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RESEARCH ARTICLE

Effect of Topical Phytosterol Fraction of *Chenopodium murale* on Induced Hypertrophic Scar in Rabbits

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Abstract

Objective: The present study aimed to evaluate the role of phytosterol fraction of *Chenopodium murale* in experimentally induced hypertrophic scar in rabbits. Methods: Surgical wound was performed on day 1 with an 8- mm biopsy punch four wounds were created on the ventral surface of the rabbit ear (in vivo) down to cartilage. Triamcinolone acetonide (TAC) and phytosterol extracts of *Chenopodium murale* are administered topically to established scars on day 31. The outcome measures include the study of histopathology of skin section, transforming growth factor beta-1(TGF-ß1) level and collagen III alpha in skin tissue. Results: The treatment groups produced high significant reduction in inflammatory scores, height, index of scar and in immunological scores of TGFß1 and scores of collagen III in comparison with induced hypertrophic scar group ($P \leq 0.001$). Significant reduction was observed also in fibroblast count as compared with induced hypertrophic scar control group ($P<0.05$).Conclusion. Phytosterol extract of *Chenopodium murale* was effective in treatment of induced hypertrophic scar through its ability to produce reduction in inflammation, fibroblast count; height and thickness of hypertrophic scar in addition to decrease in immunological scores and the results were nearly comparable to that of topical triamcinolone acetonide.

Keywords: *Chenopodium murale; Rabbits; Topical phytosterol.*

Introduction

Hypertrophic scars

Pathological wound healing process results in hypertrophic scars (HTS) and can be described as a fibro-proliferative disorder usually occurs within 4 to 8 weeks has a rapid growth phase for up to 6 months, and then gradually regresses over a period of a few years, eventually leading to flat scars with no further symptoms [1].

There are some designated characteristics that define hypertrophic scars and help to distinguish them from other kinds of pathological scars. The genesis of HTS is related to trauma, inflammation, surgery and often to skin defects caused by burns. According to literature, the incidence of HS after a surgery is 35% while such percent increases up to 80% following burn injuries [2].Hypertrophic scars usually are elevated, but do not spread beyond the border of the primary wound, this is a significant factor differentiates them from keloids, which do spread into the adjacent skin, color range of HS is between red and pink and they have a rougher surface in comparison to normal scars [3]. Symptoms of HTS are pruritus, pain, pressure, a reduced range of motion, and intolerance to heat. However, commonly the cosmetic factor presents the most bothering symptom for patients [4]. The underlying mechanisms of scar formation are complicated, and the process may be affected by multiple factors [5].

The physiologic response to wounding in adult tissue is the formation of a scar, a process that can be temporally grouped into three distinct phases: Inflammation, proliferation, and remodeling [6]. There are numerous interactions between fibrotic and anti- fibrotic growth factors extracellular matrix (ECM) components, and various enzymes [7]. In HTS tissue there are greater numbers of fibroblasts exhibiting an

alteration in their phenotype than normally found in the skin, HTS fibroblasts presents with an increased expression of TGF-ß1 than normally found fibroblasts [8]. A major role in scar production of the isoforms TGF-ß as having specifically excessive activity of the TGF-ß isoforms can lead to excessive scarring.

These are required activation prior to binding to the TGF-ß receptors (TßRI and mTßRII). TßRII is phosphorylated and on binding of the ligand [9]. Phosphorylation of the receptor complex activates the SMAD intracellular signaling pathway through the receptor Smads (Smad-2 and Smad-3) and co-Smad4. The receptor SMADs and Smad-4 cross over the nuclear membrane. Where they regulate a number of genes [10].There appears to be an alteration in TGF-ß signaling via increased phosphorylation of the receptor Smad proteins in hypertrophic-derived fibroblasts and a decreased expression of the inhibitory Smad 7 in hypertrophic scar-derived fibroblasts [11].

This study aimed to investigate our hypothesis that phytosterol fraction of *Chenopodium murale* may be successful in the therapeutic modulation of scar formation *in vivo*. *Chenopodium murale* is an annual herbaceous weed and flowering plants known as the goosefoots [12]. The extracts of the *Chenopodium murale* significantly suppressed the test fungal growth [13].Also exhibited mild to moderate inhibitory activities against different bacteria [14].

It possesses antipruritic and antinociceptive activities and the ethanolic extracts of C. album and C. murale showed anti-inflammatory activity on the rat paw edema and the cotton pellet models [15, 16]. On the other hand, phytosterol is a large group of compounds that are found exclusively in plants, they have cholesterol reduction effect and prevention against types of cancer such as colon, breast, and prostate [17], also stimulation of immunity and protection of skin [18].

Methods

A total of (32) healthy male albino rabbits between 6 and 12 months of age were used, Protocol of the current research was approved by the Institute Review Board Al-Nahrain University College of Medicine.

Rabbits were anesthetized with (IM) injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). Surgical wounds were performed on day 1 with an 8- mm biopsy punch. Four injuries created meticulously on the ventral surface of one ear down to cartilage. Removal of the perichondrial layer delayed epithelization after the hemostasis has been achieved with manual pressure; wounds covered with sterile gauze for 1 day. On day 30 the eventual scars were obtained.

Plant Material

The herb included in this study was identified and authenticated in the Department of Biology /College of Sciences/ University of Baghdad. The extraction and fractionation of herb was executed in pharmacognosy department, collage of pharmacy, Baghdad University (Iraq).

Preparation of Extracts

Powder from aerial parts of *Chenopodium murale* (250 mg) was extracted with 90% ethanol (600 ml) in reflex apparatus until complete exhaustion and evaporation to give crude fraction. Crude extract was acidified with hydrochloric acid (5%) at pH 2 then partitioned with equal volume of ethyl acetate to give two layers (aqueous and ethyl acetate) layer, ethyl acetate layer was collect, evaporated then basified with 300 ml of sodium hydroxide 5%, and extracted with chloroform to get also two layers, methanol 80% and petroleum ether was added to chloroform layer to obtain phytosterol in petroleum ether fraction [19].

Preparation of Gels Formulations

The concentration of chemical and extract was weighed and dissolved in 10 ml of absolute ethanol alcohol to prepare (solution A) after that 3 g powder of HPMC was added to the 75 ml of distilled water with stirring to get (solution B). Solutions A and B were mixed thoroughly, and the final weight was made up to 100 g [20].

The treatment groups are as follows: Group 1 Healthy animal group; Group 2 hypertrophic scar was induced, and the animals left without treatment (only base gel); Group III rabbits with induced hypertrophic scar treated with triamcinolone acetonide (TAC) 0.1% as standard drug; Group VI rabbits with induced hypertrophic scar treated topically with phytosterol extract of

Chenopodium murale 0.3%. Drug and extract were given twice daily for 21 days as formulated topical gel.

Collection of Samples

The samples were collected from each animal after anesthetized the animals at the end of the experiment (51 days) using 11 mm bunch biopsy with more than 3 mm margin of adjacent skin [21] then submitted for histological and immunohistochemical analysis.

Assessment of Histopathological Changes in Skin Sections

SEI index is the ratio of the highest vertical height of scar area between perichondrium and skin surface to the highest vertical height of normal area around the scar between perichondrium and skin surface. Each wound was measured by a blinded examiner using a calibrated eyepiece reticule [22].

The degree of inflammation and fibroblast counts was evaluated in a semi‐ quantitative manner. The degree of inflammation was evaluated according to the following scores: 0 = none; 1 = mild; 2 = moderate; and 3 = severe. Fibroblast count was evaluated according to the following scores: 0 = absence of fibroblasts; 1 = few fibroblasts; 2 = presence of disorganized fibroblasts; and 3 = presence of fibroblasts parallel to the wound surface [23].

Immunohistochemistry IHC Detection of Collagen III, TGFβ1

(I) Anticollagen III antibody: Rabbit polyclonal antibody to collagen III (Code number: MBS822102) (My Bio Source, USA). (II) AntiTGFß1antibody: Rabbit polyclonal antibody to TGFß1 (Code number: Ab236466) (Abcam, UK). Expose mouse and rabbit specific HRP/DAB Detection IHC kit (Abcam) code: GR3246437-1

Evaluation of IHC Results

Quantification of TGF‐ ß1 and collagen protein expression was evaluated under light microscopy at X20. The extent of the immunohistochemical reaction of ECM proteins, such as collagen, was measured by ranking the signal intensities according to the following scale: – (absent), + (mild), ++ (moderate), and +++ (marked) (24). Stained slides were examined to identify immunoreactivity for TGF ß1. Scoring system was done, and the score recorded was the average intensity of the expression: Absence of immunoreactivity had score 0, weak immunoreactivity had score1, moderate immunoreactivity had scored 2; and strong immunoreactivity had score 3 [25].

Statistical Analysis

Two statistical software programs used: The statistical package for the social sciences (SPSS version 22) and Microsoft Office Excel 2019. All results are presented as means ± SD. Comparison two groups was carried out using the Mann–Whitney U‐ test. Data for multiple comparisons were performed by Kruskal–Wallis test. $P \leq 0.05$ was considered significant and highly significant when $P \leq 0.01$ [26].

Results (Healing Rate)

Appearance of untreated induced hypertrophic scar Normal healing process involves three overlapping phases: Inflammation, cellular proliferation and remodeling (100% induction) at 30th day as shown in Figure 1. Group III (induced HTS in rabbits treated with triamcinolone acetonide): Healing signs were very clear starting after treatment with fading of the inflammatory sign and decrease thickness of scar (after 21 days of treatment) Figure 2a.

Group IV (induced HTS in rabbits treated with 0.3% phytosterol fraction of *Chenopodium murale*) Figure 2b: Remarkable decrease of inflammatory signs occurred after starting treatment with fading of inflammatory sign and no sign of thickness after 21 days of treatment.

Immunohistochemical and Histopathological Results

Immunohistochemical for TGF‐ ß1 and collagen III is shown in Table 1, Figures 3-5. There was an extremely high significant difference in mean of immunohistochemical scores of TGF‐ ß and collagen III between healthy control and induced HTS group enrolled in the present study ($p \leq 0.001$). All treatment groups showed a highly significant reduction in IHC expression scores of TGF‐ ß and collagen III ($p \leq 0.01$) as compared to induced HTS.

Histological results and Histopathological score reflective of scar in experimentally induced HTS was shown to be extremely high significant ($p \leq 0.001$) increased in the induced hypertrophic group in comparison to the healthy control group. All treatment groups have highly significant reduction in inflammatory degree and in scar elevation index as compared with group of induced HTS ($p \leq 0.001$).

Extremely high significant reduction was observed in TAC ($p \leq 0.001$) and significant reduction was observed in 0.3% phytosterol extract of *Chenopodium murale* ($p \leq 0.038$) in fibroblast count in comparison to induced hypertrophic scar group. All results are shown in Table 2 and Figures 6-9.

Figure 1: Gross morphological features of healing rate in the induced hypertrophic scar of rabbits during 30 day

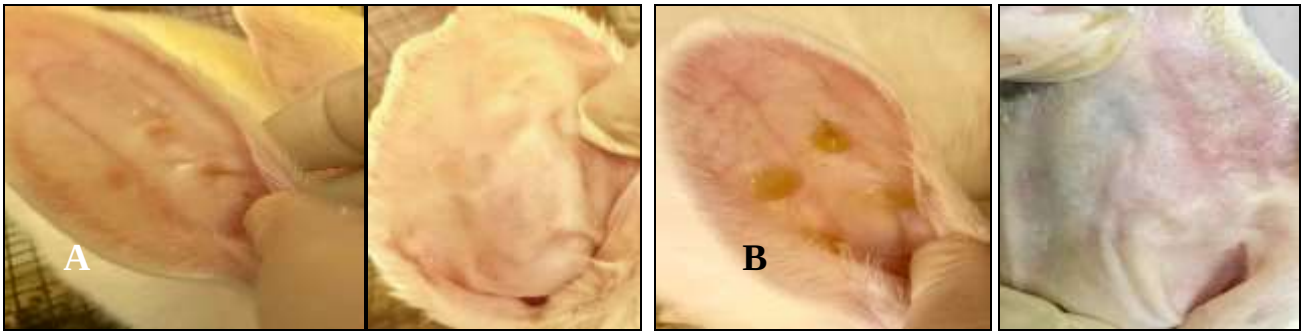


Figure 2: The application and effect of treatment groups after 21 days (a) Triamcinolone acetonide, (b) 0.3% phytosterol extract of *Chenopodium murale*

Table 1: Mean of TGF- β1and collagen III in the control and study groups

Parameters		G1 N=8	G2 N=8	G3 N=8	G4 N=8
Collagen IIIα	Mean± SD	1.0± 0.0	3.0± 0.0	1.0± 0.0	1.38± 0.52
	P value	<0.001*		<0.001*	<0.001*
TGF-β1	Mean± SD	1.0± 0.0	3.0± 0.0	1.0± 0.0	1.38± 0.52
	P value	<0.001*		<0.001*	<0.001*

Mann–Whitney U- test. SD standard deviation; p indicates the level of significance at $p \leq 0.05$; *Indicate a comparison between induced hypertrophic scar and another group. (G1) Healthy control, (G2) Induced hypertrophic scar, (G3) TA steroid, (G4) Phytosterol Extract 0.3% of *Chenopodium murale*. TGF- ß1: Transforming growth factor- beta 1

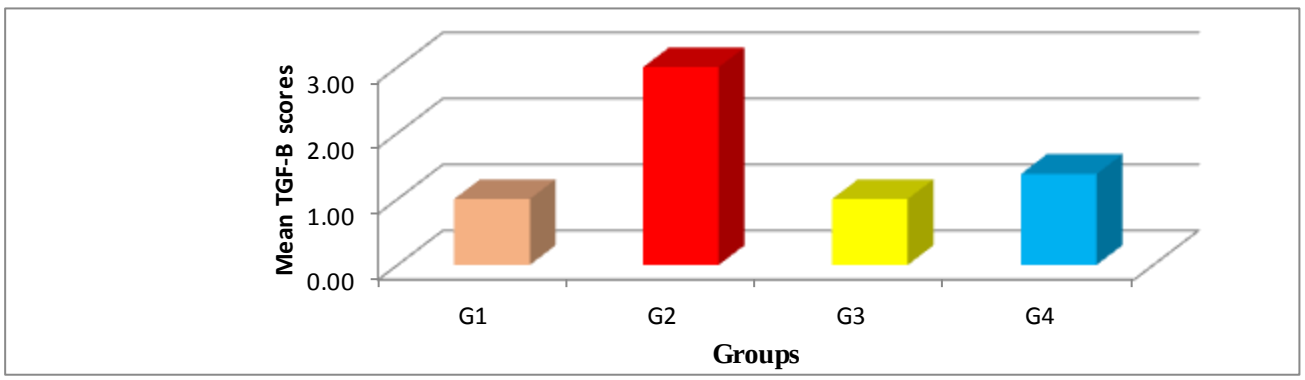


Figure 3: Mean transforming growth factor- beta 1 (TGFβ1) scores in the control and study groups. (G1) Healthy control, (G2) induced hypertrophic scar, (G3) Tiamcinolone acetonide, (G4) phytosterol 0.3% extract of *chenopodium murale*

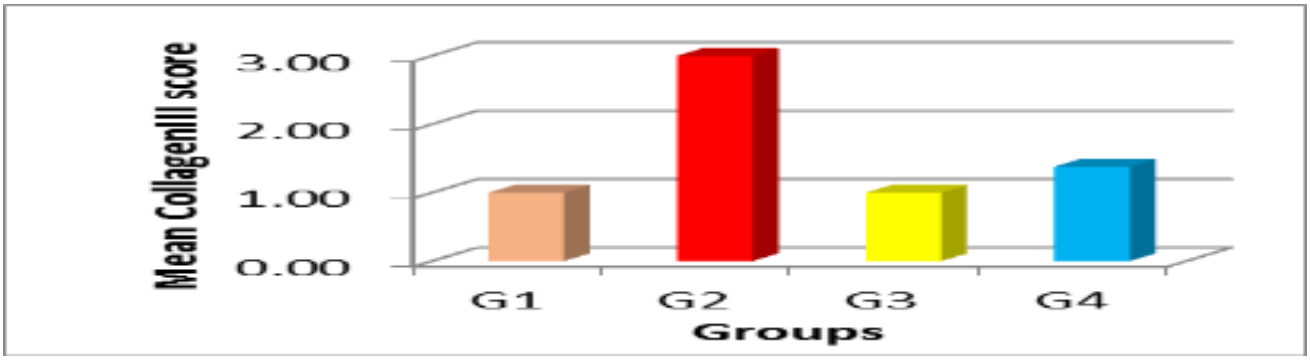
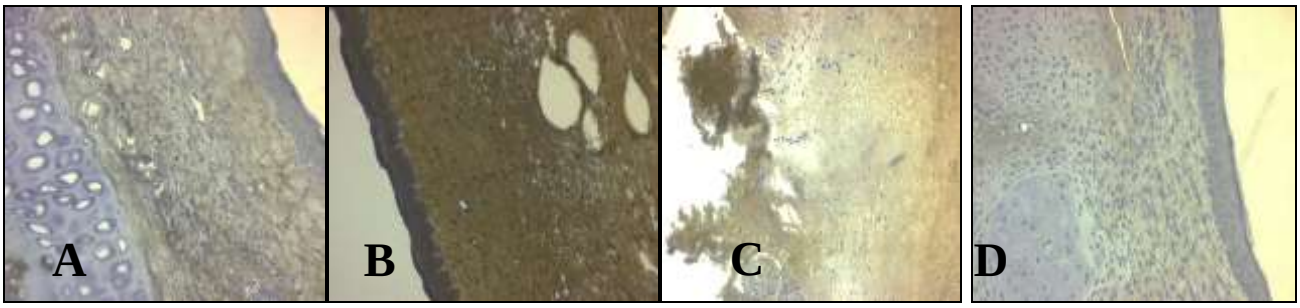
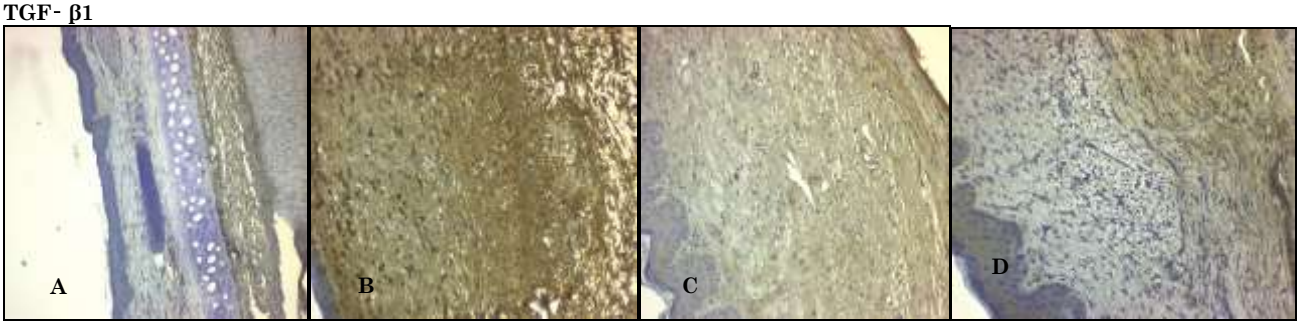


Figure 4: Mean collagen III scores in the control and study groups. (G1) healthy control, (G2) induced hypertrophic scar, (G3) triamcinolone acetonide steroid, (G4)) phytoserol 0.3% extract of *Chenopodium murale*



TGF- β1



Collagen III

Figure 5: Cytoplasmic immunohistochemically expression of transforming growth factor- beta 1 (TGF- β1) and extracellular immunohistochemically expression of collagen III of treatment groups (×20). (A)Normal tissue shown low intensity of TGF- β1 and collagen III.(B) Induce hypertrophic scar shown high intensity of TGF- β1 and collagen III. (C and D) Hypertrophic scar showed mild intensity TGF- β1 and collagen III in triamcinolone acetonide- treated group and 0.3% phytosterol treated group respectively

Table 2: Mean of histological outcome in the control and study groups

Parameters		G1 N=8	G2 N=8	G3 N=8	G4 N=8
Inflammatory degree	Mean ±SD	0.00 ± 0.00	3.00 ± 0.00	1.0± 0.0	1.0± 0.0
	P value	<0.001*		<0.001*	<0.001*
Fibroblast count	Mean ±SD	0.00 ± 0.00	2.00 ± 0.00	1.0± 0.0	1.38± 0.52
	P value	<0.001*		<0.001*	0.038*
SEI ratio	Mean ±SD	1.0± 0.0	4.14± 0.15	1.21± 0.06	1.57± 0.06
	P value	<0.001*		<0.001*	<0.001*

Mann Whitney test .SD: Standard deviation; P indicate the level of significance at ($p \leq 0.05$); ***** indicate comparison between induced hypertrophic scar and another group. (G1) Healthy group, (G2) Induced hypertrophic scar, (G3) TAC steroid, (G4) 0.3 % phytosterol fraction of the plant

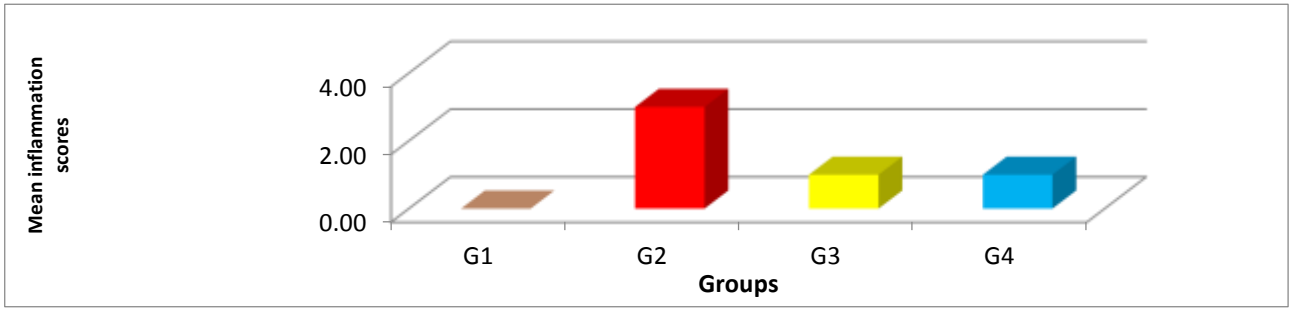


Figure 6: Mean of inflammation score in control and study groups (G1) healthy control, (G2) induced hypertrophic scar, (G3) triamcinolone acetonide steroid, (G4) phytosterol 0.3% extract of *Chenopodium murale*

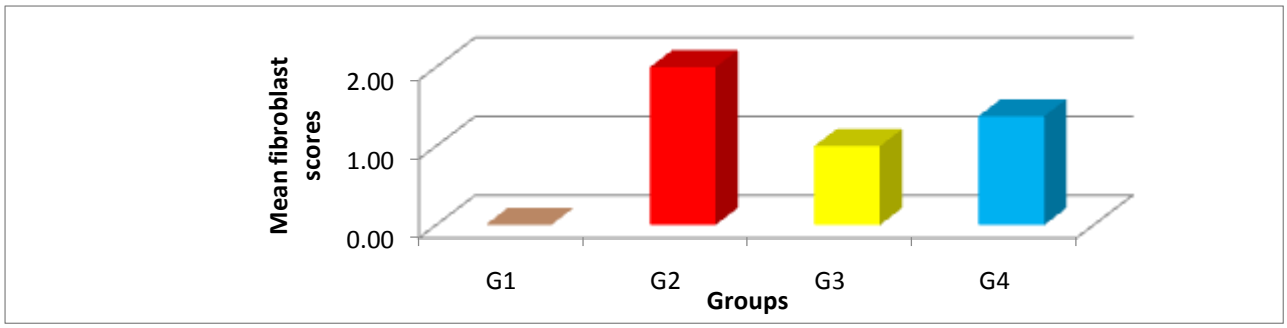


Figure7: Mean of fibroblast count of control and study group (G1) healthy control, (G2) induced hypertrophic scar, (G3) triamcinolone acetonide steroid, (G4) phytosterol 0.3% extract of *Chenopodium murale*

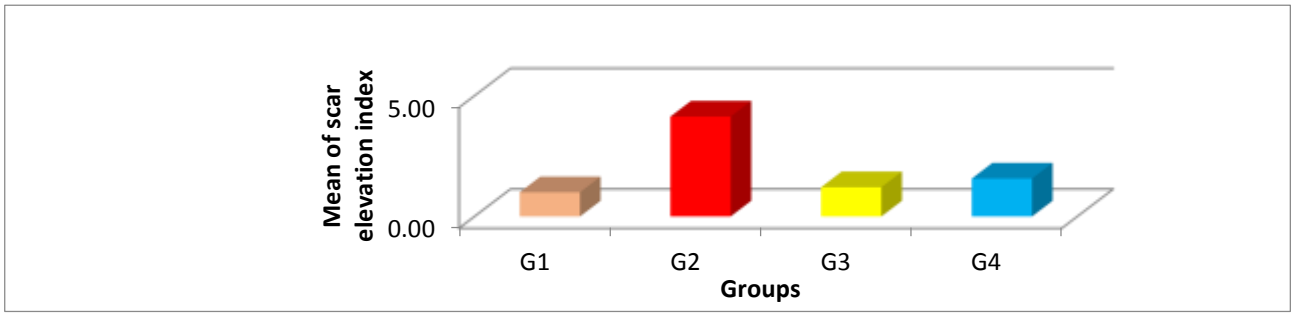


Figure 8: Mean of scar elevation index in control and study group. (G1) healthy control, (G2) induced hypertrophic scar, (G3) TA steroid, (G4) phytosterol 0.3% extract of *Chenopodium murale*

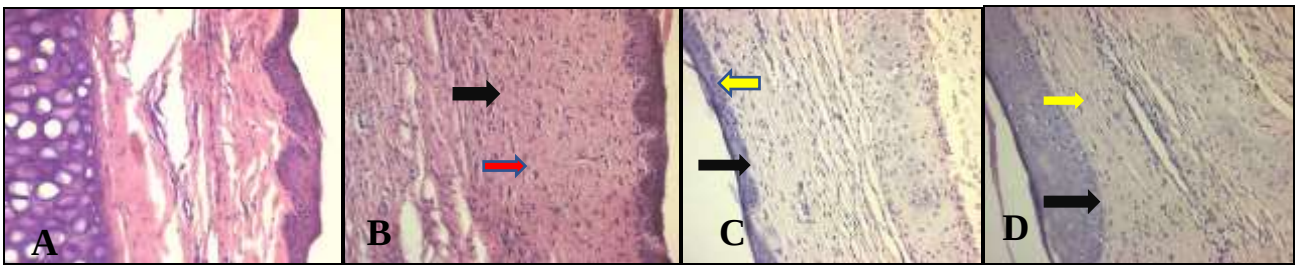


Figure 9: Some of the histological sections from all groups stained with H and E stain and examined for inflammation (black arrow), fibroblast count and arrangement (yellow arrow organized fibroblast), (red arrow disorganized fibroblast) (×20). (A) Normal dermal tissue characterized with the absence of inflammatory cell and fibroblast), (B) induced hypertrophic scar tissue represents severe inflammation and high number of polymorph nuclear cells, also dermis cellularity increases, fibroblasts were increased and arranged in a disorganized manner. (c) Hypertrophic scar of 0.1% triamcinolone acetonide group, (d) hypertrophic scar of 0.3% phytosterol of *Chenopodium murale* group

Discussion

Hypertrophic scar are the common seen pathological scars in clinics and are also the difficult challenges in plastic surgery [1]. The main characters of pathological scar are fibroblast proliferation and excessive accumulation of extracellular matrix (ECM) [7]. It is now generally accepted that the abnormal proliferation of fibroblast and excessive deposition of fibroblast-derived extracellular matrix proteins especially collagen are the main causes of hyperplasia and persistence of HTS [8].

Such pathological scarring can cause cosmetic problems, functional problems such as contractures and patients' subjective, symptoms including pruritus and pain that lead to severe functional impairment, psychological morbidity, and costly long-term care [27].

The rabbit dermal model was first described by Joseph, J., & Dyson, M [28] and subsequently improved upon by Morris *et al.*, [29]. TGF-ß can mediate fibroblast proliferation, angiogenesis, ECM synthesis, and re-epithelialization in the wound-healing process [30]. It was demonstrated that fibrogenic response to injury is mediated through angiotensin II induction of TGF-ß1 expression [31].

TGF-ß1 regulates various fibrosis-related proteins, including Type I and III collagens [9]. It can also promote the transformation of fibroblasts to myofibroblasts, which are the significant cells contributing to HTS formation and characterized by an increased synthesize of collagen and up regulation of cytokines [32].In the current study, HTS in rabbit's ear model was successful as there was significant differences between induced

HTS and normal skin in cellular response to growth factor (TGF -ß1) which is consistence with [33]. Topical triamcinolone acetonide significantly reduced TGF-ß1 as compared to induced scar non-treated group after 21 days of treatment (P<0.001) which is in accordance with (34) that found that significant differences in pro inflammatory cytokines TGF-ß1 and collagen III in rabbit ear model was found after treatment with topical triamcinolone acetonide. Carroll *et al* [35].

Studied the effect of Triamcinolone on bFGF production and TGF-ß1 inhibition by human dermal fibroblasts and found that 20 µm triamcinolone acetonide caused significant decreases in the level of TGF- ß1 for normal and keloid fibroblast cell lines; this finding supports the finding of the present study. *Chenopodium murale* is a medicinal plant which has had a role in empirical medicine in numerous countries [36].

There are no previous reports on the effect of *Chenopodium* on HTS which refer to the novelty of current study. Phytosterols in herbal extracts have protective effect against various forms of cancer by reductions in growth of various cancer cells. There have been several mechanisms proposed such as inhibition of carcinogen production, cancer cell growth and multiplication, apoptosis and reduction of angiogenesis [37]. Also, have anti-inflammatory effect that result in decrease of pro-inflammatory cytokines secretion [38].

Result of current study is in agreement with Jurjus *et al*. [39] that studied the effect of MEBO, contain beta-sitosterol as active ingredient in induced burn of rabbit's model and found persistent raise in levels of TGF-ß1 form day 2 to day 9 then dropped back on days 12 and 15. The finding of this study agrees with Noori H.S. and Abu-Raghif [40] regarding phytosterol fraction prepared from Fumaria officinalis that showed significant reduction of TGF-ß1 on HTS in rabbit's ear model.

Hypertrophic scars primarily have collagen type III in parallel position to the epidermal surface [1], results of current study have been proved that there is an elevation of collagen III expression in induced HTS group, which is consistence with finding Oliveira *et al* [41]. This research also showed significant reduction of collagen III in triamcinolone acetonide and it is in agreement with finding of Uzun *et al* [42]. In addition to decrease in TGF-ß1 after triamcinolone acetonide treatment, one of probable mechanism on ECM disposition of collagen is the effect on plasma protease inhibitors, thus allowing collagenase to degrade collagen [43]. Pytosterol extract of *Chenopodium murale* showed significant decrease in collagen III that agree with [40] regarding phytosterol fraction prepared from Fumaria officinalis showed significant decrease in collagen III on HTS in rabbits ear model. Idrus *et al* [44].

Showed the potential effect of sterols (campesterol, sitosterol, stigmasterol), in human dermal fibroblast (*vitro* media) through inhibition of the fibroblast proliferation, migration and gene expression of type I collagen, type III collagen, fibronectin [45].Regarding inflammation, the current study showed that triamcinolone acetonide significantly reduced the infiltration of inflammatory cell anti-inflammation activity of triamcinolone acetonide after 14 days treatment in wound of rats' model and registered nearly same observation of our results [46].

The effects of corticosteroids are primarily due to their suppressive effects on the inflammatory process in the wound [47]. The probable anti-inflammatory mechanism of triamcinolone against HTS primarily by suppressing of leukocyte, monocyte migration and phagocytosis, they are powerful vasoconstrictors, thus reducing the delivery of oxygen and nutrients to the wound bed [48].

In part of herb in this study phytosterol fraction of *Chenopodium murale* possess marked anti-inflammatory effect, the anti-inflammatory properties induced by plant sterols have been demonstrated in *vitro* studies [49]. B-sitosterol and campesterol significantly reduced production of prostaglandin E and prostaglandin I in liposaccharide-activated macrophage cells [18]. Triamcinolone acetonide exhibited significant change in fibroblast scores in compared to experimentally induced HTS, this finding agrees with observation of Uzun *et al* [42]. That show decrease in fibroblast count. Phytosterol fraction *Chenopodium murale* showed significant reduction in

fibroblast count, phytostrol (sitosterol; stigmasterol) and phenolic compound as antioxidant, it has been trailed for the treatment of hypertrophic, keloid, and wounds [50].Stigmasterol have cytotoxic activity in DMBA-induced papilloma in terms of both incidence of tumors and the mean number of papillomas [51].Furthermore, extract of *Chenopodium album* (leaves) has promising anti-breast cancer bioefficacy [52]. *Chenopodium quinoa* seeds, exhibited antioxidant activity the antioxidant activities of phenolic plant compounds are correlated with anticancer activity [53].

Scar elevation index (SEI) is obtained with the measurement of vertical height from perichondrium to epidermal surface in scar area and normal tissue around the scar. Triamcinolone acetonide was shown high significant decrease in height and SEI that agree with [54] phytosterol extract of *Chenopodium murale* established significant reduction of height and index of scar this result is agreed with [40] that detected decrease in scar height and index after treatment with fumaria officinalis L. in rabbits ear model.

Conclusion

Phytosterol 0.3% extract of *Chenopodium murale* was effect in reducing scar and is nearly comparable to that of triamcinlone acetonide.

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