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Localization of TNF-α in Cutaneous Incisional Wound Treated with Topical Flavonoid Extract of Hibiscus Sabdariffa in rabbits

Sura Majeed[1], Nawar Bahjet [2], Ban A.Ghani1[3], Nada Al-Ghaban[4]

College of Dentistry/University of Baghdad/Iraq[1,2,4,]
Al Rafidain University/Dentistry Department/Iraq[3]

Abstract:
The interest on plants research hadbeen increased all over the world and now there is a large body of evidence about thepotential immense of the medicinal plants to be used in a variety of traditional systems
Aim : Localization of TNF-α in Cutaneous Incisional Woundand evaluation the potency of ethanolic extract of Hibiscus sabdariffa L (flavonoids) on wound healing by a phytochemical, biochemical, histological and immunohistochemical method.
Materials and methods:
Twenty four adult New Zealand rabbitswere divided into control(12) and experimental (12)groups, incisional wound was created on facial skin and local application of FV extract will be done for experimental groups only ,all animals were sacrificed for 3,7 ,10 and 14 days, specimens were prepared for histological and immunohistochemical analysis.
Results:
As shown by examination of histological sections that healing was accelerated after flavonoid application and results of statistical analysis indicated that wound healing higher in 7and 10 days in experimental group than control group.
Findings obtained in this study showed decrease in percentage of positively stainedTNF-α in 10 and 14 days.
Conclusion: The ethanolic extract of H.Sshowed fairly goodwound-healing activity as demonstrated by histological results and TNF-αexpression by cells involved in the process in different durations.
Key words:skin wound,flavonoids,

Introduction

Following injury wound healing is beginningfor repair the skin and other soft tissues and an inflammatory response occurs and the cells below the dermis begin to increase collagen production. Later, the epithelial layer is regenerated. There are three steps in the procedure of wound healing :inflammation, proliferation, and remodeling**(1).**
At the early stages wounds contraction usually begins slowly but then become rapid on day 3 or 4. in the margin of the wound, the myofibroblasts appears to constitute the main actor for the wounds contraction**(2,9)**.
The wound epithelialization occurs primarily by the proliferation and the migration of the marginal basal cells that are lying close to the margin of the wound**(11)**. The hematoma that are present within thewounds may be replaced by a granulation tissue which usually consists of a new capillaries andfibroblasts cells**.**According to the (WHO), about 65% of the population of the worldhas incorporated the values of the plants as a methodology of the medicinal agents into their primary modality of health care**(3-5)**.

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The properties of wounds healing of the H. sabdariffa L. flower extracts may be caused by its high flavonoid content[13-16]. The Flavonoids are found to help wounds healing process mainly because of their antimicrobial properties [6,12].The tumor necrosis factor-α (TNFα) is considered as a multifunctional cytokine that can mediate inflammation, immune responses and apoptosis and it is quickly released to initiates the inflammation at the wounds tissue, but its precise an actual role in the wound healing is not yet fully understood [7-8,10].
Our present study was taken up to evaluate the efficacy of the topical application of the Hibiscus sabdariffa L. by a phytochemical, biochemical, histological and immunohistochemical method in the healing process of the wounds.

Materialsand methods

Flavonoid ethanolic extract(2 µl), anesthetic solution (Ketamine hydrochloride10% Woerden Xylazine 20 mg), formalin 10%,ethanol alcohol 96%,xylol, paraffin wax, and Hematoxylin and Eosin (H&E) stain, Anti-Tumor necrosis Factor-alpha Antibody (TNF-a) from Abcam company UK
(ab212899), Detection Kits System, Abcam Company England (ab80436).

Study design

Total of (24)New Zealand adult rabbit weighing an average of (1.5-2kg)were used after a7 days of acclimatization period to the environment of laboratory. They were given a free access to the standard rabbit food and water. The facilities of the animal care were maintained according to the national guidelines. The ethical committee of the college of dentistry at the university of Baghdad according to the no(201) had approved the study protocol. Wounds created and specimens were collected for the analysis. The rabbits were given anesthesia with a combination of xylazine (5 mg/kg) and ketamine-HCL (50 mg/kg) that is administered intramuscularly.

Surgical procedure

General anesthesia with ketamine hydrochloride 10% (50 mg/kg B.W) and xylazin 2% at a dose (5 mg/kg body weight) was given to all animals ,then under well sterilized condition incisional wounds of about 2cm were made on skin on either side of cheek ,left side was left to heal normally as control group while right side was treated with local application of FV extract,at day of scarification according to healing intervals (3,7 and 10 and14days),the specimens were put in 10%formalin and routin procedure was carried out to prepare histological H&E stained sections which were examined by light microscope.The other sections were prepared for the immunohistochemical study of the TNF-α expression by using the objective lens (power x 40)
and scoring was done as the following: cells with positive stain were counted at a 5 representative fields (x40) for all of the healing periods. The percentage of the positive cells for the interested protein was scored and it was visually estimated within the selected fields. By dividing the total number of the stained cells by the total number of the cells that are present and then multiplying the value by 100 giving the approximate percentage of the cells that are positively stained. The scores were: 0 (no stain), 1 (<25%), 2 (25-50%) 3 (>50 %) stained cells in two sections [4].

Results

Histological findings

Three days duration

Regarding control group the histological examination of skin sections at wound site of control 3days duration showsnecrotic tissue accumulation at wound surface,other view shows migration of keratinocytes at ,inflammatory cells distributed in connective tissue(figures1,2).Experimental

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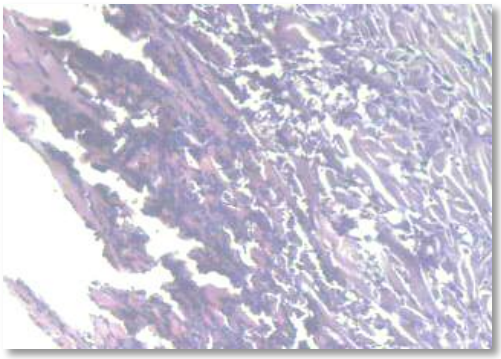
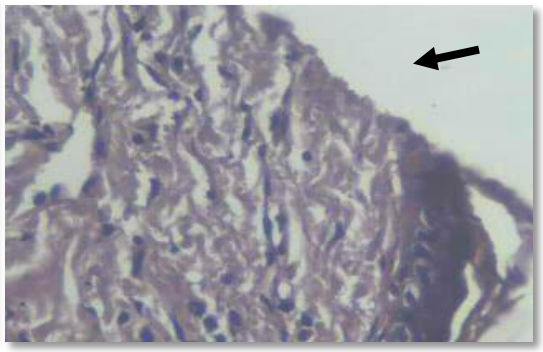
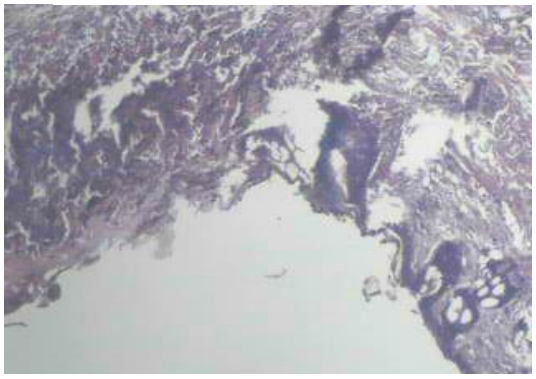
group:After 3days of local application of FV extract the histological examination illustrated thin layer of keratinocytes at surface of wound, disorganized granulation tissue ,whereas other view shows inflammatory cells recruitment is noticed at wound site ,collagen fibers associated with fibroblasts(figures3,4).



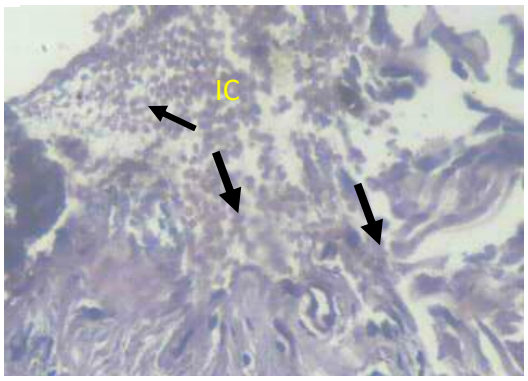
Fig(1)view shows necrotic tissue and exudates at wound edge.HEX10



Fig(2) view of controll group shows migrating keratinocttes at wound edge(arrow).HEX20



Fig(3)view of experimental group shows wound edge sealed by new proliferating cells.HEX10



Fig(4) view of experimental group shows inflammatorycell(ic) ,fibroblasts(arrow).HEX40

Seven days duration

View of control group shows thin epithelial layer at surface ,remodeling granulationtissue ,fibroblasts andcollagen fibers is noticed .Magnified view fibroblasts arranged alongside the dense fibers(figures5,6). Experimental group : wound surface of 7days duration appears sealed by newly migrating epitlium and large number of fibroblasts seen associated with well defined remodeling collagen fibers(figures7,8)

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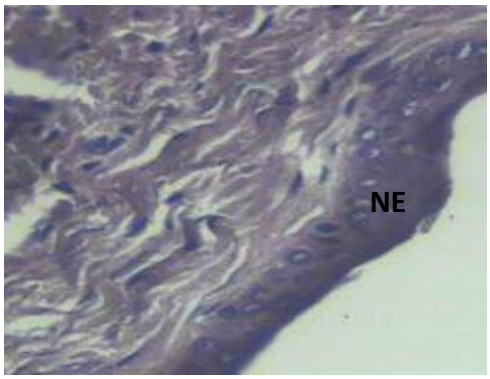
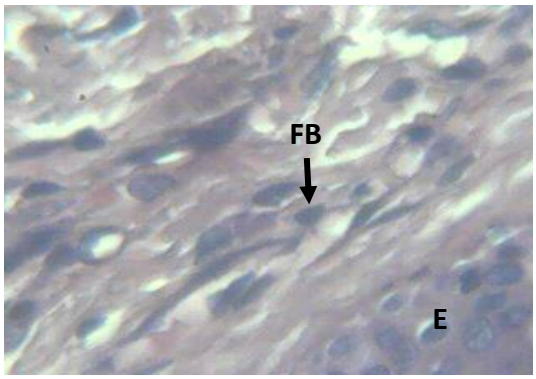
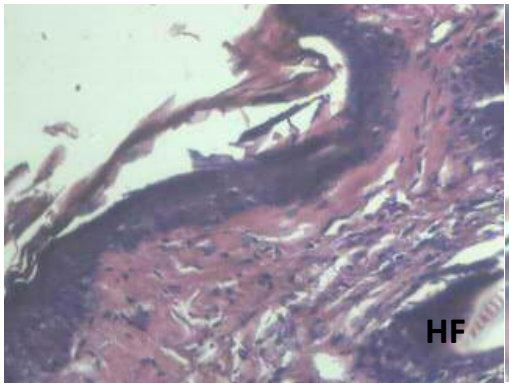


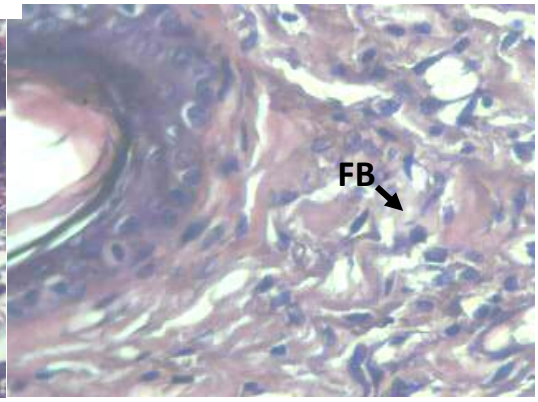
Fig(5) view of control group shows newly formed epithelium (NE).HEX20.



Fig(6) magnified view of control group shows collagen fibers and fibroblasts (FB).HEX40.



Fig(7) experimental group shows wound site covered by epithelium developing hair follicle(HF).HEX20.



Fig(8) magnified view shows remodeling collagen fibers and fibroblasts(FB),blood vesseles

Ten days duration

Control group new thin epithelium is noticed at wound surface of control group after 10 days duration,dense collagen fibers that are associated with fibroblasts ,besides new hairfollicles are seen in connective tissue and congested blood vessel(figures9,10).Experimental group:After 10 days of FV application the new epithelium seen underlined by well organized connective tissue of dermis ,hair follicles also noticed(figures11,12).

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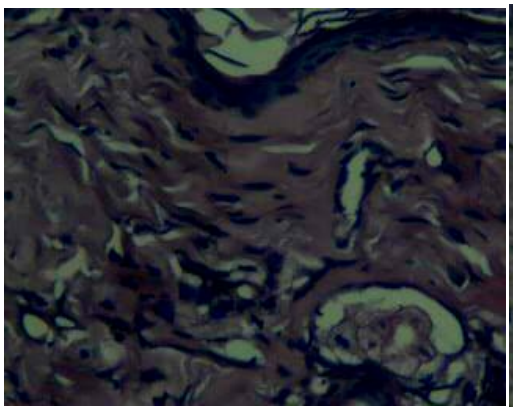
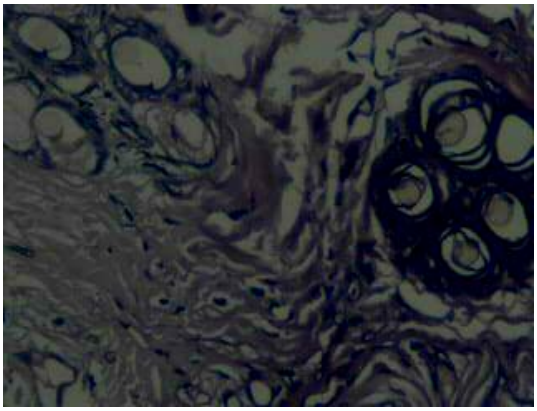
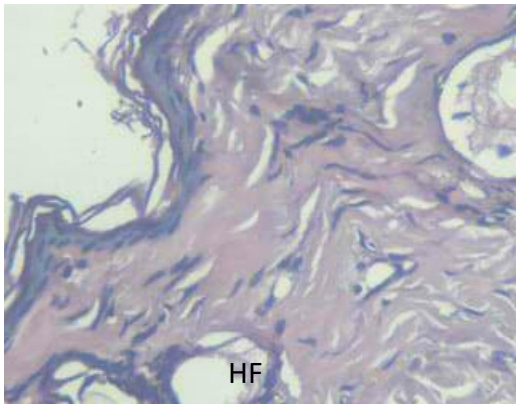


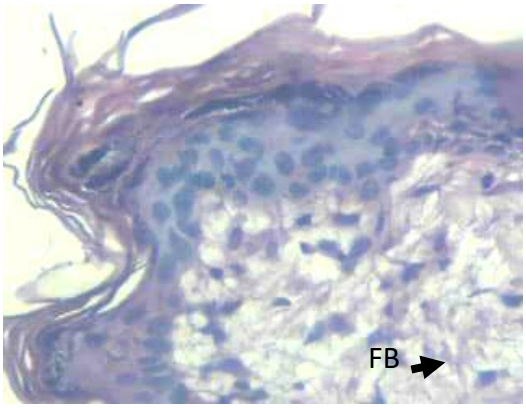
Fig(9) magnified view shows remodeling collagen fibers and fibroblasts (FB).HEX40.



Fig(10) magnified view shows remodeling collagen fibers and fibroblasts (FB).HEX40.



Fig(11) view shows dense collagen fibers and fibroblasts (FB), hair follicls(HF).HEX20.



Fig(12) view shows numerous fibroblasts remodeling collagen fibers and fibroblasts (FB).HEX20.

Fourteen days duration

Section of wound site of control group shows (figures 13,14). Experimental group: histological examination of wound site of 14 days duration shows complete closure of defect site by thickened new formed epithelium and the connective tissue is well organized , mature blood vessls(figures 15,16).

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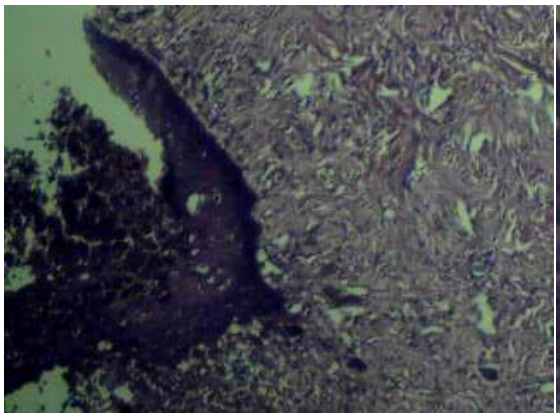
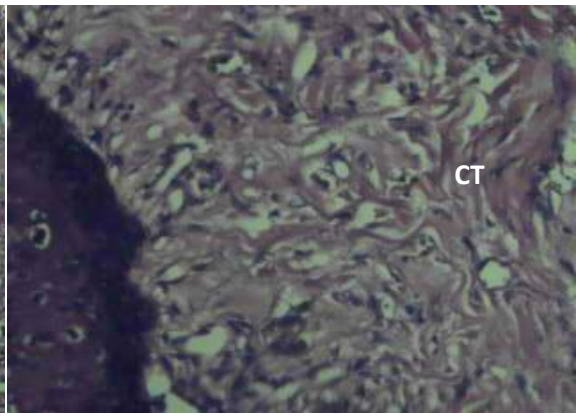
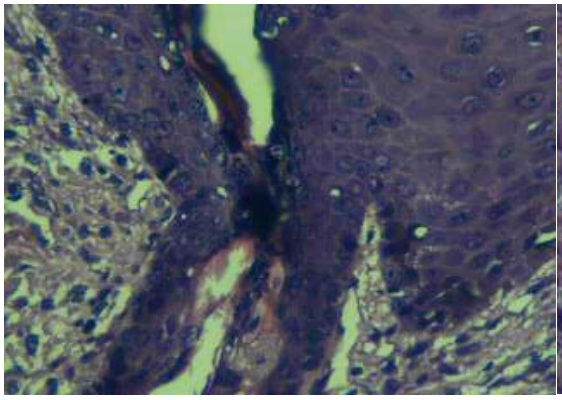


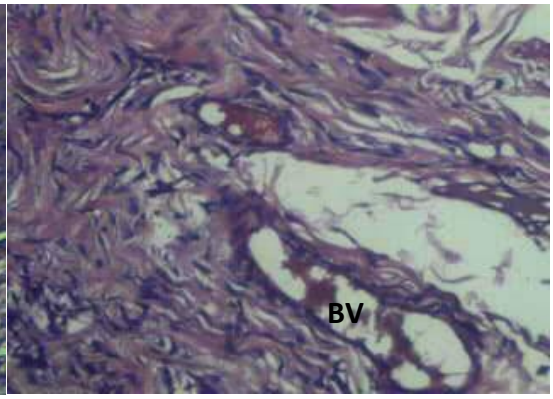
Fig(13) Control group showed incomplete Epithelial formation,HEX20



Fig(14) Control group showed connective tissue (CT) with fibroblast cell,HEX40



Fig(15) experimental group showed complete Formation of epithelial layer,HEx40

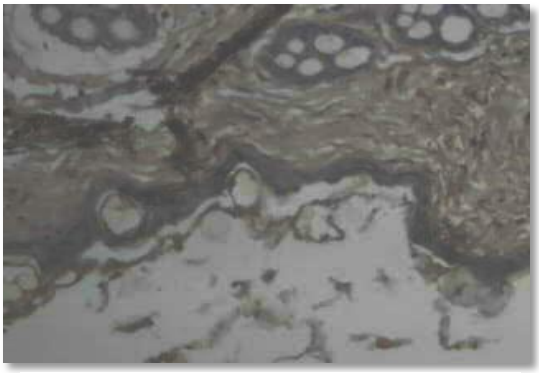


Fig(16) experimental group showed mature Blood vessel(BV),HEX40

Immunohistochemical results

Three days duration

Microphotograph views of control and experimental groups show positive immunohistochemical localization of TNF- α by epithelial cells ,fibroblasts and collagen fibers(figures21,22)



Fig(21) positive expression of TNF-α by new epithelium and fibrous tissueof dermis of control group.Hex10

Fig(22) view of experimental group indicates posive stained fibroblasts, collagen fibers and epidermis.Hex2

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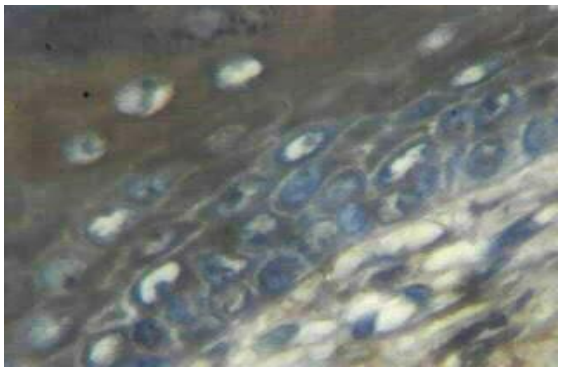
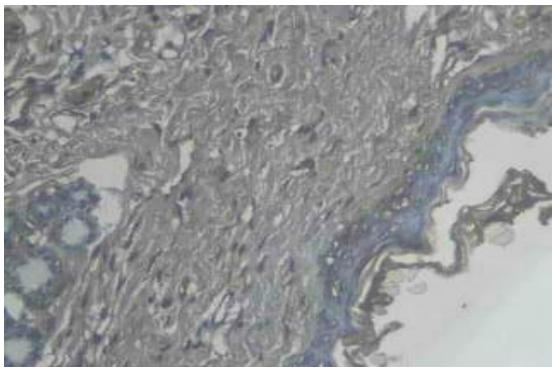
Seven daysduration

Positive expression of TNF- α by epithelial cells of epidermis and collagen fibers, of control group whereas section of experimental group shows positive expression by dermal fibroblasts collagen fibers and basal cell layer of epidermis(figures23,24).



Fig(23)positively stained dermal cells are seen in control group .Hex40.



Fig(24) view of experimental group shows positive expression by fibroblasts collagen fibers and basal cells of epidermis.Hex20.

Ten days duration

Localiztion of TNF-α is detected in epidermis and collagen fibers of dermis of control group,whereas view of experimental group showspositiveexpression by epithelial cellsofbasal layer and fibroblasts and collagen fibers of dermis(figures25,26).

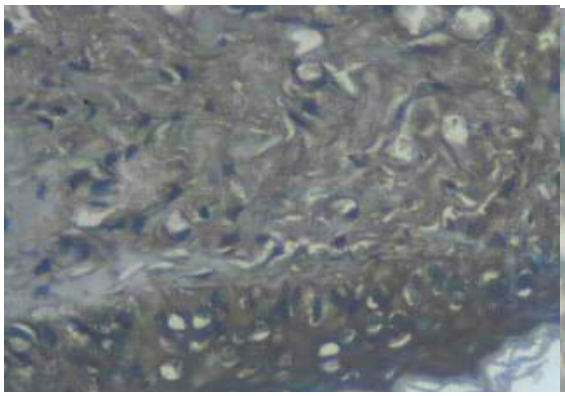
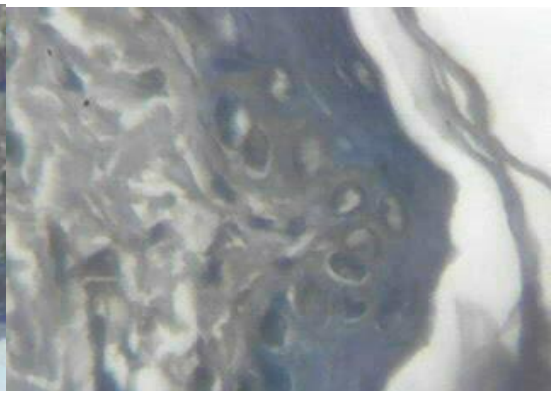


Fig (25) view of control group shows positively stained collagen fibers and epithelial cells.Hex20



Fig(26) experimental group shows positive expression in dermal fibrous tissue and epidermal epithelium.Hex40.

Fourteen days duration

After 14 days positively stained fibroblasts and collagen fibers of dermis is shown in control group section and positively stained epithelial cells as well as collagen fibers detected in experimental group(figures 27,28)

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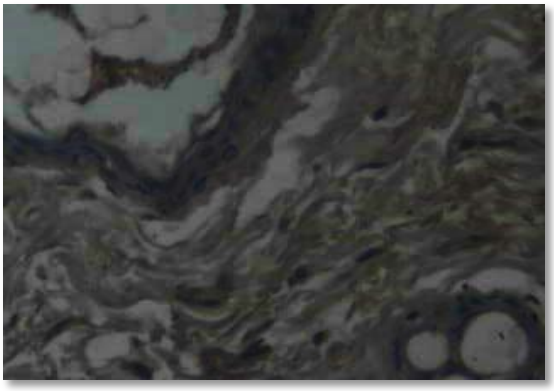
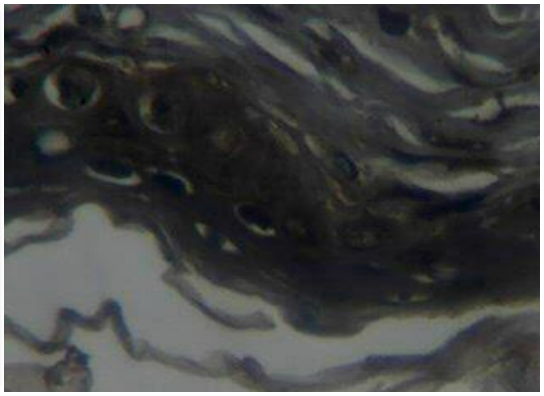


Fig(27) view of control group shows positive expression detected by fibrous connective tissue Hex20



Fig(28) view shows positive reaction detected by epithelial cells and collagen fibers in experimental group.Hex40

Scoring

The table (1) shows the variables time periods (3 days – 1week – 10days – 14days) from control and experimental**.**

Report

time periods		control	experimental
3days	Mean	774.3333	893.8333
	N	6	6
	Std. Deviation	274.58599	181.86084
1week	Mean	187.3333	383.3333
	N	6	6
	Std. Deviation	160.12079	73.57083
10days	Mean	205.1667	425.8333
	N	6	6
	Std. Deviation	151.99529	79.13891
14days	Mean	135.1667	135.5000
	N	6	6
	Std. Deviation	22.43583	35.28597
Total	Mean	325.5000	459.6250
	N	24	24
	Std. Deviation	312.82027	297.32924

p.value control = 0.00366<0.05 sig p.value experimental= 0.00108<0.05 sig

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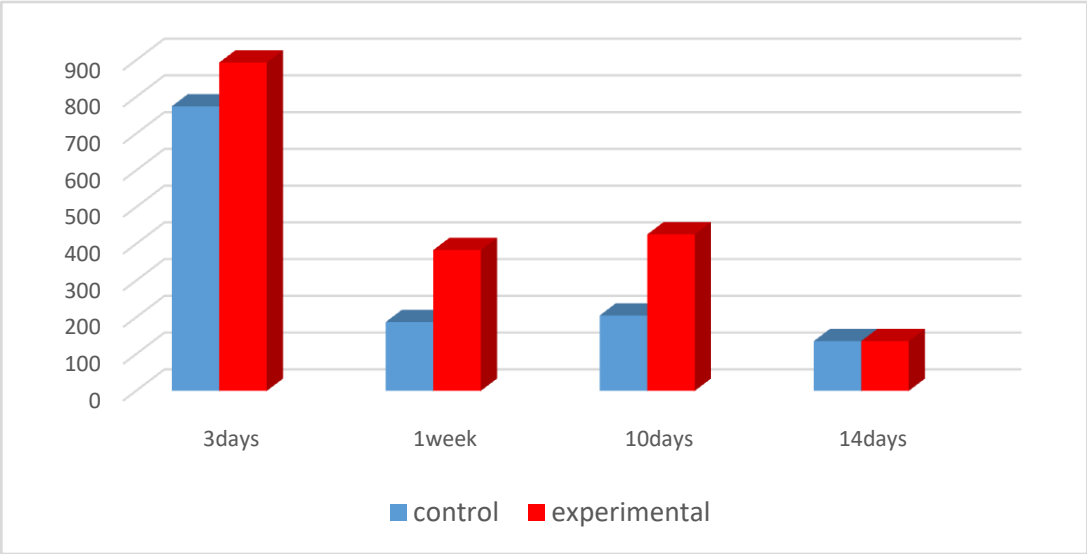

The figure (1) show variables time periods from control and experimental

The table and figure show variable time periods (3day) higher than other variants of mean and std. deviation from control and experimental.

The table (2) shows the variables time periods (3 days – 1week – 10days – 14days) from immuno control and immuno experimental.

Report

time periods		immuno control	immuno experimental
3days	Mean	.8533	.8417
	N	6	6
	Std. Deviation	.07659	.08909
1week	Mean	.7000	.7650
	N	6	6
	Std. Deviation	.07483	.05089
10days	Mean	.6100	.6133
	N	6	6
	Std. Deviation	.10488	.06623
14days	Mean	.9067	.9150
	N	6	6
	Std. Deviation	.06088	.03674
Total	Mean	.7675	.7837
	N	24	24

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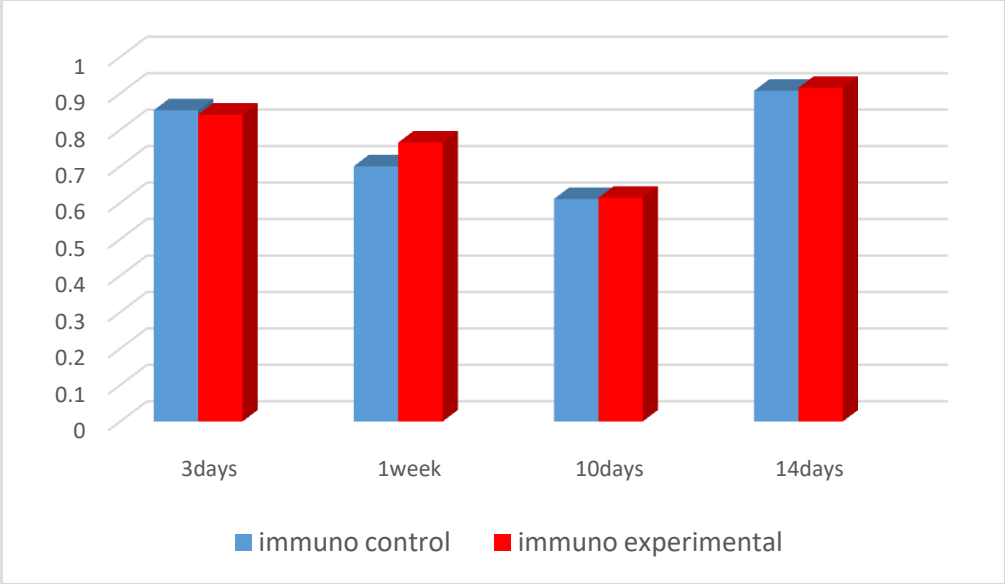
Report

time periods		immuno control	immuno experimental
3days	Mean	.8533	.8417
	N	6	6
	Std. Deviation	.07659	.08909
1week	Mean	.7000	.7650
	N	6	6
	Std. Deviation	.07483	.05089
10days	Mean	.6100	.6133
	N	6	6
	Std. Deviation	.10488	.06623
14days	Mean	.9067	.9150
	N	6	6
	Std. Deviation	.06088	.03674
Total	Mean	.7675	.7837
	N	24	24
	Std. Deviation	.14256	.12873

p.value immune control= 0.00688 sig p.value immune experimental= 0.00107 sig



The figure (2) show variables time periods from immuno control and immuno experimental.

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The table and figure show asymptotic ratio to variable time periods of mean and std. deviation from immuno control and immuno experimental.

The table (3) show one – sample test to (control, experimental, immune control, immune experimental.

One-Sample Test

	Test Value = 4					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Control	5.098	23	0.00366	325.50000	193.4076	457.5924
experimental	7.573	23	0.00108	459.62500	334.0739	585.1761
immuno control	26.374	23	0.00688	.76750	.7073	.8277
immuno experimental	29.826	23	0.00107	.78375	.7294	.8381

Discussion

This study was designed to evaluate the effect of topical application of flavonoid extract on experimentally induced incisional wound on facial skin of rabbits. Adult rabbits were selected because they provide many desired characteristics to fulfill the requirement of the study.

Histopathological examination. At day 3, dermis seen uncovered by epidermis. Defect site is filled with necrotic debris and lined by proliferating granulation tissue, The dermis shows, vascular congestion , with some extravasated erythrocytes (haemorrhages),also migrating newly proliferated keratinocytes ,numerous inflammatory cells distributed in dermis in agreement with histological resuts of **Bhaskar and Nithya ,2012**, who investigated wound-healing potency of the ethanolic extract of the flowers of Hibiscus rosa sinensis on Wistar albino rats and found that the extract increased cellular proliferation and collagen synthesis at the wound site attributed to its high flavonoid content with antimicrobial property.

As reported by **Builders etaL.,in 2013**who examined the contribution of the cytokine to the early healing process by using a mouse model with a full-thickness skin wound, and it was found that the expression of the TNF-α was immediately detected after the wound creation, it was increased during the early several hours, reaching a peak level on the first day, then it decreased to a basal level in a line with the present immunohistochemical results whichfound that the TNF-α was involved early in the process of the wound healing.

During late days 7 ,10and 14 days positive expression of this protein was detected by epithelial and connective tissue cells with variable intensities which could be due to secretion activity of increased proliferating epitheial and fibroblast cells after application of FV extract, may be explained that the TNF-α is rapidly released by the keratinocytes, vascular endothelial cells and the fibroblasts in the area of injury, and this can lead to the initiation the inflammatory phase by the promotion of therecruitment of the inflammatory leukocyte into the wound tissues [(8)].

Wound healing accelerated in experimental group than in control group because of anti-microbial and anti-bacterial effect of flavonoid extract of Hibiscus sabdariffa(17) also may be because of antioxidant effect(18)

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Conclusion

The results obtained in this study indicated that flavonoid extract from hibiscus sabdariffa had good potential activity in enhancing cutaneous wound healing.

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