

EFFECT OF B-SITOSTEROL ON HEALING OF INDUCED ORAL TRAUMATIC ULCER IN ALBINO RATS

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ABSTRACT

Background: Oral ulcerations frequently manifest as symptoms of a diverse range of diseases affecting the pediatric oral cavity, with numerous causative factors involved. The diagnostic process for these lesions can be particularly complex for clinicians, owing to the similarities in clinical and histopathological characteristics across various ulcerative conditions. So, this research aims to assess the impact of β -Sitosterol, administered orally, on the healing process of experimentally induced traumatic ulcers in albino rats.

Materials and methods: 30 adult albino rats were randomly divided into five groups. 1-Negative control group: (n=6), no ulcer or treatment, 2-Positive control group: (n=6), labial traumatic ulcers were induced, and the rats were euthanized after 2 hours. 3-Self-recovery group: (n=6), induction of traumatic ulcer in labial mucosa of rats, then euthanization after 10 days for self-recovery evaluation. 4-Olive oil treated group: (n=6), induction of labial traumatic ulcer, after 24 hours' ulcer induction, the animals were systemically administrated olive oil by oropharyngeal tube for 10 days. 5-B-Sitosterol treated group: (n=6), induction of labial traumatic ulcer, after 24 hours of ulcer induction, β -Sitosterol of dose 5m/kg, was systemically administrated by oropharyngeal tube for 10 days. Clinical and histopathological, histochemical and PCNA-immuno-histochemical assessments were used for ulcer healing evaluation.

Results: β -Sitosterol-treated group shows promising healing results in short time comparing to other study groups in albino rates.

Conclusion: oral administration of β -Sitosterol may be a valuable alternative therapy to conventional existing oral drugs used for treatment and healing acceleration of oral ulcers.

KEYWORDS: β -Sitosterol, Olive oil, traumatic ulcer, albino rats and ulcer healing.

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INTRODUCTION

The oral mucosa functions as a shield, safeguarding against harm, infectious agents, and carcinogens. It is prone to various types of lesions and conditions, with a spectrum that includes both benign and potentially serious complications^[1]. As outlined by Greenberg^[2], oral traumatic ulcers represent a distinct category of ulceration within the oral mucosa, triggered by physical injuries. Manifesting as open sores on the mucosa, these ulcers can lead to considerable pain for patients and increase the risk of severe infections and further complications. Notably, such ulcers are more commonly observed in children^[3]. The causative traumas can be thermal, chemical, or mechanical, resulting from exposure to heat, acidic substances, or physical pressure. Recurrent traumatic ulcers, such as bite injuries, are common in children with autism spectrum disorder, intellectual disabilities, or during seizure episodes^[4]. Other sources of repetitive trauma include ill-fitting dental braces or aggressive toothbrushing. Patients with nocturnal bruxism may also experience recurrent ulcers, along with symptoms like damaged tooth enamel or tongue deformations. Typically, benign and self-limiting, traumatic ulcers often appear on the lateral tongue but can also affect the gingiva and other oral mucosal areas^[5]. These ulcers usually present as sharp, well-defined lesions, often associated with trauma, especially in young children during teething^[6].

Oral traumatic ulcers most commonly occur on the buccal mucosa (28.5%), tongue (16.6%), and lower lips (8.3%). The primary management of these ulcers involves removing causative factors, treating predisposing conditions, avoiding triggers, and maintaining oral hygiene. Topical treatments focus on preventing superinfection, providing analgesia, reducing inflammation, and treating active ulcers^[8].

Chlorhexidine mouthwashes, topical antibiotics like doxycycline and minocycline, and bio-adhesive pastes containing 20% benzocaine are used for pain

relief and protection. Lidocaine and diclofenac with hyaluronic acid are also effective for temporary analgesia and anti-inflammatory action, respectively. Topical steroids like betamethasone and fluticasone are successful in treating active ulcers^[6]. In cases where local treatment is insufficient, systemic therapy is considered, taking into account the patient's age and compliance^[9].

In medical application, many medications that enhance wound healing are extensively utilised. Chemical-derived treatments have β -Sitosterol adverse effect risks and high costs^[4]. As a result, research into new β -Sitosterol capable of boosting wound healing is still required. Natural treatments for wound healing have been increasingly popular in recent years. Although numerous phytochemicals claim to have wound healing qualities including antioxidant, anti-inflammatory and antimicrobial properties, the majority of them lack well-controlled scientific data to back up their claims^[10]

Phytosterols, naturally occurring plant-based compounds, are found in a variety of sources including vegetable seeds, oils, nuts, legumes, fruits and cereals^[11]. One of these phytosterols, β -Sitosterol, known chemically as 24-ethyl-5-cholestene-3-ol, stands out as a bioactive phytosterol prevalent in plant cell membranes, bearing a structural resemblance to cholesterol produced by mammalian cells. These compounds are abundant in lipid-rich plant foods such as nuts, seeds, legumes, and olive oil. In the context of human herbal nutrition, β -Sitosterol, along with campesterol and stigmasterol, are the principal phytosterols, constituting approximately 65%, 30%, and 3% respectively^[12]. Extensive scientific literature has acknowledged β -Sitosterol for its diverse biological activities, including antinociceptive^[13], anxiolytic, and sedative properties^[14], along with anti-apoptotic^[15], immunomodulatory^[16], antimicrobial^[17], anticancer^[18], anti-inflammatory effects. It is also noted for its protective role against non-alcoholic fatty liver diseases^[19], lipid-

lowering capabilities ^[20], hepatoprotective properties ^[21], benefits in respiratory diseases ^[22], wound healing effects ^[23], antioxidant ^[24] properties, and anti-diabetic activities ^[25].

Research conducted by Babu and colleagues highlighted that β -Sitosterol, when applied in a model of 1,2-dimethylhydrazine-induced colon carcinogenesis in rats, leads to an increase in both enzymatic and non-enzymatic antioxidants ^[26] through the activation of the estrogen receptor/PI3-kinase pathway, suggesting that β -Sitosterol acts as a scavenger of reactive oxygen species (ROS) ^[27].

Novotny and colleagues have identified phytosterols, including β -Sitosterol, as significant contributors to human nutrition and as agents with potential anticancer properties. The advent of advanced instrumental analytical techniques has opened new avenues for exploring the behaviour and mechanisms of β -Sitosterol's action within the human body. It is understood that β -Sitosterol influences numerous metabolic systems. However, its application in cancer therapy is hindered by two primary limitations: its comparatively low activity and its structural similarity to cholesterol, which is a common component in the daily diet. This similarity may cause an overlap between β -Sitosterol's cholesterol-modulating and anticancer activities ^[28].

Within the pharmaceutical industry, the distinct biological and physicochemical characteristics of β -Sitosterol are greatly unique. Studies have shown that β -Sitosterol exhibits extensive anti-inflammatory properties in various tissue types. Its effectiveness is evident in different models of inflammation, such as inflammation related to chronic obesity, lung inflammation induced by ovalbumin, colitis caused by 2,3,4-Trinitrobenzene sulfonic acid, and rheumatoid inflammation observed in mice ^[29]. However, there is a noticeable gap in research regarding its anti-inflammatory influence on the healing process of oral ulcers. Therefore, this research is focused on exploring

the effects of β -Sitosterol on the healing of labial mucosal traumatic ulcers.

Null Hypothesis: oral administration of β -sitosterol has no effect on the healing of oral traumatic ulcer.

MATERIALS AND METHODS:

Ethical Approval

This study received the necessary ethical clearance from the Faculty of Dentistry's Institutional Research Ethics Committee (REC) at Suez Canal University, Egypt (Ethics Approval Reference: 702/2023). The research procedures adhered strictly to the guidelines established by the World Health Organization in 2011.

Sample size determination:

The calculation of the required sample size was performed using the G*Power statistical power analysis program (version 3.1.9.7), in line with the guidelines provided by Faul et al. (2007). The experimental design necessitates the use of 30 rats, divided equally into five distinct groups. This number of subjects is sufficient to detect a significant effect size of 0.70, ensuring an actual power (1- β error probability) of 0.8 (80%) and a significance level (α error probability) of 0.05 (5%) for conducting a two-sided hypothesis test.

Sample selection and grouping:

In this experiment, thirty male albino rats, each weighing between 150 to 200 grams, were utilized. They were accommodated in groups of three per cage throughout the study duration, with unrestricted access to standard chow and tap water. The rats were maintained in climate-controlled environments, with room temperatures set between 21 to 23°C and relative humidity levels kept at 60-65%. Additionally, their housing conditions included a consistent cycle of 12 hours of light followed by 12 hours of darkness.

All rats were numbered from 1 to 30, and then were randomly divided by the web site by <https://www.randomizer.org/> into five groups as follows:

- 1- **Negative control group:** (n=6), no ulcer or any treatment was done.
- 2- **Positive control group (Zero-day ulcer):** (n=6), labial traumatic ulcers were induced, and the rats were euthanized after 2 hours.
- 3- **Self-recovery group:** (n=6), induction of traumatic ulcer in labial mucosa of rats, and animals were euthanized after 10 days [30] for self-recovery evaluation.
- 4- **Olive oil treated group:** (n=6), induction of labial traumatic ulcer, after 24 hours' ulcer induction, the animals were systemically administrated olive oil (a volume equivalent to drug in group IV) by oropharyngeal tube for [31] 10 days [32].
- 5- **β -Sitosterol treated group:** (n=6), induction of labial traumatic ulcer, after 24 hours of ulcer induction, β - sitosterol, dissolved in olive oil of dose 5m/kg [33] systemically administrated by oropharyngeal tube [31] for 10 day [32].

Preparation and characterization of β -Sitosterol

β -Sitosterol A.R (C₂₉H₅₀O) (Euromedex, France) was supplied in powder form and dissolved in olive

oil (32,33) in a concentration of 0.25% (4.14 mg/10 ml). The olive oil was obtained from The Green Gold Olive Oil Company, Egypt. The prepared β - sitosterol was characterized using UV-visible spectroscopy.

Traumatic ulcer induction procedure

Initially, a clinical assessment was carried out to determine the integrity of the labial mucosa in the area of interest. The albino rats assigned to the positive control, self-recovery, olive oil, and β -sitosterol groups were anesthetized using a freshly prepared mixture of ketamine (90 mg/kg, Medistar, Ascheberg, Germany) and xylazine (10 mg/kg, Riemser, Greifswald, Germany), administered intraperitoneally. Subsequently, each rat underwent an oral cavity antiseptis procedure using 0.12% chlorhexidine, applied with individual, single-use cotton swabs. Tissue Punch biopsy was used for inducing traumatic circular ulcer of 4 mm diameter with 1 mm depth in labial mucosa (34,35) (Fig.1).

Assessment methods:

- 1- **Clinical observation:** of ulcer healing had been done by photographs at the 3rd, 7th, and the 10th days of experiment by Samsung Galaxy S22 Ultra rear camera phone (12 MP). Upon completion of the experiment, the rats were



Fig. (1) A photograph showing (A) Normal intact labial mucosa (B) The traumatic ulcer created by punch biopsy (Zero-day ulcer)

humanely euthanized using the method of cervical dislocation.

2- Histopathological, Histochemical and PCNA-Immunohistochemical examination: labial mucosal samples were obtained at the end of experiment (after euthanizing at 10th day) and fixed in 10% buffered formic acid.

- Preparation of tissues for histopathological and Histochemical evaluation:

The collected specimens were subjected to standard processing techniques and sectioned into slices measuring 4–6 μ m in thickness. For histological analysis, these sections were stained using haematoxylin and eosin (H&E). Additionally, Masson's trichrome and Periodic Acid-Schiff (PAS) staining methods were employed for detailed histochemical examinations.

- The immuno-histochemical detection system of proliferating cell nuclear antigen (PCNA):

Adhering to the instructions provided by the manufacturer, the ultra-vision mouse tissue detection system was utilized for this study. This system is inclusive of a monoclonal antibody designed to couple with the primary antibody, specifically the PCNA monoclonal antibody. The constituents of the kit together establish a streptavidin-biotin based immunoenzymatic antigen detection system. The procedure involves sequential incubation: initially with a primary antibody that is not conjugated and specific to the target antigen, followed by the addition of a biotinylated secondary antibody that interacts with the primary antibody. Subsequently, this complex is treated with an enzyme-conjugated streptavidin and DAB chromogen. A section's positivity or negativity was determined by the presence or absence of brown nuclear staining.

To quantify PCNA staining, the percentage of area showing positive staining was determined using

light microscopy at a standardized magnification (x 200). An image analysis system was used to calculate the Positive Index (PI), aiding in evaluating the percentage area of cells that stained positively. This analysis was performed with the aid of a Leica Quin 500 computer system (located in Wetzlar, Germany), which includes a color video camera, a color monitor, and a CPU from an IBM personal computer, all linked to the microscope. The image analysis system was initially calibrated automatically to convert pixel measurements provided by the software into actual micrometers.

Statistical analysis:

The study's data was rigorously collected, processed, organized into tables, and analyzed statistically using relevant tests. The Shapiro-Wilk test was employed to assess the normality of the sample distribution. The calculation of descriptive statistics involved the use of mean and standard deviation (SD). The differences among the groups for each variable were evaluated using One-way Analysis of Variance (ANOVA) tests. To determine the statistical significance across the groups, Bonferroni's post hoc test was applied. The threshold for statistical significance was established at a P value of 0.05. The entire statistical analysis was conducted using the SPSS software (version 26.0, for Windows) from the Statistical Package for the Social Sciences, IBM Corp, Armonk, NY.

Characterization of β -Sitosterol:

Using UV-visible spectroscopy analysis, β -Sitosterol dissolved in olive oil was analysed by measuring UV-vis spectra of the solution. The β -sitosterol spectrum of the sample was taken in the range of 200–800 nm using Thermo scientific UV-vis spectrophotometer. The maximum values for β -Sitosterol and olive oil were at the expected ranges as reported by previous researchers (625 nm and 400 nm respectively) (36, 37) (Fig. 2)

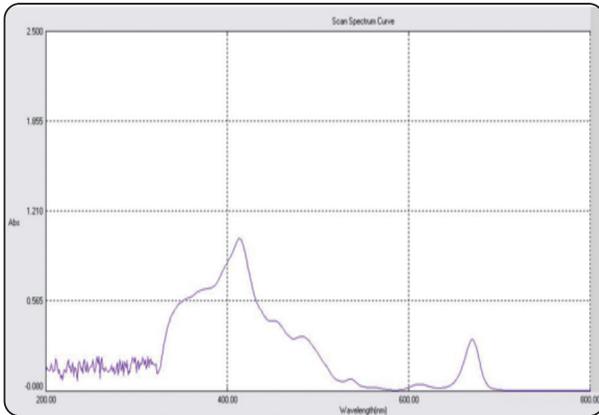


Fig. (2) Ultraviolet spectral analysis of β -Sitosterol dissolved in olive oil between 200-800 nm

RESULTS

Clinical observation:

The rates were clinically examined throughout the experiment to follow up the healing process. Clinical records for ulcers were done by photographs at the 3rd, 7th, and the 10th day.

Negative control group: The normal labial mucosa revealed intact mucosal surface (**Figure 1**).

Positive control group: the ulcer appeared erythematous with raised edges (**Figure 1**)

Self-recovery group: animals showed delayed healing through the days of experiments where, the ulcer exposed with eroded surface and necrotic margin and contraction of ulcer size had been noticed.

Olive oil treated group: had eroded surface till day 7, while at day 10 appeared with white peel at the ulcer site. However, **β -Sitosterol treated group** showed marked healing clinically in labial mucosa from day 7 with decreasing in the ulcerated surface; however, labial mucosa at site of ulcer appeared clinically normal with intact mucosal surface at day 10 (figure 3).

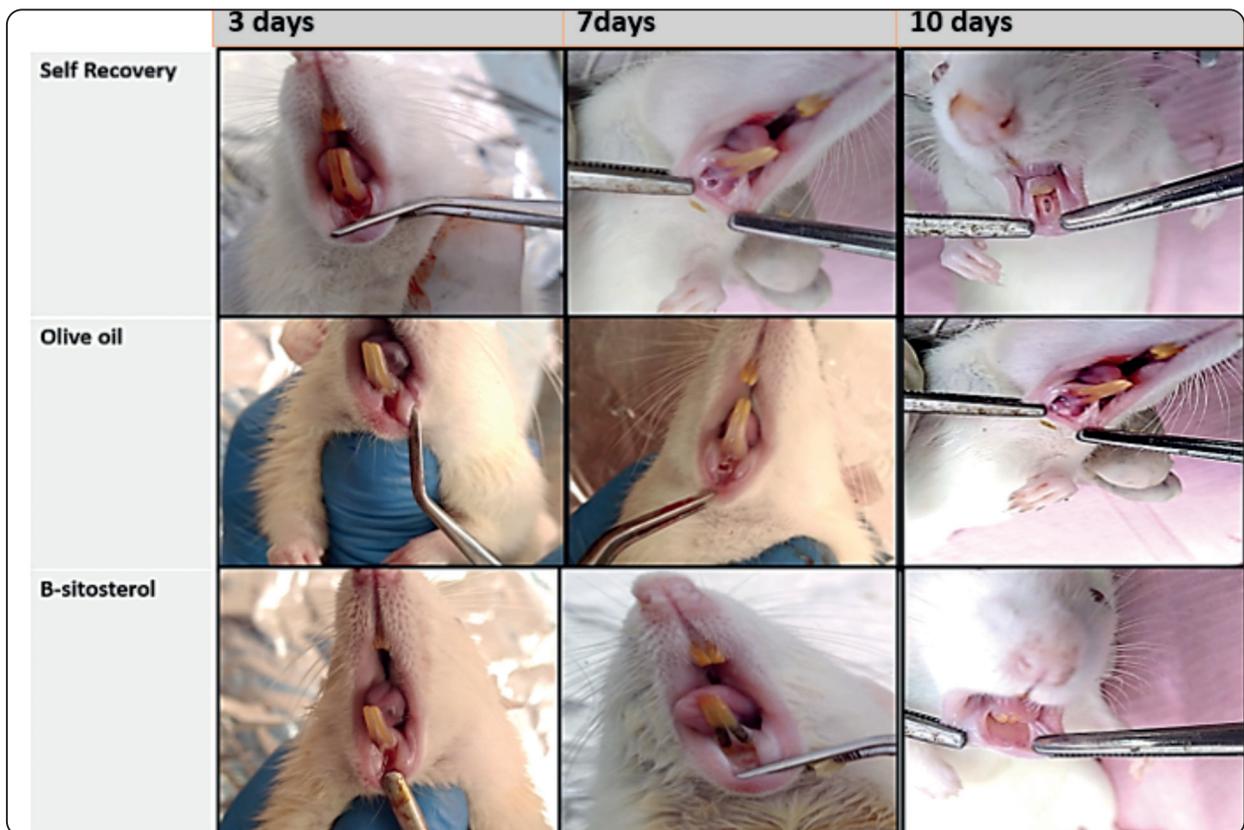


Fig (3) Photographs of traumatic ulcer of self-recovery and treated groups at 3, 7, and 10 days.

Histopathological, Histochemical and Immunohistochemical observations:

A- Histopathological (H&E) observations (Figure 4):

Negative control group: For the negative control group, examination of the normal labial mucosa displayed a keratinized stratified squamous epithelium characterized by regular, wide, and short rete ridges. The underlying lamina propria contained well-organized collagen fibers and blood vessels of relatively normal size.

Positive control group (zero-day group): In the positive control group, corresponding to the zero-day group, there was a complete loss of epithelium over the ulcerated area. This was accompanied by infiltration of inflammatory cells and the presence of blood vessels in the thickened lamina propria

below. The periphery of the ulcer was demarcated by keratinized stratified squamous epithelium.

Self-recovery group: For the self-recovery group, a period of 10 days' post-ulcer induction revealed initial signs of re-epithelialization. This was evident through the formation of an epithelial basal layer and prickle cell layer, which contributed to the reduction in ulcer size. The lamina propria underneath showed signs of inflammation with loosely arranged fibers. Additionally, the keratin layer of the adjacent mucosa was observed to be detached.

Olive Oil Treated group:

A reduction in ulcer size was noted, indicative of initial re-epithelialization characterized by the formation of basal and prickle cell layers. The lamina

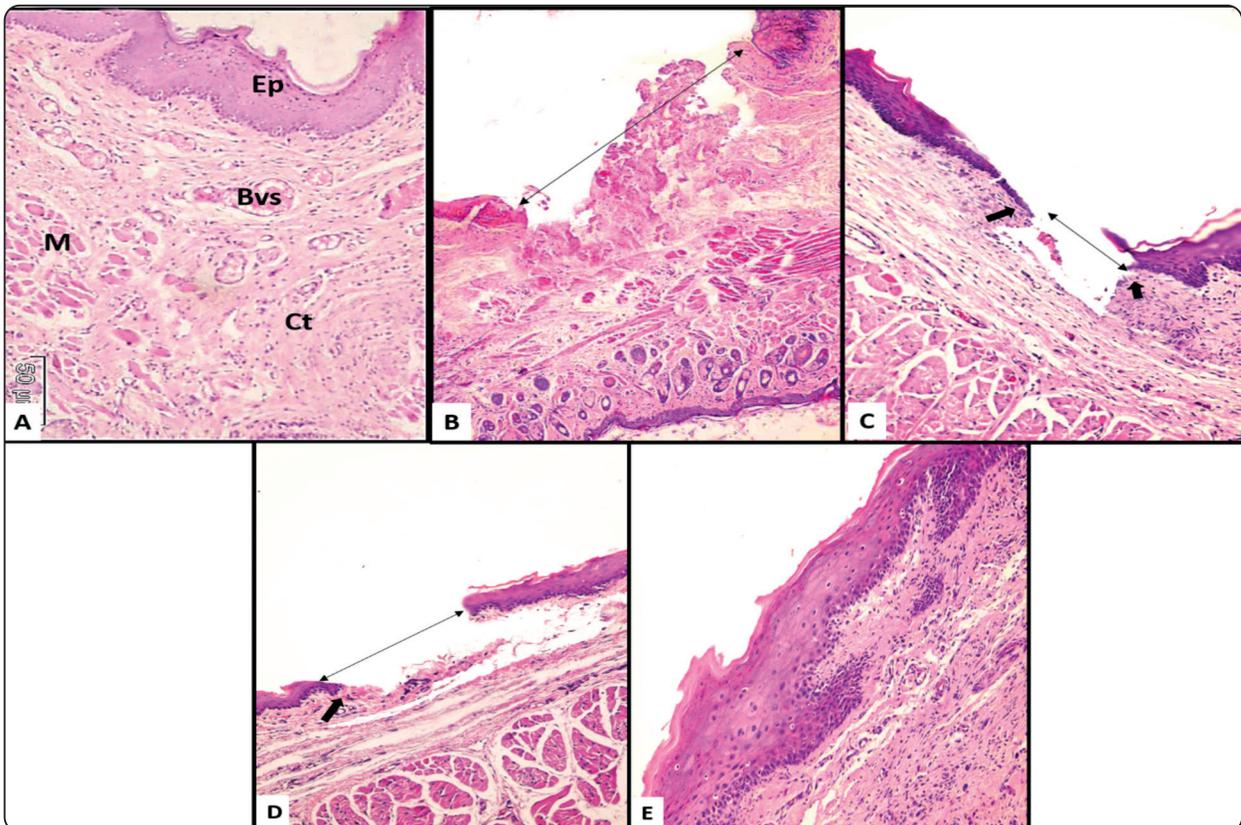


Fig (4) A photomicrograph of H&E-stained sections showing (A) negative control group, (B) Zero-day group, (C) self-recovery group, (D) Olive oil group and (E) β -Sitosterol group. (Ep) epithelium, (Ct) connective tissue, (M) muscles, (Bvs) blood vessel, Ulcer size (Double head arrows), re-epithelialization (black arrows) (H&E mag. X200)

propria beneath exhibited signs of inflammatory cell infiltration. It appeared more organized yet thicker and fibrotic, with localized areas showing fiber dissociation.

β -Sitosterol Treated group:

Observations revealed the formation of a new, continuous epithelial layer covering the ulcer, characterized by keratinized stratified squamous epithelium with the development of short rete ridges. The lamina propria beneath this layer was notably dense and exhibited a nearly well-organized structure, containing numerous newly formed blood vessels and infiltrations of inflammatory cells.

PAS histochemical observations (Figure 5):

In negative control there was intense positive PAS reaction, *in the zero-day ulcer and self-recovery*

group, there was a weak reaction of PAS staining at the surface of the mucosal epithelium surrounding the induced ulcer and negative PAS stain in the ulcer area. *In Olive oil treated group* there was intense positive PAS staining at margin surrounding the ulcer area that showed re-epithalization while ulcer area showed negative PAS stain. In the *β -Sitosterol treated group* the mucosa surface which completely covering the ulcer showed intense positive reaction to PAS stain (**figure 5**).

Masson's trichrome observations:

In negative control group showed densely backed collagen fibres (blue) and normal sized blood vessels (Red). *Zero-day ulcer group* showed areas of loosely disorganized collagen fibres with area fibres content loss at ulcer site while at margin of ulcer the fibres content appeared dense (Blue) with

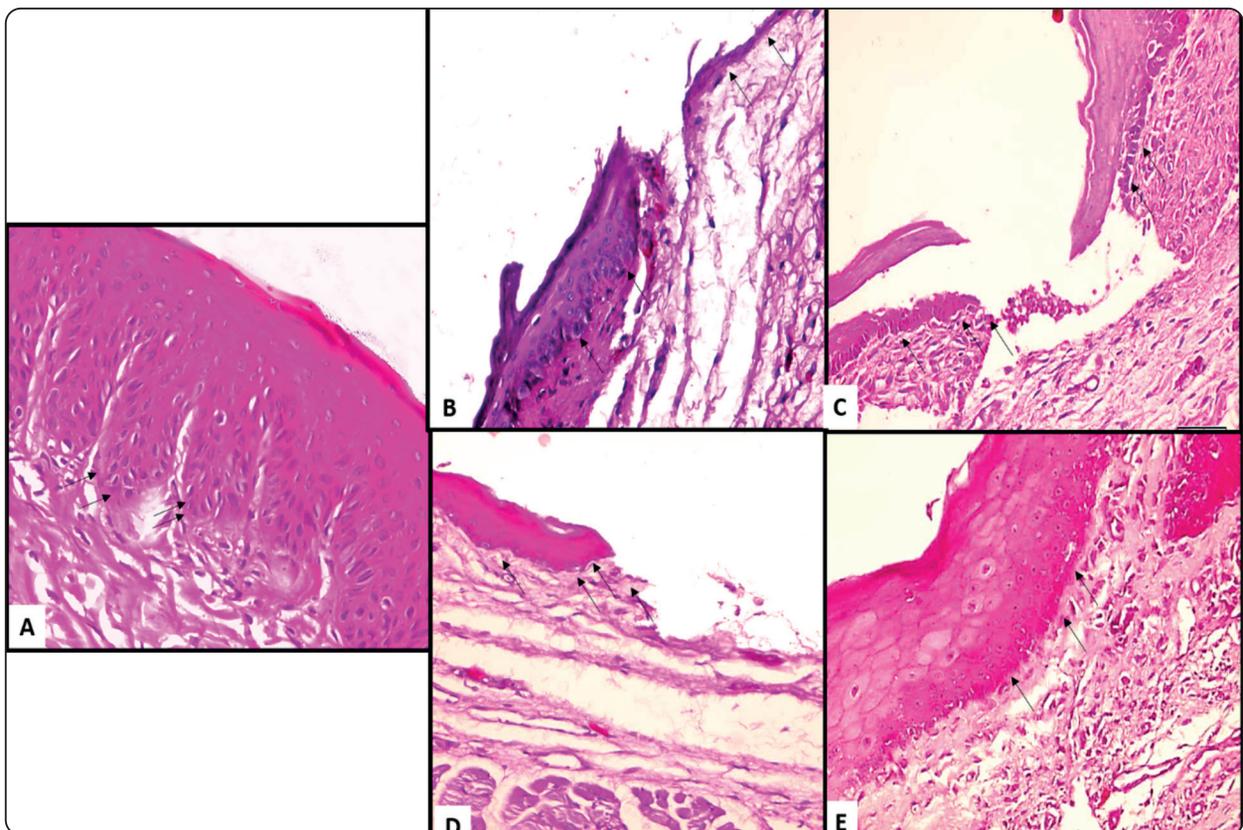


Fig. (5): A photomicrograph of PAS stain showing intense PAS reaction (A) negative control, (B) zero-day ulcer with negative PAS reaction, (C) self-recovery group with positive PAS reaction, (D) Olive oil group with positive PAS reaction and (E) β -Sitosterol group with PAS positive reaction. (PAS Mag. X400)

multiple blood vessels (Red). *Self-recovery group* showed loosely arranged fibres with inflammatory cells and *Olive oil* groups revealed mucosa with inflammation in the sub epithelial region (Red) and both *Self-healing recovery and olive oil treated groups* demonstrated reduced in collagen content under mucosal wound (Faint blue). Also, focal areas

of complete fiber loss and dilated blood vessels (red). However, the *β -Sitosterol group* showed intact mucosal surface (Red), increase in fibres content (Blue) that appeared more organized and high density in deep sub epithelial with new blood vessels formation (Red) Fig (6).

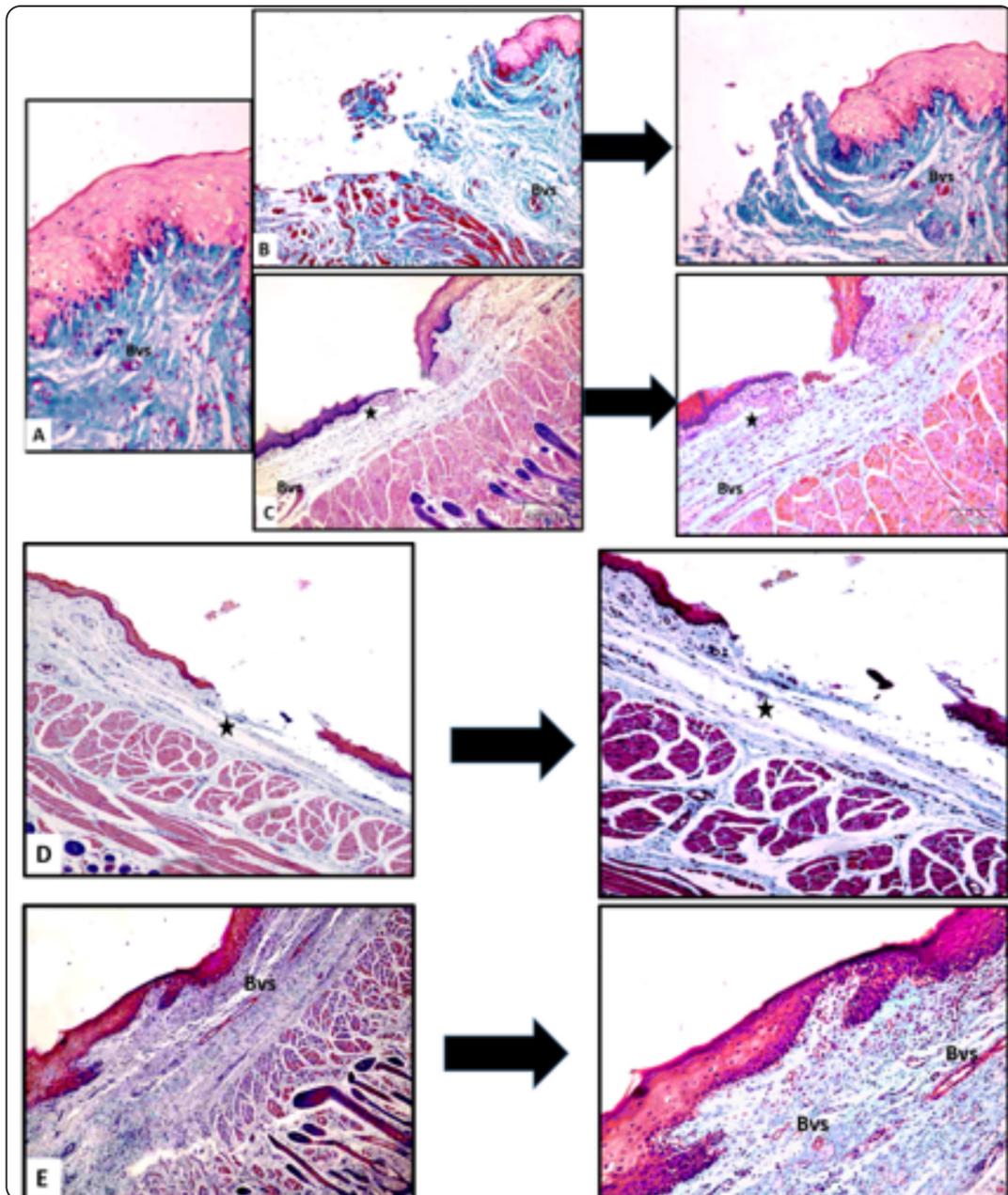


Fig (6): A photomicrograph showing Masson trichrome stain of (A) negative control, (B) zero-day ulcer group, (C) self-recovery group, (D) Olive oil group and (E) β -Sitosterol group with their magnifications. Epithelium (Red), Fibres (Blue), blood vessels (BVs), area of fibre loss (star) (MT Mag. X.100,200).

PCNA Immuno-histochemical observations: (Figure 7)

Negative control group showed positive PCNA-immuno-expression in basal and supra basal cells. Also, connective tissue showed detectable positive reaction. **Zero-day ulcer group** and **self-recovery group** showed positive immuno-expression in the basal cells at epithelial margin of ulcer with

detectable positive cell in the connective tissue. **Olive oil group** showed weak nuclear-positive reaction basal epithelial cells at margin of ulcers with no detectable positive reaction in connective tissue cells. The **β -Sitosterol group** showed positive reaction in the nucleus basal cell of epithelium and at closure site of ulcer with delectable positive reaction in connective tissue.

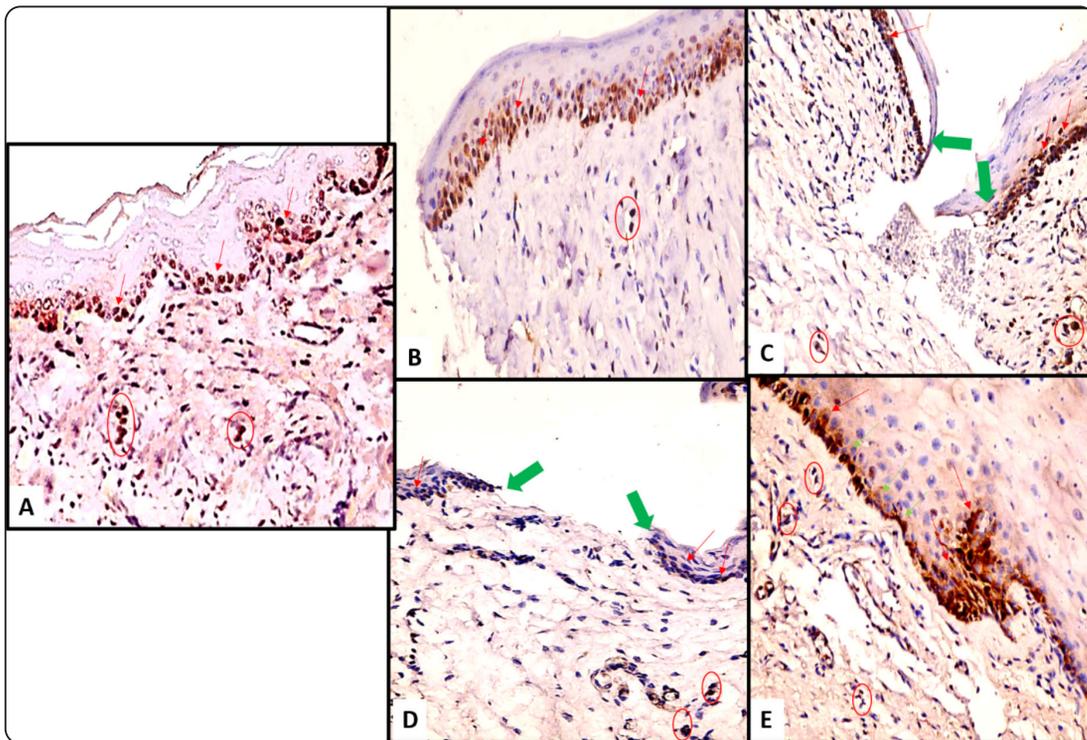


Fig (7): A photomicrograph showing epithelial and connective tissue nucleolar anti-PCNA reaction. (A) negative control, (B) zero-day ulcer, (C) Self- recovery, (D) Olive oil and (E) β -Sitosterol. Note: positive epithelial cells (Red arrows), positive connective tissue cell (red circle), regenerated epithelial cells (green arrows) (Mag. X. 200).

Statistical results:**PAS stain statistical analysis**

The intensity values of the PAS stain, as measured using ImageJ software (refer to Table 1), revealed a significant variation among the study groups. This was determined through a one-way ANOVA analysis ($F=62.93$, $P<0.001$). Upon pairwise comparison, significant differences were noted between the

negative control, positive control, and self-recovery groups. However, no significant difference was observed between the negative control and the β -Sitosterol group, as well as between the positive control and olive oil groups. The highest mean values were seen in the β -Sitosterol group, followed by the negative control, olive oil, and positive control groups, respectively, while the self-recovery group exhibited the lowest value.

TABLE (1) PAS histochemical stain

Groups	Mean	Std. Deviation	F	P value
Negative control	25.5 ^a	1.47		
Positive control	17.94 ^b	2.00		
Self-recovery	14.68 ^c	1.25	62.93	<0.001**
olive oil	19.73 ^b	1.20		
β -Sitosterol	26.44 ^a	1.69		

******, and different letters means significant difference between groups at $P<0.05$

TABLE (2) Masson's Trichrome histo-chemical stain

Groups	Mean	Std. Deviation	F	P value
Negative control	53.21 ^a	2.10		
Positive control	32.18 ^b	1.72		
Self-recovery	26.71 ^c	1.82	231.07	<0.001**
Olive oil	23.81 ^d	1.41		
β -Sitosterol	50.94 ^a	3.46		

******, and different letters means significant difference between groups at $P<0.05$

Masson's trichrome stain statistical analysis

Analysis of the data presented in Table 2 indicates a significant disparity among the groups studied, specifically in relation to Masson's Trichrome staining. This was ascertained using a one-way ANOVA ($F=231.07$, $P<0.001$). In the pairwise comparisons, a notable difference was observed between the negative control group and all other groups, with the exception of the β -Sitosterol group. The highest mean values were observed in the negative control group, succeeded by the β -Sitosterol, positive control, and self-recovery groups, in that order. The group treated with olive oil exhibited the lowest mean value.

PCNA stain statistical analysis:

Table 3 presents results indicating a substantial variance among the groups studied in terms of PCNA and PCNA for connective tissue (PCNA-CT) expression, as determined by a one-way ANOVA ($F=274.78$, $P<0.001$ for PCNA; $F=66.32$, $P<0.001$ for PCNA-CT). Upon pairwise comparison, there was a significant difference observed between the negative control group and all other groups for PCNA, except in the case of β -Sitosterol. In contrast, for PCNA-CT expression, significant differences were noted among the groups, except between the self-recovery and olive oil groups. Regarding both PCNA and PCNA-CT expression, the highest mean values were seen in the negative control group, followed in order by the β -Sitosterol, positive control, and self-recovery groups. The olive oil group registered the lowest mean value in these assessments.

TABLE (3) PCNA immune histochemical stain

	PCNA	PCNA-CT
Negative control	112.50 \pm 0.55	18.67 \pm 2.94
positive control	107.50 \pm 3.15	10.83 \pm 1.47
Self-recovery	85.17 \pm 1.72	5.33 \pm 1.63
olive oil	82.67 \pm 2.5	3.67 \pm 1.21
β -Sitosterol	110.83 \pm 1.94	13.50 \pm 1.38
F test	274.78	66.32
P value	<0.001**	<0.001**

DISCUSSION

Currently, the therapeutic drugs that are not only expensive but also not particularly efficient, discovering new alternative therapies, researchers are interested in exploring novel options derived from natural resources. Hence, the present study demonstrated the effect of oral administration of

β -Sitosterol on the induced traumatic ulcers in labial mucosa of Albino rats.

In this study, Albino rats were chosen as the experimental model due to their cost-effectiveness and the valuable insights they offer. While the information obtained from utilizing rats is straightforward, it holds the potential to stimulate additional research within this field of study [38].

Systemic route of administration was the choice of current study as topical treatment might be not applicable as in cases of inaccessible site of ulcers, multiple ulcers or handicapped child systemic therapy can be used [9].

Clinical monitoring through photographic documentation at 3, 7, and 10-day intervals revealed the persistence of unhealed ulcers at day 10 in the self-healing group. This observation aligns with the findings of Altenburg et al., who noted that ulcers resulting from acute trauma typically resolve spontaneously without complications within 14 days [39]. Therefore, a 10-day period was selected for the euthanasia and subsequent histopathological, histochemical, and immunohistochemical analysis. This timeframe was chosen to investigate the histological alterations occurring prior to the natural healing of the ulcer, drawing from previous research indicating notable histological changes at this stage following traumatic ulcer induction [31].

The healing process of oral ulcers, encompassing inflammation, proliferation, remodelling, and maturation stages, mirrors that of other wound types. These stages are interrelated, with the proliferation phase being heavily influenced by inflammation and pivotal in determining the maturation/remodelling phase. This phase includes fundamental wound healing activities such as cell migration, proliferation, re-epithelialization, extracellular matrix and collagen formation, and angiogenesis [40]. For a comprehensive histological assessment, in addition to H&E staining for descriptive histology, we employed PCNA immunostaining to evaluate

cellular proliferation, PAS staining to examine basement membrane regeneration, and Masson's trichrome staining to observe the formation of new collagen.

The ulcer's clinical follow-up during the trial revealed that at 3 day, the healing wasn't evident in all groups of experiments clinically, these result was compatible with Arundina et al, study that reported 7-day treatment duration was sufficient for Fibroblast growth factor (FGF), Vascular Endothelial Growth Factor (VEGF), Platelet-derived growth factor (PDGF), and collagen-1 (COL-1) expression, which are necessary growth factors for traumatic ulcer healing [41]. β -sitosterol promoted the healing process over an interval of 7 and 10 days when compared to the self-recovery and olive oil groups.

The histopathological results confirm the clinical results as β -Sitosterol group showed sealing of epithelial gaps in oral mucosal wounds with newly formed continuous epithelial lining and short, little rete-ridges. Continuity of basement membrane and reepithelization along oral mucosal wound in β -sitosterol group was demonstrated by significant rise in PAS reaction revealed that complete epithelial sealing at ulcer site compared to olive oil and self-recovery group that showed low significant rise of PAS at margin of ulcer indicating weak re-epithelization in these groups. These results were compatible with Hennessey study reported that the fluidity of membrane-bound enzymes and their activity are regulated by phytosterols [42]. phytosterols also have been shown to have anti-inflammatory [43], anti-pyretic, and immune-stimulating properties [44]. They are also effective in lowering proton and sodium ion leakage from cell membranes. The stability of phospholipid monolayers is improved by phytosterols. Phosphatidyl-choline bilayers' water permeability is said to be decreased by them [45]. Martel-Estrada et al., revealed the β - sitosterol ability of enhancing re-epithelialization process in wounded areas which

aids the ability to heal, together with the anti-inflammatory and antimicrobial activity^[46]. Also, a study by Xiao et al., reported anti-gastroulcerative activity of β -Sitosterol-glycoside and its aglycone in rats^[47].

The histopathological examination revealed that the lamina propria in the β -Sitosterol group exhibited new blood capillaries and collagen formation with a dense distribution of fibers and inflammatory cell infiltration. This was in contrast to the self-recovery and olive oil groups, which showed a less dense fiber distribution and areas of fiber dissociation. These findings are supported by the Masson's trichrome staining results, which indicated a significant increase in collagen content in the β -Sitosterol group compared to the self-recovery and olive oil groups. These observations align with the research by Cui et al., which indicated that β -Sitosterol has three analogs contributing to wound healing: inhibiting Na^+/K^+ -ATPase, promoting cell proliferation, and enhancing cell migration and collagen synthesis, all of which are conducive to wound healing^[48]. Additionally, β -Sitosterol has been recognized for its role in promoting hyaluronic acid biosynthesis, enhancing skin barrier function^[49], and exerting therapeutic angiogenic effects on damaged blood vessels^[50].

Despite β -Sitosterol being a predominant sterol in virgin olive oil, the negative histopathological outcomes observed in the current study align with findings from Trancoso et al., who reported that short-term supplementation with extra virgin olive oil can increase oxidative damage and pro-inflammatory responses, thereby impairing acute wound closure^[51]. Oxidative stress is a significant impediment in wound healing, generally hindering tissue remodelling^[52].

PCNA, a cellular protein, is utilized as an indicator of cell proliferation. Essential for DNA replication and repair, it is present during all stages of cell proliferation in both normal and

cancerous cells. Its inhibition is known to impact cell division, underscoring its importance in cellular processes^[53].

The investigation revealed that PCNA levels in the β -Sitosterol group did not significantly deviate from those in the negative control group, indicating that the rate of cell proliferation in the β -Sitosterol group is on par with normal conditions. In contrast, the groups undergoing self-recovery and treated with olive oil displayed notably lower PCNA immunoreactivity than the negative control, suggesting a decrease in cell proliferation rates. This observation is in line with Kasirzadeh et al.'s research, which found that β -Sitosterol can alleviate sepsis-induced lung damage by inhibiting the NF- κ B pathway, leading to reduced inflammation, altered apoptosis, and modulation of claudin-4 and claudin-5, crucial for maintaining the integrity of alveolar epithelial cells. On the other hand, olive oil's effect on lipid peroxidation and the resulting oxidative DNA damage involves processes that could potentially affect inflammatory responses. They also affect transcription factor activity, gene expression, and signal transduction, which can result in modifications to metabolism, cell proliferation, and angiogenesis^[54]. Moreover, they alter the immune system, which can cause abnormalities in immune function and an imbalance in the production of inflammatory cytokines^[55]. The previous study confirmed the current investigation results of olive oil group.

β -Sitosterol plays a pivotal role in regulating the levels of antioxidant enzymes during pathogenesis, effectively reducing the generation of free radicals in vitro^[56], the previous study could confirm the results of current study that suggested that 5mg/kg of β -sitosterol have ability to accelerate the wound healing through its anti-inflammatory and relieving the oxidative stress in the cell and help in tissue repair.

RECOMMENDATION

More research is required to determine the precise mechanism of action of β -Sitosterol that help to recognize its properties. To confirm the most effective treatment approach, further research should try various delivery routes, concentrations, and dissolving solutions. Further research in suitable dissolved media is needed to use β -Sitosterol in emulsion or gel form for topical application trials.

CONCLUSION:

β -Sitosterol holds potential as an effective alternative to traditional oral medications currently employed in the treatment and expedited healing of traumatic oral ulcers.

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