Evaluation of the Role of Propolis and a New Herbal Ointment in Promoting Healing of Traumatic Oral Ulcers: An Animal Experimental Study

Abstract
Most of the currently used medications for the treatment of oral ulcers focus primarily on providing symptomatic relief and preventing secondary infections. Not much attention has been paid in the past on agents that can promote healing of these lesions. In the current study, we have tested and compared the efficacy of such wound healing promoting agents i.e. Propolis (product obtained from Bees) and a newly developed herbal ointment (containing extracts of Azadirachta indica (Neem), Linum usitatissimum L.(Linseed) oil, and resin of Shorea robusta) in experimental rabbits.

Methodology: Traumatic oral ulcers were inflicted in experimental rabbits (New Zealand white) with the help of a punch biopsy instrument. The animals were then divided into three groups each group consisted of 12 animals (n = 12), Group I was the untreated control group and Group II was topically treated with nonalcohol extract of propolis and Group III was treated with newly developed herbal ointment. Histological healing scores of the ulcers were evaluated on day 7 and day 14. Data were statistically analyzed using descriptive and inferential statistic methods. Results: In Group III treated with newly developed herbal ointment, significantly higher healing scores, in comparison to the untreated control group (Group I) and propolis (Group II) was observed on day 7 and 14. Improved healing scores in prapolis-treated group were observed than the untreated control group. However, the difference was not statistically significant on both day 7 and day 14. Conclusion: With the newly developed herbal ointment, superior healing of traumatic oral ulcers was observed.

Keywords: Azadirachta indica, herbal ointment, histopathological healing, Linum usitatissimum, propolis, Shorea robusta, traumatic oral ulcers

Introduction
Traumatic oral ulcers are one of the common types of ulcers encountered in clinical practice. Injuries of the oral mucosa could result from physical, chemical, or thermal trauma. Such ulcers generally, heal quickly within a few days without any complications. However, chronic ulcers caused by chronic trauma from sharp edges of teeth, restorations, and appliances-like ill-fitting dentures, orthodontic appliances, etc., may take a longer time to heal. The prevalence of such traumatic ulcers varies from 3.5% to 15.6% in different studies conducted worldwide.

Clinically, mucosal wound healing in the oral cavity occurs by 5–7 days. This is achieved through acute, robust inflammatory response, with the recruitment of neutrophils, macrophages, and lymphocytes to the wound site, which is followed by the inflow of fibroblasts, ECM synthesis, and reorganization. This is critically important in the oral cavity, which is colonized and contaminated by numerous microorganisms. It is, hence, crucial for the oral mucosa to heal healthily and quickly as possible.

The current approach for the treatment of oral ulcers primarily deals with providing symptomatic relief and/or preventing secondary bacterial infections through the use of topical anesthetics and antibiotics. Not much emphasis has been given in the past on the use of agents, which can promote healing of oral ulcers. Moreover, the research conducted in this field is still scanty.

Medicinal plants, herbal derivatives, and animal products, namely turmeric, neem (Azadirachta indica) extracts, Aloe vera, honey, beeswax, have shown immense potential for the management and treatment of ulcerative disorders, as they contain a variety of antioxidants, anti-inflammatory, and wound-healing molecules. The use of herbal products in the management of oral ulcers is not only backed by various in vitro studies but also by a few in vivo and clinical trials. However, this research has been often limited due to a lack of scientific evidence and standardized methodology in the design of the study.

In this study, we evaluated the role of a newly developed herbal ointment containing the extracts of Linum usitatissimum L.(Linseed) oil, propolis (product obtained from Bees), and the resin of Shorea robusta in comparison to the nonalcohol extract of propolis, and a control group with no treatment.

The treatment was evaluated on day 7 and day 14 using histological healing scores.

Results:

- In Group III treated with newly developed herbal ointment, significantly higher healing scores were observed in comparison to the untreated control group (Group I) and propolis (Group II) on day 7 and 14.
- Improved healing scores in propolis-treated group were observed compared to the untreated control group.
- The difference was not statistically significant on both day 7 and day 14.

Conclusion:
With the newly developed herbal ointment, superior healing of traumatic oral ulcers was observed.
of wounds. A good number of such products have been used by tribal and folklore in many countries for the treatment of wounds and ulcers. Recently, these products are gaining popularity in the field of research and medicine.\[10\]

In the recent past, agents like propolis, which is a golden-dark brown resinous substance derived from beeswax, has been reported to promote the healing of oral ulcers in comparison to conventionally used medications.\[11\] A. indica locally called as “Neem” is a plant widely found in India. Almost all the parts of this plant have medicinal properties, but leaves are most commonly used. The extract from neem leaves has been used widely in the Ayurveda (ancient Indian system of medicine) for treating various diseases such as gastrointestinal infections, skin diseases, and ulcers.\[12\] Similarly, Shorea robusta bark resin is known for its antibacterial, astringent, and cooling properties. It has also been found useful in treating wound ulcers, burns, and neuralgia.\[13\] So also oil derived from seeds of Linum usitatissimum L. (Linseeds) is used as a common base for liniments. Crushed linseeds are applied in the form of a poultice for the relief of local inflammations, ulcers, boils, and carbuncles.\[14\]

Given the medicinal properties of these plants, a herbal ointment was prepared by Pathak and Borkar in 2006. The herbal ointment containing extract of A. indica (Neem) leaves, L. usitatissimum L. (Linseed) oil and resin of Shorea robusta (Shaal) experimentally found to be more effective as compared to silver sulfadiazine in promoting healing of burn ulcers in experimental rabbits.\[15\]

The current research was conducted on experimental rabbits to develop a new medicament which can be put to clinical use. Besides, the standardization of variables such as size and depth of ulcer would not be possible in humans. The rabbits in this work could generate information that can be used for further researches in this area to develop clinically effective protocol for the management of oral ulcers. Against the said background, it was envisaged, to compare the efficacy of new herbal ointment and propolis in promoting healing of experimentally inflicted oral ulcers in rabbits.

**Objectives**

1. To evaluate the healing of traumatic oral ulcers in untreated experimental rabbits based on wound healing scores and wound contraction scores
2. To evaluate the healing of traumatic oral ulcers in experimental rabbits treated with alcohol-free propolis (Y.S. Eco bee farms) extract based on the above-said parameters
3. To evaluate the healing of traumatic oral ulcers in experimental rabbits treated with new herbal ointment (laboratory preparation) based on said parameters
4. To compare the above three groups with each other.

**Methodology**

**Study design (type of study): Experimental randomized researcher blind animal study**

The present study was undertaken in Sharad Pawar Dental College, Sawangi (Meghe), Wardha, in the animal house DMIMS, Sawangi (Meghe), Wardha. All the Biochemical parameters were carried out in Central Research Laboratory, DMIMS, Sawangi (M), Wardha. The approval of the Institutional Animal Ethical Committee was obtained vide their letter no. DMIMSDU/IAEC/2013–14/53 dated 29/03/14. Subsequently, the approval of the Institutional Ethical Committee (IEC) was obtained vide their letter no DMIMS (DU)/IEC/2012–13/144 dated 22/02/13.

Based on the results obtained from a pilot study, the sample size was estimated for the expected event rate between 15% and a protective fold of 70% using the formula described by Wang and Bakhai.\[16\] As per the calculations, the sample size came to 11.52 per group. Hence, twelve animals were selected as the sample size per group (n = 12).

To eliminate researcher selection bias allocation of animals to each group was made through random allocation by the computer-assisted method. Furthermore, the person performing the biopsy procedure and the histopathologist performing the histopathological analysis of the biopsy samples were kept blinded from the distribution of groups of experimental animals to minimize bias in the study.

**Procedure for preparation of herbal ointment**

New Herbal Formulation was prepared in the Central Research Laboratory of Datta Meghe Institute of Medical Sciences (Wardha) under sterile conditions. A total of 20 g of Neem leaves were boiled in 500 ml of Linseed oil and then allowed to cool at room temperature. The solution was then triturated with the powdered resin of 100 g of Shorea robusta. The contents were washed 100 times with distilled water to obtain pure form of new herbal formulation. The preparation was done as per the previous study by Pathak and Borkar.\[15\]

**Sample selection**

Thirty-six healthy male/female, New Zealand White Rabbits were selected for the study. All the animals were between 6 months and 3 years of age, weighing between 2.5 kg and 3.5 kg. Pregnant and diseased rabbits were excluded from the study. All the animals were kept on a prescribed laboratory diet and were kept in Cages under standard conditions of light, humidity, and temperature. None of the animals were sacrificed or died during the study. The average weight (group wise) was recorded at the beginning and the completion of the study [Table 1].

**Procedure**

Atraumatic ulcer was inflicted on labial mucosa of each of the experimental animals with the help of a 5-mm punch biopsy instrument. Surgical procedures were performed under general anesthesia by intramuscular administration
of 0.1 ml of ketamine hydrochloride (Hypnoket) combined with 0.05 ml of xylazine hydrochloride (Xylazine), per 100 g of body weight of the animal. Then, the rabbits were randomly divided into three groups ($n = 12$).

- Group I: no drug was administered to the animals of this group (untreated control)
- Group II: Animals of this group were topically applied with alcohol-free extract of Propolis (Y.S. Eco bee farms) from day 1 to day 14 daily, three times a day at eight hourly intervals (morning 6 am, 2 pm, and 10 pm)
- Group III: Animals of this group were topically administered with new Herbal ointment (containing extract of $A. indica$, Shorea robusta, and Linseeds) from day 1 to day 14 daily, three times a day at eight hourly intervals (morning 6 am, 2 pm, and 10 pm).

**Estimation of wound contraction**

Wound contraction was monitored by measuring the progressive changes by tracing the raw wound area on a transparent paper on day 1, 7th, and 14th Post ulcer days. The tracing was then transferred to a 1 mm² graph sheet, from which the wound surface area was calculated. The calculated surface area was used to calculate the percentage of wound contraction, taking the initial size of wound as 100% by using the following equation.$^{[17]}$  

Wound contraction (%)  

$$\frac{\text{Initial Wound Size} - \text{Specific Day Wound Size}}{\text{Initial Wound Size}} \times 100$$

**Histopathological estimation of wound healing**

Biopsy specimens taken from edges of the healing ulcer on day 7 and day 14 from all groups and were stained with a Picrosirus stain for collagen and hemotoxyllin and Eosin stain for tissue inflammation. A histological examination of each specimen was done. Scoring criteria of Sultana et al. in 2009 were used to compare the quality of healing of wounds [Table 2].$^{[18]}$

Total healing score of each case was calculated by adding the score of individual criteria. Lower scores indicated poorer wound healing, while higher scores indicated a better healing process [Figure 1a and b].

**Statistical analysis**

All the specimen (36/36) were included in statistical analysis which was done by using descriptive and inferential statistics using one-way ANOVA for the analysis of variance between and within the groups [Tables 3 and 4], Dunnett $D$-test was applied for comparison of scores between the groups [Tables 5 and 6]. Software used in the analysis was STATA 15.0 version by STATA CORP LLC, TEXAS, and $P < 0.05$ at 95% confidence interval was considered as statistically significant.

**Results**

Mean histologic healing scores and wound contraction scores on day 7 and day 14 for all the groups are given in [Table 7]. On both days 7 days and day 14, in herbal ointment treated group (Group III). Superior healing scores on both parameters (histologic healing and wound contraction) were observed, which was statistically significant as compared to Group I (untreated control) and Group II (propolis) [Tables 5 and 6, respectively].

**Discussion**

In the current study, wound healing by the second intention was chosen because it resembles a clinical condition that is frequently encountered in cases of traumatic oral ulcers.$^{[1-4]}$ An experimental period of 14 days was kept, as most oral wounds, even if infected, would show complete or nearly complete healing by the end of this period.$^{[9]}$ Rabbits were selected for the study as the studies conducted on animals are of low cost and provide useful information that could be used for further clinical studies. It is difficult to eliminate biases in human studies concerning their behavioral variables, and also to standardize and maintain the same living conditions during the entire experiment. Furthermore, standardization of variables such as size and depth of ulcer would not have been possible to achieve precision in human studies as it was achieved in rabbits.

The surgical mucosal wounds were made in the labial mucosa of all animals using a 5-mm punch-biopsy instrument before being removed with a scalpel from the rabbit’s labial mucosa. This method is very effective for treating ulcers of uniform diameters.$^{[11]}$
Healing score was calculated based on histopathological examination by using the method of Sultana et al.[18] On day 7 and day 14 in Group III (treated with Herbal ointment), significantly superior healing than Group II (treated with propolis) and Group I (Control) was observed. The healing status further improved in general in all the groups from day 7 to day 14. Group II in comparison to Group I showed slightly higher healing scores on both day 7 and day 14; however, the difference between these two groups was not statistically significant for both days. The slightly improved healing scores with Group II on day 7 and day 14 can be attributed to the biological and pharmacological properties of propolis such as tissue regenerative properties, antimicrobial properties, antioxidant activity, and antiulcer activity. Ali and Dahmoush (2012)[11] and M. Abdulrhman et al. (2012)[17] reported that propolis promotes the healing of oral ulcers.

Furthermore, on comparison of wound contraction scores on day 7, Herbal ointment (Group III) produced 68.53% wound contraction, which was significantly higher than 55.09% for the control group (Group I) and 54.97% for Propolis (Group II). Furthermore, on day 14, wound contraction in Group III, which was significantly higher (92.26%) than Group I (80.64%) and Group II (84.52%). Propolis produced slightly more superior wound contraction than untreated control on day 14, but the difference was not statistically significant. Thus it was observed that in terms of both criteria of histologic healing scores and wound contraction scores. Herbal ointment proved more effective in promoting healing of oral ulcers than propolis as well as the untreated control group. These observations are in accordance with findings of Pathak and Table 2

| Histopathological findings                                      | Score                                           |
|-----------------------------------------------------------------|-------------------------------------------------|
| Amount of granulation tissue                                    | Profound - 1, moderate - 2, scanty - 3, absent - 4 |
| Inflammatory infiltrate                                         | Profound - 1, moderate - 2, few - 3             |
| Collagen fiber orientation                                      | Vertical - 1, mixed - 2, horizontal - 3          |
| Amount of early collagen                                        | Profound - 1, moderate - 2, minimal - 3, absent - 4 |
| Amount of mature collagen                                       | Profound - 1, moderate - 2, minimal - 3          |
| Dilated blood capillaries and endothelial cells proliferation    | Profound proliferation - 1, moderate proliferation - 2, capillary dilatation only - 3 |

Table 3: Analysis of variance of means of histological wound healing scores using one way analysis of variance

| Day   | Source of variation | Sum of squares | DF | Mean square | F     | P           |
|-------|---------------------|----------------|----|-------------|-------|-------------|
| Day 7 | Between groups      | 280.83         | 5  | 56.16       | 38.34 | 0.0001 (S)  |
|       | Within groups       | 96.66          | 66 | 1.46        |       |             |
|       | Total               | 377.50         | 71 |             |       |             |
| Day 14| Between groups      | 97.73          | 5  | 19.547      | 21.89 | 0.0001 (S)  |
|       | Within groups       | 58.91          | 66 | 0.893       |       |             |
|       | Total               | 156.65         | 71 |             |       |             |

S: Significant

Table 4: Analysis of variance of means of wound contraction scores using one way analysis of variance

| Day   | Source of variation | Sum of squares | DF | Mean square | F     | P           |
|-------|---------------------|----------------|----|-------------|-------|-------------|
| Day 7 | Between groups      | 2774.16        | 5  | 554.83      | 45.79 | 0.0001 (S)  |
|       | Within groups       | 799.62         | 66 | 12.11       |       |             |
|       | Total               | 3573.79        | 71 |             |       |             |
| Day 14| Between groups      | 1629.47        | 5  | 325.89      | 34.93 | 0.0001 (S)  |
|       | Within groups       | 615.66         | 66 | 9.32        |       |             |
|       | Total               | 2245.14        | 71 |             |       |             |

S: Significant

Table 5: Intergroup comparison of means of histological healing scores of Group I to III with using Dunnet test

| Day   | Group   | Mean difference (I–J) | SE    | P         | 95% CI   |
|-------|---------|-----------------------|-------|-----------|----------|
|       | Group I |                       |       |           |          |
| Day 7 | Group II| 0.33                  | 0.49  | 0.941 (NS)| −0.93    | 1.60     |
|       | Group III| 4.50                 | 0.49  | 0.0001 (S)| 3.22     | 5.77     |
|       | Group III| 4.16                 | 0.52151| 0.0001 (S)| 2.69     | 5.63     |
|       | Group III| 0.66                 | 0.38  | 0.296 (NS)| −0.32    | 1.66     |
|       | Group III| 2.66                 | 0.38  | 0.0001 (S)| 1.67     | 3.66     |
|       | Group III| 2.00                 | 0.38238| 0.0001 (S)| 0.92     | 3.07     |

Group I: Untreated control group; Group II: Treated with propolis extract; Group III: treated with new herbal ointment; CI: Confidence interval; SE: Standard error; S: Significant; NS: Not significant
Table 6: Intergroup comparison of means of wound contraction scores of Group I to III with using Dunnet test

| Day   | Group       | Mean difference (I–J) | SE  | P        | 95% CI             | Lower bound | Upper bound |
|-------|-------------|-----------------------|-----|----------|--------------------|-------------|-------------|
| Day 7 | Group II    | Group I               | −0.12 | 1.42     | 1.000 (NS) | −3.78 | 3.53        |
|       | Group III   | Group I               | 13.43 | 1.42     | 0.0001 (S) | 9.77  | 17.09       |
|       | Group III   | Group II              | −13.55 | 1.37     | 0.0001 (S) | −17.44 | −9.67       |
| Day 14| GROUP II    | Group I               | 3.87  | 1.24     | 0.012 (S)  | 0.65  | 7.08        |
|       | Group III   | Group I               | 11.61 | 1.24     | 0.0001 (S) | 8.40  | 14.82       |
|       | group III   | Group II              | −7.74 | 1.13     | 0.0001 (S) | −10.94 | −4.54       |

Group I: Untreated control group; Group II: Treated with propolis extract; Group III: treated with new herbal ointment; CI: Confidence interval; SE: Standard error; S: Significant; NS: Not significant

Table 7: Mean scores for wound contraction, and histological healing for Group I through Group III

| Day   | Group       | Mean (SD) | Wound contraction | Histological healing |
|-------|-------------|-----------|-------------------|----------------------|
| Day 7 | Group I     | 55.09 (3.95) | 8.58 (0.79)     |                      |
|       | (control)   |           |                   |                      |
|       | Group II    | 54.97 (4.67) | 13.08 (1.56)     |                      |
|       | Group III   | 68.53 (2.77) | 13.75 (1.21)     |                      |
| Day 14| Group I     | 80.64 (4.15) | 14.33 (0.98)     |                      |
|       | Group II    | 84.52 (3.87) | 17.00 (0.85)     |                      |
|       | Group III   | 92.26 (2.65) | 17.83 (0.83)     |                      |

Group I: Untreated control group; Group II: Treated with propolis extract; Group III: Treated with new herbal ointment; SD: Standard deviation

Borkar, wherein they found that the said herbal ointment produced significantly better healing in comparison to silver sulfadiazine on burn wounds in rabbits. The superior healing properties of herbal ointment can be attributed to the effective medicinal properties of its constituents such as tissue regenerative properties, anti-microbial properties, antioxidant activity, and antiulcer activity.

Limitations of the study

The present study was conducted in animals; hence, the findings cannot be directly utilized for treatment in humans. This will require further clinical studies.

Conclusion

The herbal ointment used in this study was found to be more effective as compared to propolis, the presently preferred drug, used for promoting healing of oral ulcers. The herbal ointment after clinical trials is likely to emerge more effective and economic therapy as compared to propolis for the treatment of oral ulcers.

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Conflicts of interest

There are no conflicts of interest.

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