also the first layer of the dermis layer demonstrates a high number of pro-inflammatory cells. Group III show No hyperkeratosis in the longitudinal section of rat's skin. The rete ridges were eliminated in response to the treatment with a corticosteroid. Scattered inflammatory cells are still noticed in the sub- epidermal and distal dermal layers. Longitudinal section in group IV show significant changes and limitation of keratinocyte proliferation compared with the induced psoriatic group. The thickness of the epidermis was almost normal, with a well-defined stratum Basale layer of cells. No elongated rete ridges, nor hyperkeratosis or acanthosis were observed. The hair follicles with their associated sebaceous glands were deeply oriented within the distal part of the dermis. Scattered inflammatory cells. The rat tissue in group V demonstrated distinct histological preservation for the skin's architecture, close to the normal picture. Although the papillary layer is normal and none of the animals in this group had hyperkeratosis, the stratum corneum layer was nonetheless thicker than normal skin. Few inflammatory cell infiltrates were visible in the tight connective tissue of the dermis layer, but there were more inflammatory cell aggregations around the hair follicles and hair bulbs. Group VI shows histological images with a slight improvement, but the epidermal layer thickening was still visible. The dilated blood vessels and the absence of a well-defined stratum basale are also characteristics noticed in this group. As shown in figure (4).

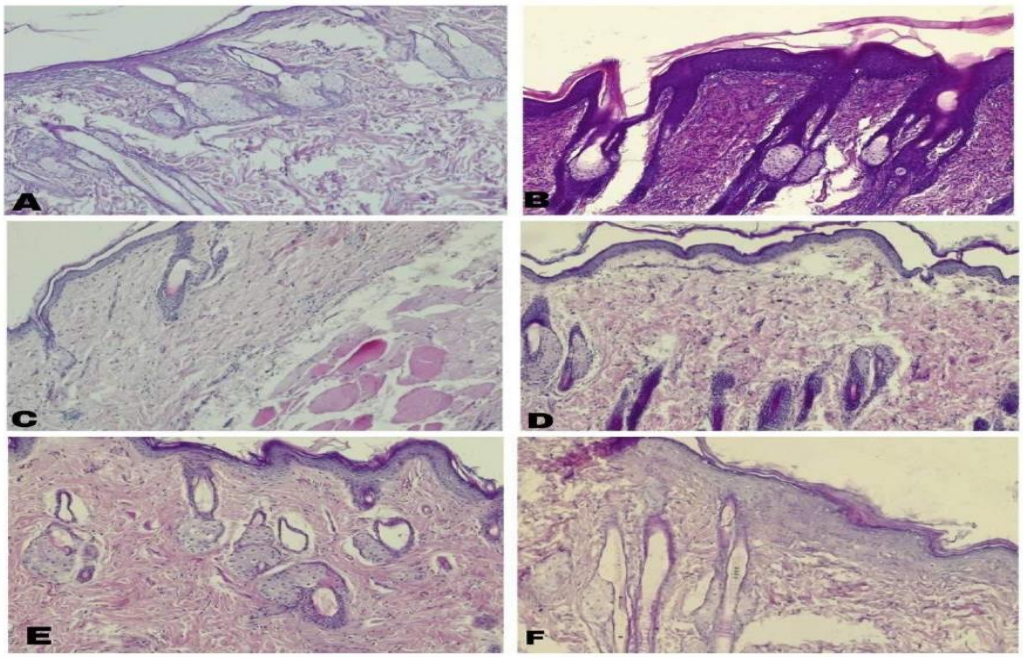


Figure 4: Histopathologic pictures of the different animal groups (A: control group, B: induction group, C: clobetasol group, D: topical aescin group, E: the combination of topical aescin and clobetasol group, F: oral aescin group). X40

Discussion:

Psoriasis is an immune mediated skin disease [21] that causes itchy, scaly patches, most commonly on the knees, elbows, trunk and scalp [22]. The present study aimed at investigating the effects of topical and oral use of aescin on psoriasis through its anti-inflammatory, antioxidant, anti-edematous and venotonic effect and compare these effects to the effects of clobetasol. In the present study imiquimod was selected to induce psoriasis, then upon administration of 0.04g/kg of aescin gel topically and 10mg/kg of oral aescin the Ki-67, TNF-α and many of the psoriatic histopathologic features were analyzed using immunohistochemical and histopathological studies and found to be decreased. Higher reduction was noticed with the use of topical aescin (Ki-67 score: 19%) (TNF-α score: 6), than with oral aescin (Ki-67 score: 23%) (TNF-α score: 8) and the highest reduction was seen with the use of a combination of aescin gel and clobetasol ointment (Ki-67 score: 8%) (TNF-α score: 2). Ki-67 is a non-histone nuclear protein that is detectable in the nuclei of all dividing cells of any tissues, not only in neoplastic cells but also in non-neoplastic lesions that are characterized by uncontrollable proliferation of cells [23]. A Recent study showed that the use of the index of Ki-67 antigen expression with immunohistochemical techniques is one of the most popular indicators of cell proliferation today [24]. In the current study, the level of ki-67 decreased after aescin therapy, suggesting that aescin may at least partially have an anti-proliferative impact, these results came in agreement with previous studies [25]. TNF-α is a potent inflammatory cytokine that plays important roles in the initiation and maintenance of inflammation by stimulating the production of other pro-inflammatory cytokines including IL-6 [26]. Additionally, it was noticed that aescin administration reduced the levels of TNF- in inflammatory tissues, and these finding was consistent with earlier studies showing that aescin affects immune cells and their functions through contracting their release at the vascular level [27]. In the present study aescin was able to change the histopathologic features of the psoriatic skin. The results of this *in vivo* experiment led us to the conclusion that aescin, both topically and orally, could reduce experimental cutaneous inflammation which is related to the suppression of inflammatory mediators. The current study showed that the use of a combination of topical aescin and steroid (clobetasol) significantly potentiate the anti-inflammatory effects of one another, Which is also consistence with previous studies that showed that steroid exert its anti-inflammatory effect by combining with glucocorticoid receptors and aescin in part can increase the expression of glucocorticoid receptors in the skin [28].

Conclusion:

the effects of aescin in controlling psoriasis was proven successfully in reducing its signs through its anti-inflammatory, venotonic and antioxidant properties as shown by decreasing the levels of Ki-67, TNF-α expression and through altering the histopathological features of the skin. The study showed higher beneficial effect of topical aescin when applied directly on the site of psoriasis lesion than oral aescin which was used systemically. The study also showed an additive effect between aescin and clobetasol in controlling psoriasis.

Conflicts of Interest:

no conflicts of interest to declare.

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