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Anti-Inflammatory Activity of *Gingko biloba* Extract in Cotton Pellet-Induced Granuloma in Rats: A comparative Study with Prednisolone and Dexamethasone

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Abstract

The current study was designed to evaluate the anti-inflammatory effect of GKB in the rat model of granulomatous inflammation. Thirty rats were distributed into five groups: The first group served as negative control group that received distilled water (DW) only without inducting inflammation, positive control group; treated with DW with the induction of inflammation and they were assigned to cotton pellet-induced granuloma, ginkgo biloba (GKB) treated group (200mg/kg/day), dexamethasone-treated group (1mg/kg), and Prednisolone treated group (5mg/kg). All the treatments were given orally for seven consecutive days. On day eight, the rats were anesthetized and the pellets together with granulation tissue were carefully removed and made free from extraneous tissue. The weight and the percent of the exudate and granuloma were determined and samples of the tissues were sent for histopathological examination. Blood samples were collected by cardiac puncture and used for the analysis of the inflammatory markers: TNF-alfa, IL10, VCAM-1, and hs-CRP. The study revealed a significant reduction in the weight and the percent of exudate (p-value = 0.019), (17%) and granuloma (p-value = 0.013), (20%) by GKB which was comparable to that produced by prednisolone. All the treatment groups showed a significant reduction in serum TNF-α, VCAM-1, and hs-CRP concentration compared to the positive control. The histopathological finding revealed pronounced improvement. In the current study, GKB was effective in attenuating the level of inflammation by decreasing the exudate, granuloma, and inflammatory markers. The underlying mechanisms could be the inhibitory effect on the expression of the inflammatory cytokines and endothelial adhesion molecule. These findings suggest GKB as a good contender to be tested in the treatment of inflammatory diseases.

Keywords: GKB extract, Dexamethasone, Prednisolone, Granuloma, Inflammatory markers.

التاثير المضاد للالتهابات لمستخلص الجينكوبيلوبا في نموذج الورم الحبيبي المستحدث في الجرذان: دراسة مقارنة مع كل من الدكساميثازون والبريدنيزولون

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الخلاصة

تم تصميم الدراسة الحالية لتقييم التأثير المضاد للالتهابات لـ GKB في نموذج الجرذان للالتهاب الحبيبي. تم تقسيم ثلاثين جرذاً إلى خمس مجموعات: مجموعة السيطرة السلبية: عولجت بالماء المقطر فقط بدون تحريض للالتهاب ، مجموعة السيطرة الإيجابية. تم علاجهم بالماء المقطر فقط وتم تعيينهم على الورم الحبيبي الناجم عن حبيبات القطن ، والمجموعة المعالجة بالجنكوبيلوبا (GKB) (٢٠٠ مجم / كجم / يوم) ، والمجموعة المعالجة بالديكساميثازون (١ مجم / كجم) ، والمجموعة المعالجة بالبريدنيزولون (٥ مجم / كجم). أعطيت جميع العلاجات عن طريق الفم لمدة سبعة أيام متتالية. في اليوم الثامن ، تم تخدير الجرذان وإزالة الكريات مع الأنسجة الحبيبية بعناية وجعلها خالية من الأنسجة الدخيلة. تم تحديد الوزن والنسبة المئوية للإفرازات والورم الحبيبي وأرسلت عينات من الأنسجة للفحص التشريحي المرضي. تم جمع عينات الدم عن طريق ثقب القلب واستخدمت لتحليل علامات الالتهاب TNF-alfa :و IL10 و VCAM-1 و hs-CRP. كشفت الدراسة عن انخفاض ملحوظ في الوزن والنسبة المئوية للإفرازات (القيمة الاحتمالية = ٠،٠١٩) ، (١٧٪) والورم الحبيبي (القيمة الاحتمالية = ٠،٠١٣) ، (٢٠٪) بواسطة GKB وهو ما يقارن بتلك التي ينتجها بريدنيزولون. . أظهرت جميع المجموعات العلاجية انخفاضًا كبيرًا في تركيز TNF-α و VCAM-1 و hs-CRP في الدم مقارنةً بمجموعة السيطرة الإيجابية. لوحظ تحسن كبير في النتائج النسيجية المرضية. في الدراسة الحالية ، نجح GKB في تقليل الالتهاب عن طريق تقليل الإفرازات والورم الحبيبي وعلامات الالتهاب. يمكن أن تكون الآليات الأساسية هي التأثير المثبط على التعبير عن السيتوكينات الالتهابية وجزيء الالتصاق البطاني. تشير هذه النتائج إلى أن GKB مرشح جيد لعلاج الأمراض الالتهابية.

الكلمات المفتاحية: مستخلص GKB ، ديكساميثازون ، بريدنيزولون ، ورم حبيبي ، علامات التهابية

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Introduction

Inflammation is a part of the body's defense mechanism against any harmful stimuli such as tissue injury and infections [1]. It is a beneficial process by which the host's immune system eliminates these harmful agents. The inflammatory process initially starts to get rid of the causative agent and the process will be followed by repair mechanisms on the molecular and tissue levels initiating the healing process and restoring homeostasis [2]. Disrepair occurs when the body's defense mechanisms fail to control inflammation, it will eventually result in complications and long-lasting damage [3]. There are two types of inflammation: acute and chronic. Acute inflammation is the initial fast response of the body to foreign agents, it is rapid in onset, starting within minutes to hours, and resolving within hours to few days after the offending agent has been eliminated successfully. While chronic inflammation is a slow response of the body to foreign stimuli, it is slow in onset, starting after days, and has a long duration of years [2].

There are a bunch of medications for the management of inflammatory reactions like steroids, nonsteroidal anti-inflammatory drugs [4]. Steroids are broadly used medications in various diseases for their anti-inflammatory effects. In the biological system, during inflammation, a high level of unbound cortisol will be available since the affinity for binding to their receptor will be attenuated giving more chance to the free form to fight against inflammation [5]. Despite the great effectiveness of steroids in inflammatory diseases; unwanted effects may halt their uses such as musculoskeletal, metabolic, and endocrine adverse effects, infections, cardiovascular, neuropsychiatric, GIT, and dermatological adverse reactions [6]. Therefore, we need to seek new medications that possess anti-inflammatory activity with minimum unwanted reactions.

Growing evidence proved the role of medicinal plants in the management of many diseases [7].

Ginkgo biloba (GKB) is broadly used as nutraceutical in the developed countries. This plant possesses antioxidant, immune-modulating, and anti-inflammatory activities. Additionally, ginkgo biloba extract is also used in many neurological disorders [8], cardiovascular disease [9], diabetes mellitus [10, 11], and gastrointestinal diseases such as ulcerative colitis and acute pancreatitis [12- 14].

Furthermore, GKB is effective in ameliorating hippocampal neuronal death secondary to ischemia through its anti-inflammatory and antioxidant properties [15]. Another study proved the effectiveness of GKB in the chronic inflammatory condition in colons of mice by suppressing the macrophage activation and down regulating the inflammatory mediators [16].

All of the previous studies suggest GKB to be evaluated for the anti-inflammatory activities; accordingly, the current study was intended to test the anti-inflammatory activities of GKB in the rat model of granulomatous inflammation.

Methodology

Experimental animals

Thirty Wistar rats with age range 8-10 weeks age of both genders weighing (150-200g) were purchased from the College of Medicine/ Hawler Medical University and housed in The College of Pharmacy/University of Sulaimani from March to September 2020. The rats were kept in well-ventilated plastic cages, temperature (25±2C°), and 12h light-dark cycle. They had free access to food and water and were fed standard pellet chaw. The experiment was approved by the ethical committee of the College of Medicine, University of Sulaimani (Certificate number 193 on September 20th, 2020). The study was performed following the Canadian Council on Animal Care (CCAC) guidelines, 1998.

Study design

Thirty rats were used and allocated into the following groups (Six rats in each group):

1. Negative control group: Treated with DW without induction of inflammation.
2. Positive control group: Treated with DW with the induction of inflammation and they were assigned to cotton pellet-induced granuloma.
3. GKB group: Treated with GKB (200mg/kg/day) orally for the study of anti-inflammatory activity of GKB in rat model cotton pellet-induced granuloma.
4. Dexamethasone treated group: Treated with dexamethasone (1mg/kg) orally as a standard anti-inflammatory agent, in a rat model of cotton pellet-induced granuloma.
5. Prednisolone treated group: Treated with Prednisolone (5mg/kg) orally as a standard anti-inflammatory agent, in a rat model of cotton pellet-induced granuloma.

The method used for the induction of inflammation was Winter and porter method [17, 18]. In this method of inducing inflammation, (10±1mg) cotton pellets were used after sterilization for 30 minutes in an autoclave at 120°C, four pellets were implanted subcutaneously into the ventral region of the anesthetized rat, two on each side. Ginkgo biloba (200mg/kg), dexamethasone (1mg/kg), prednisolone (5mg/kg), and vehicle distilled water (0.2ml/100gm body weight) were given one hour before the induction and the treatment sustained for seven successive days. On eighth day, the animals were anesthetized and the cotton-pellets together with granulation tissue were sensibly removed and made free from unnecessary tissue.

The weight of the wet cotton-pellets was determined, and then dried using an incubator for 18 hr at 60°C, the dried pellets were weighted again; and the amount of exudate was calculated by subtracting the constant weight of dried pellets from the weight of wet pellets. The weight of granulation tissue was calculated by subtracting the weight of cotton pellets (10mg) from the weight of dried pellets. The following equations were used to calculate the percentage of inhibition of exudate and granulation tissue formation [19]:

Exudate inhibition (%) = {1 – Exudate in treated group / Exudate in controls} c x 100.

Granuloma inhibition (%) = {1 – granuloma in treated group / granuloma in controls} x 100.

Histotechnique procedure

The histological protocol was started at the endpoint of the experiment. Initially, animals were fasted for at least 10 hours before sacrificing and then euthanized in a humane practice. Successively, after animal sacrificing necropsy started by collecting tissue samples for histological preparation. Tissue sections were stained with Harris's hematoxylin and eosin and then viewed under a bright field light microscope. In brief, granuloma skin samples were immobilized into tissue cassettes then fixed with 10% neutral buffered formaldehyde solution for 48 to 72 hours. Then, sections were dehydrated via passing through series of ascending ethanol alcohol (60%, 70%, 90%, and 100%), followed by three steps of xylene clearance. Thereafter, skin tissues were infiltrated and embedded in melted paraffin blocks using an automated wax embedder at (60 -70°C). Paraffinized tissues were sectioned to 4 µm using a semi-automated rotary microtome (Leica-Germany). After that, tissue sections were hunted on glass slides and dried with the aid hot plate tissue holder. Later on, the mounted tissue sections were deparaffinized and cleaned with xylene solution for 30 minutes and air-dried. Finally, Harris's hematoxylin and eosin solution was used for staining tissue sections; cleaned with xylene, and then coverslipped.

Histological semi-quantitative measurement

A semi-quantitative measure of granuloma histological sections from the skin of each animal was measured in µm and statistically evaluated as a mean percentage. Moreover, inflammatory exudate, area of granuloma and proliferated fibrous connective tissue were estimated in four fields tissue sections under medium power magnification (200X), then the mean average was measured in µm and calculated statistically in percentage. Tissue sections were examined under the light microscope (NOVEL XSZ-N107, China) using an image analyzer software (AmScope Ver. 3.7) with aid of a microscope digital camera (MU300, 2019). The mean percentage of the calculated values were expressed as the following lesion scores (score 0-10% as no lesions; score 10-50% as mild; score 50-75% as moderate; score 75-100% as severe lesions).

Statistical analysis

The data were analyzed by using GraphPad Prism 7.00 software (Graph Pad Software Inc., San Diego, CA, USA). Unpaired *t*-test and one-way ANOVA followed by Tukey's multiple comparisons test were utilized for statistical evaluation of the differences between the means. $p<0.05$ were considered statistically significant.

Results

Effect of GKB extract on the weight and percent of inhibition of exudate and granuloma in cotton pellet-induced granulomatous inflammation

Ginkgo biloba extract inhibited exudate formation in a rat model of granulomatous inflammation (Figure 1A), achieving (17%) with the use of 200mg/kg GKB; while prednisolone produced (22%) inhibition but it was still less than that produced by dexamethasone 1mg/kg which was (33%).

Figure 1B shows that GKB significantly reduced the weight of exudate (p-value = 0.019) compared to the positive control, which was less than that produced by prednisolone (p-value = 0.006) while the greatest reduction in weight of exudate produced by dexamethasone 1mg/kg (p-value < 0.001).

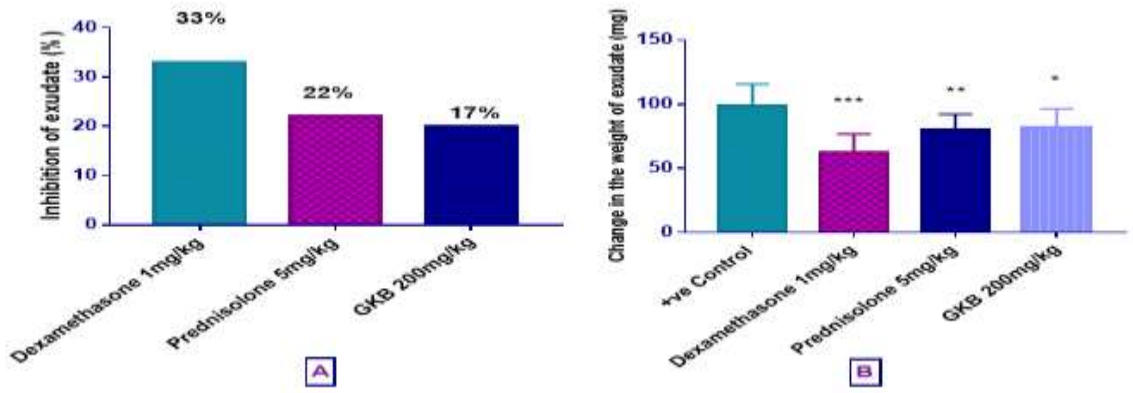

Figure 1: Effect of GKB extract on the percent (A) and weight (B) of exudate in cotton pellet-induced granulomatous inflammation. Values were presented as mean ± S.D (n= 6 animals in each group); values with (*) are significantly different from the positive control using ANOVA and post hoc test (* p < 0.05), (p < 0.01), and (*** p < 0.001).**

Ginkgo biloba also inhibited granuloma formation. Figure 2A shows that GKB 200mg/kg dose resulted in (20%) inhibition, while prednisolone 5 mg/kg inhibited granuloma formation by (23%), meanwhile, dexamethasone 1mg/kg significantly reduced the formation of granuloma by (55%). Figure 2B indicates that GKB significantly reduced the weight of granuloma when compared to a positive control (p-value = 0.013) which was comparable with that produced by prednisolone 5mg/kg (p-value = 0.04) but, still significantly less than that produced by and dexamethasone 1mg/kg (p-value = 0.0001).

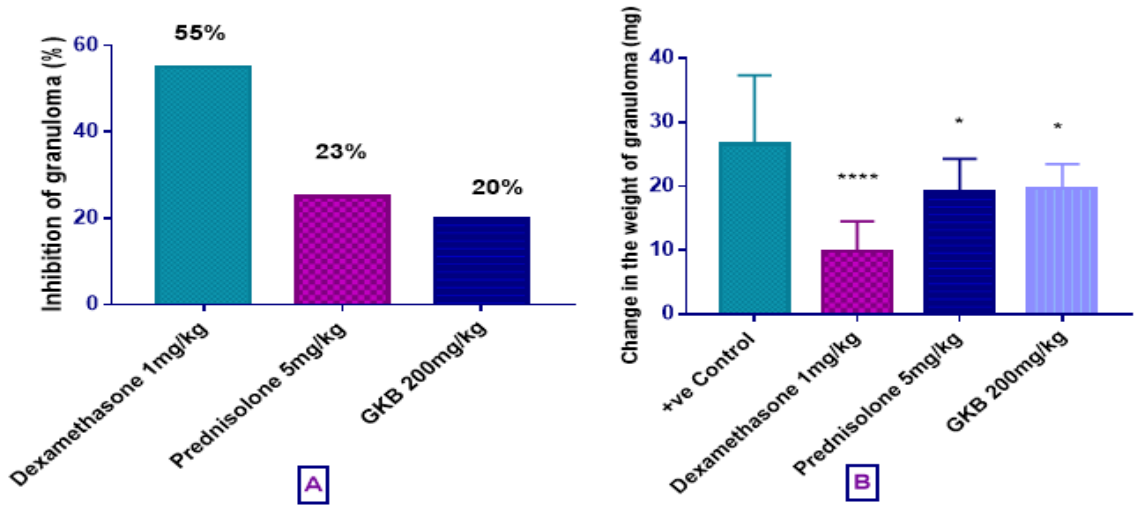

Figure 2: Effect of GKB extract on the percent (A) and weight (B) of granuloma in cotton pellet-induced granulomatous inflammation. Values were presented as mean ± S.D (n= 6 animals in each group); values with (*) are significantly different from the positive control using ANOVA and post hoc test (* p < 0.05), (p < 0.01), and (*** p < 0.001).**

Effects of Ginkgo biloba on the inflammatory markers in granuloma model of inflammation

In the granuloma model of inflammation, Figure 3A shows a significant attenuation (p = 0.02) in the level of serum TNF-α compared with the positive control (22.43 ± 3.098 vs 37.98 ± 4.867) was achieved by the use of GKB 200 mg/kg. Dexamethasone and prednisolone also resulted in significant (p = 0.02) and (p = 0.004) reduction in serum TNF-α level (22.73 ± 1.93 vs 37.98 ± 4.867) and (18.43 ± 3.287 vs 37.98 ± 4.867) respectively. Figure 3B revealed a non-significant reduction in the level of IL-10 in the positive control group when compared to the negative control. The use of GKB, dexamethasone, and prednisolone produced no significant change when compared to the positive control.

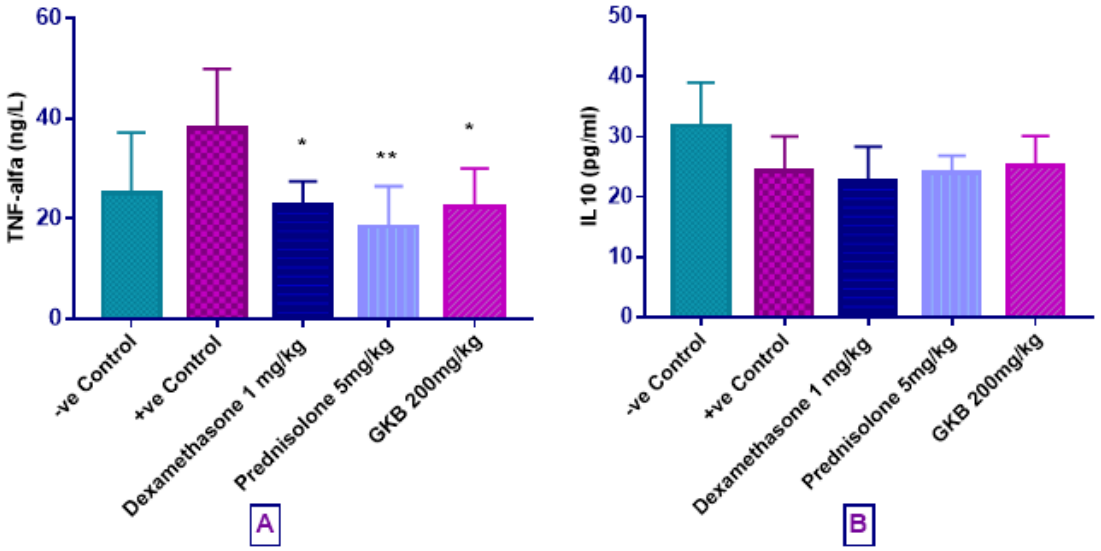

Figure 3. Effects of GKB extract on the inflammatory markers TNF-alfa (A) and IL10 (B) in granuloma model of inflammation. Values were presented as mean ± S.D (n= 6 animals in each group); values with (*) are significantly different from the positive control using ANOVA and post hoc test (* p < 0.05), and (p < 0.01).**

The result presented in Figure 4A shows that the level of VCAM-1 in the positive control group was non-significantly increased when compared to the negative control. Ginkgo biloba 200 mg/kg revealed a significant (p = 0.006) reduction in the level of VCAM-1 compared to the positive control (1.94 ± 0.2731 vs 3.367 ± 0.286); more significant (p = 0.0005) and (p = 0.0001) reduction was observed with the use of dexamethasone and prednisolone (1.617 ± 0.1887 vs 3.367 ± 0.286) and (1.533 ± 0.1174 vs 3.367 ± 0.286) respectively. Figure 4B displays a significant (p = 0.029) increase in the level of hs-CRP in the positive control in comparison with the negative control (28.33 ± 4.773 vs 15 ± 2.236) and the use of GKB, dexamethasone and prednisolone resulted in resulted in a significant (p = 0.005), (p = 0.003), and (p = 0.015) decrease compared with the positive control (8.02 ± 1.98 vs 28.33 ± 4.773), (10 ± 0 vs 28.33 ± 4.773) and (10 ± 0 vs 28.33 ± 4.773) respectively.

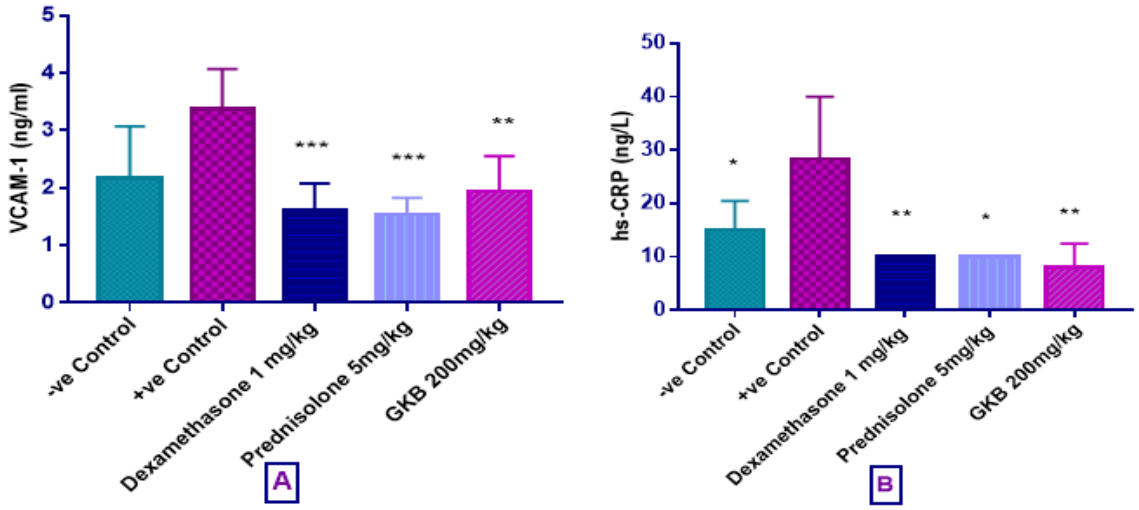

Figure 4.Effects of GKB extract on the inflammatory markers VCAM-1 (A) and hs-CRP (B) in granuloma model of inflammation. Values were presented as mean ± S.D (n= 6 animals in each group); values with (*) are significantly different from the positive control using ANOVA and post hoc test (* p < 0.05), (p < 0.01), and (*** p < 0.001).**

Histopathological findings

Primarily, table 1 reveals morphometric assessment and lesion scoring of foreign body granuloma indued with cotton pellets inserted subcutaneously. Generally speaking, animals in group G2 (granuloma positive control) show significant augmentation in the area of granuloma in comparison to G1 (control negative group) with no granuloma lesion, demonstrated by profound deposition of eosinophilic inflammatory exudate together with diffuse infiltration of inflammatory cells. Moreover, a small area of dystrophic calcification can be seen within the granuloma lesion. On the other hand, animals treated with prednisolone 5mg/kg (G5) and dexamethasone (G4) 1mg/kg reveal a statistically significant and obvious reduction in the area of granuloma and inflammatory exudates together with an increasing amount of proliferated fibrous connective tissue in comparison to G2, indicating the process of healing since it was more prominent in G5 animals treated with prednisolone. Additionally, and remarkably, animals in G3 who received 200mg/kg Ginkgo biloba demonstrated a significant $P<0.05$ decrease in the extent of granuloma lesions evident by dropping in the amount of inflammatory exudates and proliferation of more collagen fibers. Therefore, accordingly, treatment with 200mg/kg of Ginkgo biloba show actual therapeutic effect against experimentally induced granuloma lesion, however, it is more effective in animals treated with 5mg/kg prednisolone and 1mg/kg dexamethasone, respectively. All the results were shown in table 1 and Figure 5.

Table 1 .Histological evaluation of granuloma lesion

Experimental Groups N=5	Area of Granuloma * (Mean %)**	Inflammatory exudate (Mean %)**	Connective tissue thickness * (Mean %)**	Lesion Scoring (0 -100%)	Lesion Grading
(G1) CNG†	0.1 % ^{A}#	1.6 % ^{A}	8.4 % ^{A}	0-10 %	No lesion
(G2) GRC	96.2 % ^{E}	89.3 % ^{E}	78.5 % ^{D}	75-100 %	Severe
(G3) GR+ GKB 200mg	62.3 % ^{B}	64.7 % ^{B}	68.1 % ^{C}	50-75 %	Moderate
(G4) GR+ DEX 1mg	56.3 % ^{D}	53.4 % ^{D}	62.8 % ^{D}	50-75 %	Moderate
(G5) GR+ PRD 5mg	52.8 % ^{C}	51.4 % ^{C}	73.5 % ^{C}	50-75 %	Moderate

Notes: *Area of granuloma, inflammatory exudate and connective tissue thickness were estimated by (µm). **Each value represents mean percentage (n=6). #Statistical comparison among groups: Mean values with different capital letters have significant differences at ($P < 0.05$). †G1: Control negative group; G2: Granuloma positive control; G3: Granuloma and Ginkgo biloba 200mg/kg; G4: Granuloma and dexamethasone 1mg/kg; G5: Granuloma and prednisolone 5mg/kg.

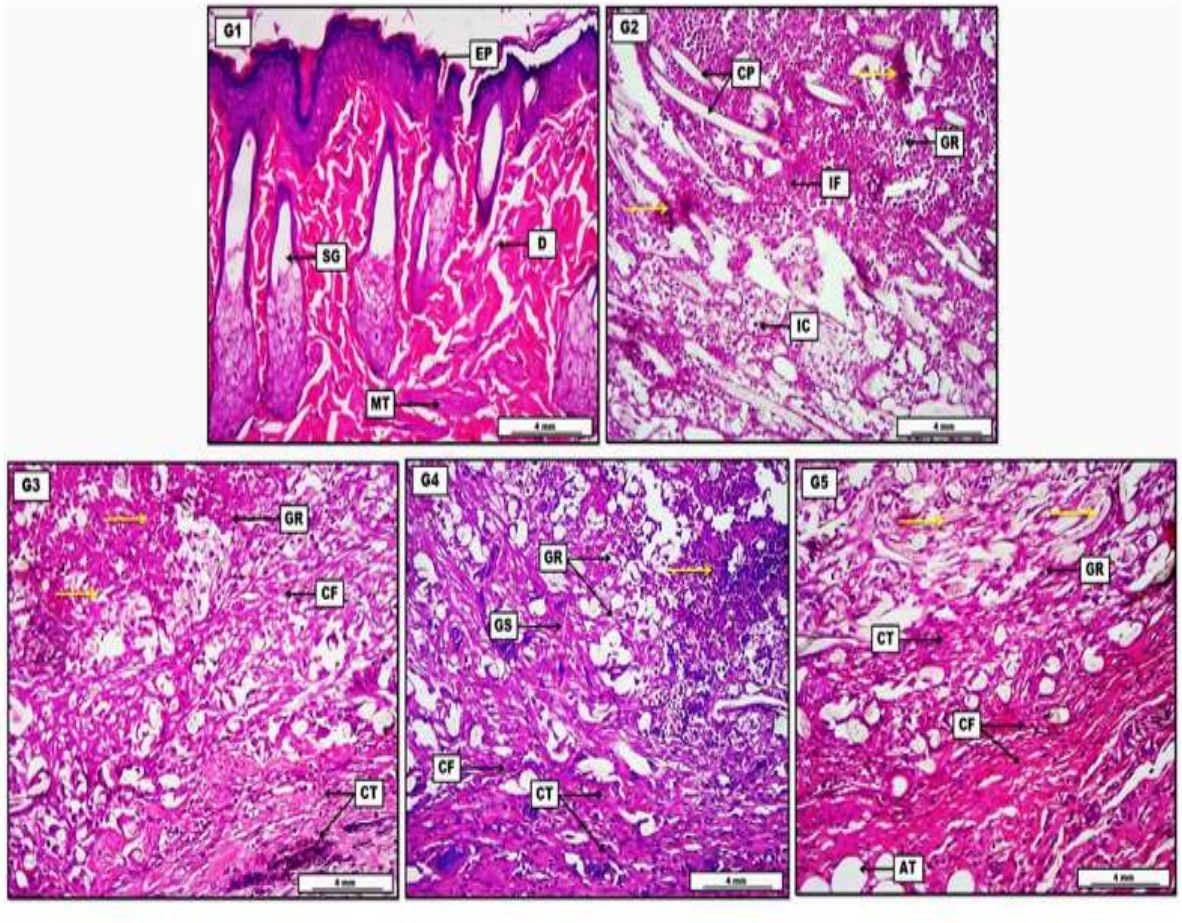

Figure 5.Photomicrograph of skin from groups; (G1) Control group, show distinctive structural arrangement of the epidermis (EP) and dermis (D), with the presence of several sebaceous glands (SG) attached to hair follicles together with some muscle tissue (MT) seen in the skin section. (G2) Granuloma positive group, demonstrate the area of granuloma (GR) infiltrated with significant amount of inflammatory exudate (IF) mixed with inflammatory cells (IC), together with the presence of several longitudinal sections of cotton pellets (CP). Slight deposition of calcium crystals within the granuloma area (yellow arrows) indicates local tissue destruction. (G3) Granuloma with Ginkgo biloba (GKB) 200mg/kg group, reveal significant reduction in the area of granuloma (GR) together with the number of inflammatory cells and exudate (yellow arrows). Granuloma area is replaced with deposition of eosinophilic connective tissue (CT) consisting of many proliferated collagen fibers (CF) and inflammatory cells. (G4) Granuloma with dexamethasone 1mg/kg group, show significant reducing in the amount of inflammatory exudate (yellow arrow), the granuloma area (GR) is contained by eosinophilic deposition of ground substance (GS) together with proliferated collagen (CF) and connective tissue (CT). (G5) Granuloma with prednisolone 5mg/kg group, show significant reduction of inflammatory exudate within the area of granuloma (GR), in addition to, deposition of connective tissue (CT) and proliferation of more collagen fibers (CF) which infiltrate the granuloma. Various sections of cotton pellet (yellow arrows) can be seen within the granuloma given section, along with some amount of adipose tissue (AT). H&E. Scale bars: 4 mm.

Discussion

The effectiveness of steroids is well-documented in inflammatory diseases [20, 21], however, prolong steroid therapy could be associated with numerous undesirable effects [6, 22]. The reason behind choosing dexamethasone and prednisolone as standard steroids for comparison; was the fact that each of the aforementioned steroids has been used in similar cases of inflammation. Dexamethasone has been proven to be effective in reducing formalin-induced paw edema and granulomatous inflammation in various animal studies [18, 23, 24], and prednisolone appeared to have a successful role in reducing the size and extent of the lesion in patients with idiopathic granulomatous mastitis [25] and patients with solitary cysticercus granuloma [26]. Ginkgo biloba extract for the first time in the current study tested in granulomatous inflammation and was efficacious in producing a significant reduction in the inflammatory response. Nutraceuticals are increasingly being used as an alternative medicine for a variety of diseases, [27] since they contain many pharmacologically active constituents [28], such as vitamin E, carotenoids, and polyphenols. Additionally, the presence of dietary supplements like proteins, vitamins, and minerals,

besides these phytochemicals render these nutraceuticals to become very effective in the prevention and treatment of chronic inflammations [27, 29], Ginkgo biloba extract contains flavonoids including quercetin, kaempferol, isorhamnetin, and glycoside that act as free-radical scavengers preventing lipid peroxidation and oxidative stress. It also contains terpenoids that inhibit platelet aggregation and improves memory [30]. The data in the current study shows the effectiveness of GKB extract in decreasing both the amount of exudate and granuloma although the effect was less than that produced by dexamethasone and prednisolone; the anti-inflammatory effect of GKB was more obvious on the inflammatory markers. The significant decrease in the level of TNF-α produced by GKB mainly contributed to the radical scavenging properties, down-regulating some of the inflammatory mediators and inflammatory responses through the modulatory effect on the expression of many inflammatory mediators [8, 14]. Targeting TNF-α may augment the down regulation of the immune and inflammatory responses, because of its pivotal role in the stimulation of ROS production, expression of other pro-inflammatory mediators, activation of leucocytes, and eventually amplification of the inflammatory cascades [14, 16]. Ginkgo biloba was also effective in producing a significant decrease in the level of VCAM-1 compared with the positive control which could be attributed to the antioxidant properties that in turn modulate the redox-sensitive transcription pathways and minimize the expression of endothelial adhesion molecule including VCAM-1 via suppressing TNF-α–induced expression of VCAM-1 and ICAM-1 [31]. In contrast to the study that showed no association between taking supplements contain ginkgo biloba and the level of hs-CRP [32]: the current study revealed that the level of hs-CRP has been attenuated by the use of GKB and the two steroids used in the study. This finding was in tune with other studies done on patients with metabolic syndrome where the use of ginkgo biloba as adjuvant therapy was shown to be effective in reducing the level of hs-CRP [33]. Ginkgo biloba extract has a crucial role in dropping oxidative stress and inflammatory response [34,35].

Furthermore, another underlying mechanism for the anti-inflammatory effects of GKB could be attributed to the inhibitory action on the expression of inflammatory cytokines through suppressing the inflammatory signaling pathway toll-like receptor 4/ nuclear factor kappa-light-chain-enhancer of activated B cells (TLR4/NF-κB) [36].

Conclusion

In conclusion, GKB was effective in diminishing inflammation as reported by decreasing the exudate, granuloma, and inflammatory markers. The underlying mechanisms could be the inhibitory effect on the expression of the inflammatory cytokines and endothelial adhesion molecule. These findings suggest GKB as a good contender to be tested for the treatment of inflammatory diseases.

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Conflicts of Interest

The authors report no conflicts of interest in this work.

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