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Chitosan Gel Containing *Hypericum perforatum* Extract Inhibiting Effect on Oxidative Stress in Burn Wounds of Rats

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ABSTRACT

The current study aimed to examine the impacts of topical sole and a combination of chitosan gel (Chit) and ethanolic extract (Ext) of *Hypericum perforatum* on inhibiting oxidative stress chain in burn wounds of Wistar rats. Three circular burn wounds on the back of the rats were induced in this study. The experimental groups included Sham, Chit, Silver Gel, Ext 5%, Ext 10%, Chit/Ext 5%, and Chit/Ext 10%. In addition to the evaluation of the healing process, total antioxidant capacity (TAC), malondialdehyde (MDA), and hydroxyproline levels were assessed. The results of the current study demonstrated that Chit/Ext treatment, especially at the 10% concentration, significantly reduced MDA, and remarkably enhanced TAC and hydroxyproline levels compared to the other groups. Based on the current study findings, it can be concluded that topical use of Chit and Ext can accelerate the healing process of second-degree burn wounds by inhibiting oxidative stress and inflammation and promoting angiogenesis and collagen production.

Introduction

Burn injuries directly/indirectly result in morbidity is a worldwide concern.[1,2] During the last decades, burn wound management has been recognized as one of the most challenging issues.[3,4] In the burn injuries different pathological mechanisms play key roles in the area including, inflammation, ischemia, and oxidative stress, leading to delay in healing process.[1,5] At the cellular and molecular level, inflammatory cells accumulation, the area vascular damage, and ischemia, result in reactive oxygen species (ROS) over-generation in the area. Increase of ROS in the burn wounds causes numerous problems including vascular permeability, dermal structural derangement, and activation of cell death mechanisms such as apoptosis in the important healing cells like fibroblasts.[6,7] The key goals in burn wound treatment, introduced as wound healing acceleration, inflammation and oxidative stress control, and pain alleviation.[4,8]

Despite improvements in burn wound management especially during recent years resulting in enhanced clinical outcomes, burn wounds still remained as one of the most devastating injuries with high morbidity and mortality.[9] The use of natural medicine is increasing both in general population and scientific area. Also, it has been unconventionally referred to as alternative and complementary method. These treatment methods are often derived from natural sources and facilitate improvements in health and well-being by supporting the body's intrinsic healing processes.[10,11]

Hypericum perforatum L. (HPM) is a well-known traditional medicine, used in various disease treatment such as depression, ulcers, burns, and hemorrhoids.[12] The HPM is one of the most used traditional medicines in cuts and burns treatment.[12,13] Indeed, testing HPM in an *in vitro* condition demonstrated that HPM extract contains antioxidant, swelling behavior, antibacterial, and *in vitro*

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wound healing capability. Moreover, not only no cytotoxic impact of HPM on fibroblasts populations was observed, but also HPM administration improved fibroblasts proliferation and function.[14] In addition, the HPM administration has been revealed to be effective in both topical and oral approaches in diabetic rats' wound healing process,[13-17] while HPM use in niosomal drug delivery method to treat dog models, also showed the beneficiary impacts of HPM in wound heling process.[18] These controversies in form of HPM administration demonstrates a doubt regarding the type of use of this drug in treatments.

In 2014, Sayar and colleagues revealed that using HPM cream on second-degree burn wounds in rats accelerated reepithelization process, increased angiogenesis process, and fibroblasts immigration to the wound area.[19] Daryabeigi and colleagues reported no significant difference between HPM and silver sulfadiazine treatment on rats' second-degree burn wound healing process 35 days post-treatment.[20]

An ideal wound dressing must provide a moist healing environment, and protect the area from bacterial contaminations. At the same time, it should promote cellular migration and proliferation, tissue repair, and remodeling.[21] Chitosan is a well-known natural alkali polysaccharide, which can be derived from chitin from crustacean shells.[22,23] Chitosan covers all aforementioned features and is also its low cost, non-toxic and natural. For instance, it has been revealed that chitosan inhibits wound infection by promoting inflammatory cells function in the area.[24] Moreover, chitosan does not result in any kind of allergy or reaction in the area.[25] Containing these factors has resulted in its wide use of it in various fields, especially in wound healing cases.[26,27]

The current study was conducted to investigate the chitosan gel containing HPM potential oxidative stress inhibitory role in burn injuries. In order to achieve this goal, malondialdehyde (MDA), and total antioxidant capacity (TAC), as the main oxidative stress and antioxidant markers were examined. Moreover, hydroxyproline is a well-known marker for collagen production in the wound injuries. Indeed, collagen synthesizes is a result of constituent cell growing in the area. Higher the levels of hydroxyproline indicates faster rate of healing wound.[28] Thus, here in the current study hydroxyproline level was also examined to evaluate different type of treatments healing process.

Materials and Methods

Extract Preparation of HPM

The maceration method was used to prepare the ethanolic extract of HPM aerial parts (leaves, stem, petals, and flowers). The dried Plant was purchased from a local market (Mashhad, Iran), cleaned, powdered, and soaked into 70% ethanol for 48 h at room temperature. The mixture was passed through paper filter (pore size of 11 µm), and the extract was freeze-dried to dry completely.[29]

The total phenolic content of the HPM extract was determined by the Folin-Ciocalteu method. A standard Gallic acid curve was prepared at a concentration 20-160 mg/ml in water. These dilutions and HPM extract were mixed with of Folin-Ciocalteu reagent (at ratio 1:1) for 2h at 30 °C. Then, the absorbance of all samples was read at 765 nm by UV spectrophotometry (Shimadzu, Japan). The amount of polyphenol percentage was calculated as follows: A: Sample absorption, b: Standard curve width, m: Standard curve slope, W: Sample weight, V: Sample volume, D: Dilution coefficient.

Preparation of Chitosan Hydrogel and Loading of the Extract

In order to prepare hydrogels containing 5 and 10% extract, 2 gr of chitosan powder (Cat: 448869, Sigma-Aldrich, Germany) with low average molecular weight was added to 100 ml of 1% acetic acid solution while rotating. After 1 hour, it was sonicated for 10 minutes to remove the air bubbles. The extract was added to the chitosan hydrogel while it was rotating slowly and the resulting gel was esterified for 2 hours until the 5 and 10% extract-containing gels were prepared.[30] pH was adjusted to 5-5.5.

Animals and Experimental Model

A total of 105 male mature Wistar rats (300 ± 20 g) were included in this study. During the 21 days of experimental period, all the animals were kept in a standard condition to minimize the stress factors, while ad libitum access to food (rat pellet) and water was provided, based on the guidelines of Ferdowsi University for research using animal models. All the experimental protocols were evaluated and approved by the Faculty of Veterinary Medicine's ethical committee, Ferdowsi University, Iran (Ethical Number: IR.UM.REC.1400.370).

After adaptation period, the animals were randomly divided into seven groups (N=15 rats /group). The model induction was performed as previously described.[31] In brief, the rats were anesthetized using 80 mg/kg ketamine and 20 mg/kg xylazine (IM) mixture. After shaving the hair on the back of the animal, a 2 cm brass rod was used to induce burns. The brass rod was heated in boiling water for 5 minutes, then placed on the back skin of each rat without any pressure for 30 seconds.[32] In this way three rows of circular burns on the back of the animal parallel to midline were created. Forty-eight hours post inducing a full thickness burn injury, necrotic tissue was removed. The animals were subcategorized into (A) Sham group, received only normal saline, (B) Chit group,

received only chitosan gel without extract, (C) Silver Gel, received only silver sulfadiazine ointment, (D) Ext receive only 5% extract of tea grass, (E) Ext, receive only 10% extract of tea grass, (F) Chit/Ext received chitosan gel containing 5% tea grass extracts and (G) Chit/Ext, received chitosan gel containing 10% tea grass extracts. Treatment applied topically and bandaged in all animals. Dressings and treatments were repeated daily during experimental period.

Macroscopic Evaluation of the Wound

On the zero day (the first day of the burn) a photo was taken. Then, on the second day a photo was taken and necrotic tissue was removed, and the treatment of the groups began. The fifth, ninth, fourteenth, seventeenth, and 21st days images were taken as well. After scanning, the area was measured in the Image J software and the percentage of living area was calculated, as previously described.[31]

Tissue MDA and TAC Levels

After unfreezing the tissues at room temperature, the samples were homogenized using an ultrasonic homogenizer. In order to refine the tissue remains, the samples were transferred into the microtubes and centrifuged for 20 minutes at 5000 rpm. The supernatant was separated and used for the measurement of total antioxidant capacity (TAC) and malondialdehyde (MDA). Both TAC and MDA examinations were conducted according to Navand Salamat commercial kits manual (TAC: NS-15013; MDA: NS-15023; Navand Salamat Co., Urmia, Iran), and read by a DANA-3200 ELISA Reader (Garni Medical Engineering Co., Tehran, Iran).

Hydroxyproline Examination

The skin samples were prepared for analysis by first homogenized in a homogenizer with a 150 mM KCl solution at a pH of 7.4. For breaking down the samples, 0.5 ml of the homogenate was treated with 1 ml of 6 N HCl and heated at 120 °C for 8 hours. In order to oxidize the free hydroxyproline present in the samples, a mixture of 50 µl of citrate/acetate buffer (composed of 5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH = 6) and 1 ml of chloramine-T solution was added to 50 µl of the samples. This mixture passed 20 minutes at room temperature and was combined with 1 ml of Ehrlich's solution, which consisted of 2.5 g of 4-dimethylaminobenzaldehyde, 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid. The sample-Ehrlich's solution mixture was then subjected to a 15-minute incubation at 65 °C in a water bath. After cooling down to room temperature, the spectrophotometric absorbance was measured at 550 nm using a Bio Tek Instruments EPOCH2TC spectrophotometer. To determine the concentration of hydroxyproline in the skin samples, a standard curve was established using hydroxyproline standards ranging from 0 to 10 µg/ml, following the method described by Woessner in 1961.[33] The hydroxyproline concentration was expressed as µg hydroxyproline/mg of protein. To determine the protein content of the samples, the Lowry method, as outlined by Lowry *et al*. in 1951, was employed.[34]

Statistical Analysis

Normality and homogeneity of the results were evaluated by Kolmogorov-Smirnov and Levene's tests. All quantitative findings were analyzed using the ANOVA and Tukey post hoc correction tests. The GraphPad software (version 9.4, USA) was used to perform the statistical analysis. All data were presented as Mean ± SD and the p-value less than 0.05 was considered as statistically significant.

Results

The amount of total polyphenols in the ethanolic extract of HPM aerial parts was calculated as 37 ± 1.2 mg/g in dry plant material using the standard gallic acid curve. In a study, the total polyphenol contents in the ethanolic extract of HPM aerial parts was also obtained to be 64.4 mg ± 1.5 g equivalents of gallic acid.[35] The induced burning wound area was evaluated using Image J software at 5, 9, 14, 17, and 21-days post-surgery (Figure 1). The results demonstrated no statistically significant changes between experimental and control groups on day 5. However, SilverGel and Chit/Ext 10% treated groups wound area diminished significantly on day 9, 14, 17, and 21 of experiment compared to the Sham and other treated groups ($p < 0.05$; Figure 2). Moreover, Ext 10% and Chit/Ext 5% treated animals demonstrated a remarkable wound area reduction on day 21 of experiment compared to sham and Chit treated animals ($p < 0.05$; Figure 2). The Chit/Ext 10% and Sliver Gel treatment reduced MDA level in rats compared to the sham group at all three experimental days ($p < 0.05$). However, Ext 5, 10, and Chit/Ext5 % treated animals showed a significant ($p < 0.05$) down-regulation of MDA, only at day 21 (Figure 3). Further analysis of tissue TAC demonstrated a remarkable ($p < 0.05$) increment of TAC in Chit/Ext 10% and Sliver Gel groups compared to others (Figure 4). Hydroxyproline analysis illustrated that in both Silver Gel and Chit/Ext 10% treated groups significantly ($p < 0.05$) higher levels of hydroxyproline were detected on days 9 and 21 compared to the other treated and sham groups (Figure 5).

Discussion

During the last decades, second-degree burn injuries have become an important public health issue, caused

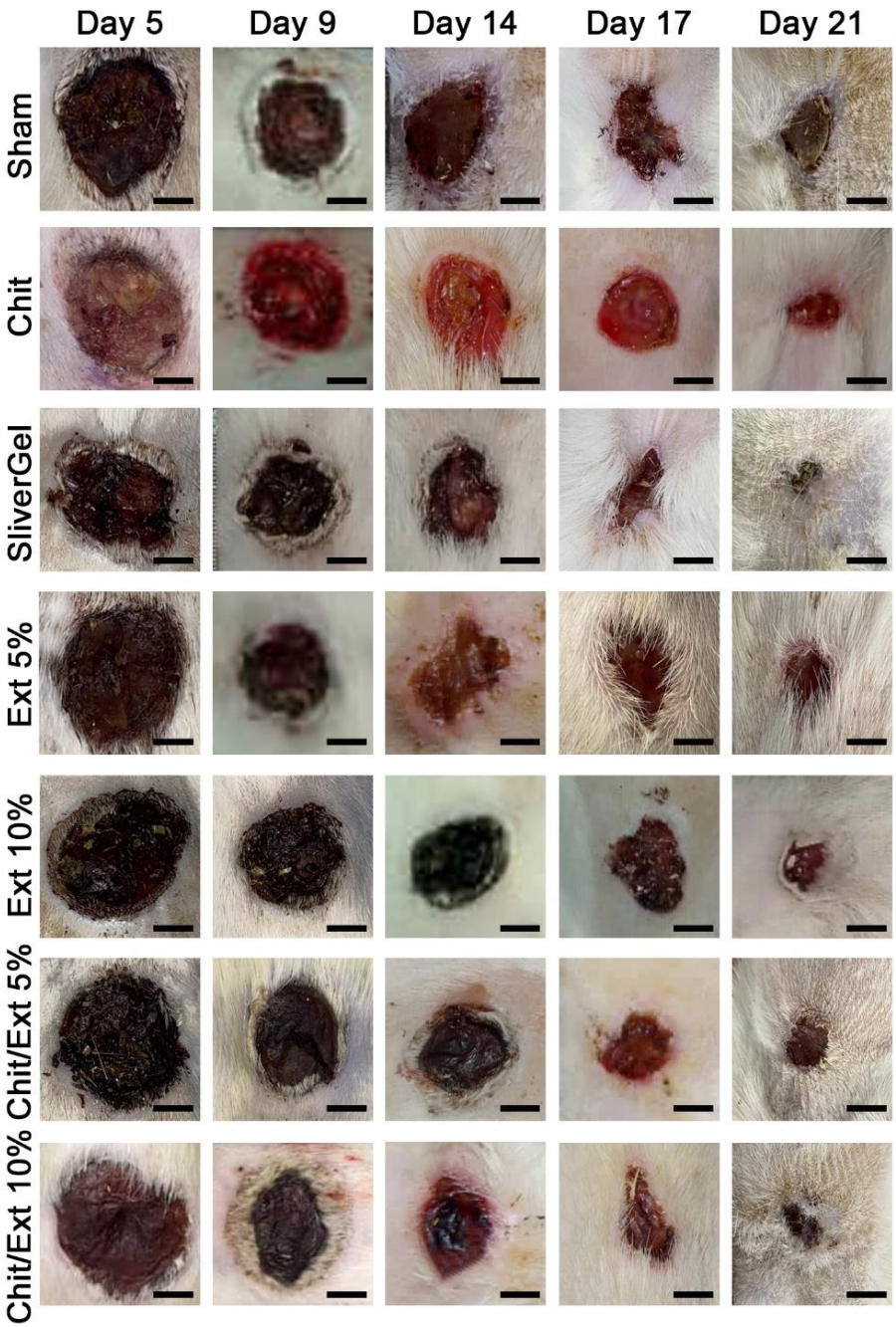

Figure 1. Wound macroscopic images at day 5, 9, 14, 17, and 21 post surgery, demonstrating the wound healing process.

mainly due to hot water and liquids.[36] These injuries may cause different problems, including psychological, functional, appearance, and life quality issues, as well as fatal complications.[37] Thus, numerous researchers have focused on improving this condition using natural compounds combined with new therapeutical approaches to accelerate the healing process. In line, different herbs possible beneficial effects have been tested on burn wounds, such as *Camellia sinensis,*[38] *Arnebia euchromia*,[39] nettle extract,[40] and licorice.[41]

It has been revealed that burn injuries by increasing ROS generation and accumulation cause oxidative stress. Although oxidative stress at physiological level is beneficiary for wound healing process by increasing vascular regeneration in the wound area, high levels of ROS may cause delay in wound healing process.[42]

It has been previously reported that olive oil extract of *Hypericum perforatum* accelerates the healing process due to its antioxidant, antibacterial, and anti-inflammatory potentials.[43] The polyphenolic compounds of this plant have several properties including antioxidant, antimicrobial, skin protection from UV rays, and skin repair.[44,45] Indeed, Hypericum spp components, especially flavonoids, play antioxidant role, making their extract highly efficient in wound's oxidative stress control. Hypericum spp components, especially flavonoids, play antioxidant role, making their extract highly efficient in wound's oxidative stress control.[46] Thus, improved TAC and scavenging of ROS by Chit/Ext combination exhibited by diminished MDA levels,

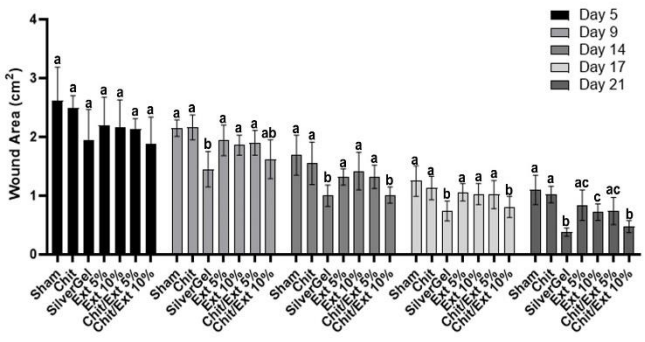

Figure 2. Wound area size (cm²) in Sham and different treated groups. All data are presented in Mean ± SD, (n = 15/each group). Letters are presenting statistically significant ($p < 0.05$) differences between groups.

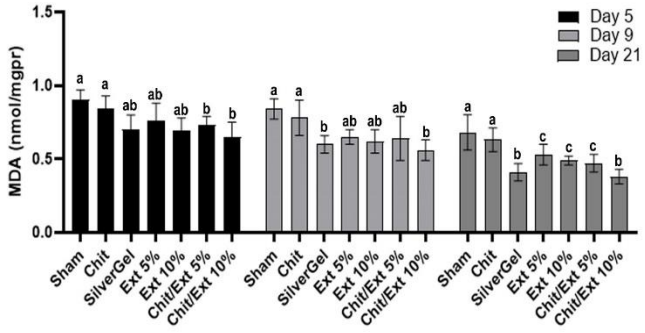

Figure 3. Malondialdehyde (MDA) level in Sham and different treated groups. All data are presented in Mean ± SD, (n = 15/each group). Letters are presenting statistically significant ($p < 0.05$) differences between groups.

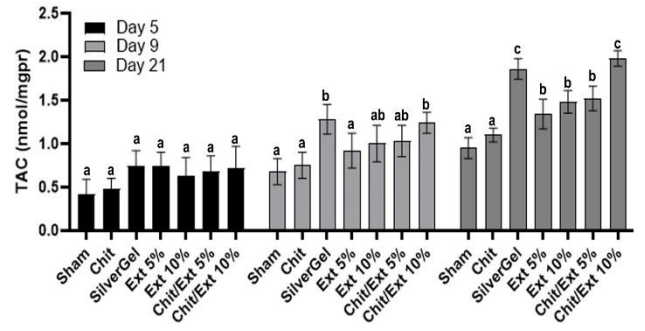

Figure 4. Total antioxidant capacity (TAC) level in Sham and different treated groups. All data are presented in Mean ± SD, (n = 15/each group). Letters are presenting statistically significant ($p < 0.05$) differences between groups.

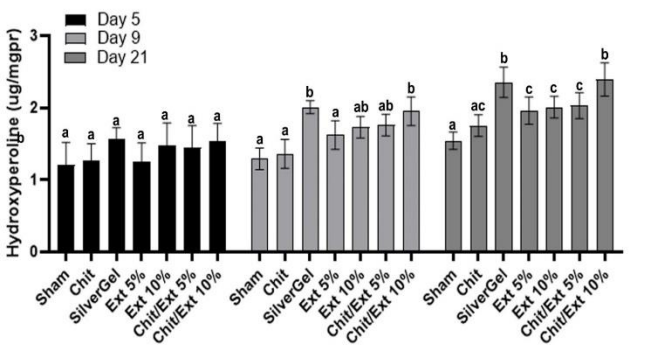

Figure 5. Hydroxyproline in Sham and different treated groups. All data are presented in Mean ± SD, (n = 15/each group). Letters are presenting statistically significant ($p < 0.05$) differences between groups.

demonstrate oxidative stress control in the current study-induced wounds.

The critical role of collagen in the wound healing process has been highlighted in recent decades, especially with the widespread use of collagen as a scaffold to accelerate the healing process of various tissue damages.[47] Collagen types I and III have been reported as the main components of a healthy skin structure, with the ratio of these two collagens determining the wound healing process.[48,49] The accumulation of collagen in the wound area was evaluated using hydroxyproline, and higher collagen accumulation was observed in the Chit/Ext and Silver Gel groups compared to other groups. Similarly, Damlar and colleagues demonstrated in 2016 that *Hypericum perforatum* administration could significantly increase collagen accumulation in xenograft wounds.[46] Moreover, Altıparmak and Eskitaşçıoğlu reported the positive impact of topical *Hypericum perforatum* on collagen accumulation in diabetic surgical wounds.[13] In line with these findings, the Ext 5% and 10% groups demonstrated higher levels of collagen accumulation compared to the control group. However, the combination therapy of Chit and Ext significantly improved this positive effect, particularly in the Chit/Ext 10% group. Thus, the findings of the current study demonstrate that the synergistic use of Chit in combination with *Hypericum perforatum* improves the wound healing process, at least in the case of burn injuries. These findings are in consistent with previous reports that topical application of *Hypericum perforatum* reduced immune cell infiltration into the wound area, improved fibroblast population and activity in diabetic surgical wounds.[13] In line with these molecular results, the macroscopic evaluation of wound area in different groups also demonstrated the beneficial impact of Chit/Ext 10% at the clinical level. Reduced wound area was observed in both SilverGel and Chit/Ext 10% groups, which this clinical finding was in line with our molecular findings.

The results of the current study demonstrated that topical *Hypericum perforatum* extract and chitosan combination therapy was able to inhibit the burn wounds-induced oxidative stress by reducing MDA level and increasing TAC. Reduced oxidative stress in the burn wound injuries may improve recovery period. Moreover, *Hypericum perforatum* extract and chitosan treatment increased fibroblast accumulation, leading to higher collagen secretion. These findings demonstrate that the combination of *Hypericum perforatum* extract and chitosan is able to control the oxidative stress of the burn wounds as effectively as Silver Gel, a commercial product.

Acknowledgments

This study is a part of the first author's DVCs thesis.

Conflict of Interest

None to declare.

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