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Original Article

Effect of Glycyrrhizin and Sappan Wood Extract on Chemically-Induced Oral Mucosal Ulcer: An *In Vivo* Animal Study

Ariiq Azmi Rofiqi Sulkhan[1], Abdul Mun'im[2], Sutriyo[1,*]

ABSTRACT

Introduction: Stomatitis is the most common oral mucosal lesions characterized by round ulcers with yellow-white color and usually heals up to 14 days. Many recent studies have demonstrated that glycyrrhizin (GL) and *C. sappan* extract (CSE) exhibits anti-inflammatory, anti-ulcer and antioxidant action but no study has demonstrated the effect on the oral mucosal ulcer. **Objectives:** To evaluate the effect of GL and CSE in experimentally chemically induced oral mucosal ulcers using rats. **Methods:** Male Sprague-Dawley rats were randomly distributed into seven groups: the control group, the comparison group of 0.1% triamcinolone acetonide (TCA), a single treatment group of 3% GL and 3% CSE, combination groups of 3% GL + CSE (1:1, 1:2, 2:1). The oral ulcer model was induced by 15 µL of 50% acetic acid. The clinical healing was evaluated by measuring the ulcer size and body weight from day 0–14 and evaluate the leukocyte number on days 0, 4, 9 and 14. Histological examination was conducted at the end of the treatment. **Results:** The group of 0.1% TCA and GL:CSE (2:1) showed greater ulcer closure (>80%) and decreased leukocyte number since day 4 ($p<0.05$). Body weight loss was observed after ulcer initiation and started to increase after day 4 of treatment. While the histological examination showed similar tissue regeneration profile only from the GL:CSE (2:1) group with the healthy oral mucosa. **Conclusion:** Combination treatment of GL:CSE (2:1) enhanced the closure of oral mucosal ulcer and demonstrate complete tissue regeneration.

Key words: Stomatitis, Licorice, *Caesalpinia sappan*, Triamcinolone Acetonide.

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INTRODUCTION

Stomatitis is recognized as the most common oral mucosal disease characterized by symptoms of inflammatory conditions such as the presence of vesicles, erosion, aphthae, and ulceration.[1] The ulcers usually affect the nonkeratinized mucosa of buccal, labial, surface of the tongue, non-attached gingiva, or soft palate.[2] It presents as painful round shallow ulcers with circumscribed margins, a fibrin clot that covered the lesions, a yellow-white appearance and heals within 10 to 14 days.[3] This oral ulcer also called recurrent aphthous stomatitis that occurs recurrently because the etiology and pathogenesis remain unclear.[4] Many factors may be involved to cause oral ulcers such as traumatic injury, infection, hormone imbalance, immune dysregulation and medications.[5] The current treatment like topical corticosteroids, analgesics and antibiotics are intended to reduce the pain and ulcer duration. However, frequent and long-term exposure to these drugs may further lead to more adverse effects and often drug resistance.[6,7]

Natural medicines have become increasingly used in many countries. These become popular among consumers who search for natural ways as an alternative therapy and the general feeling that natural medicines are safer than synthetic drugs. Treatment of stomatitis with natural medicine might be best based on their anti-inflammatory, antioxidant, antistress, membrane protective activity because the oral mucosa membrane is susceptible to stress by contact with teeth, food and fluid intake and oral hygiene.[7] The natural agents such as licorice (*Glycyrrhiza glabra*) and sappan wood (*Caesalpinia sappan*) have already reported having anti-ulcer, anti-inflammatory, antioxidant, antibacterial, and antiviral properties.[8,9]

Licorice has been used worldwide as a sweetener and a flavoring additive in food and medicine and has been given Generally Recognized as Safe (GRAS).[10] The major active compound and abundantly present in the root of licorice is glycyrrhizin (GL). Many studies have reported that the anti-inflammatory activity of licorice is majorly influenced by that compound.[11] The other study showed that licorice enhances immune function and potentially increases mucosal immunity and anti-inflammation effects in the peripheral tissues of pigs.[12] Licorice mouthwash in the comparison study with 0.2% chlorhexidine both showed an equally effective treatment against chronic gingivitis up to 4 weeks in a total of 104 individuals.[13]

On the other hand, an ethanolic fraction of *C. sappan* showed significantly higher inhibition of oral pathogenic bacteria that cause dental caries and gingivitis than 0.12% chlorhexidine.[14] In that study, the polar fraction of *C. sappan* has the highest antibacterial activity. Another study of the hydroalcoholic extract of *C. sappan* reported that the extract exhibits gastroprotective activity and healing potential of ulcer lesions.[14] The main constituent that plays an important role in the *C. sappan* pharmacological activity is brazilin which is reported to have strong antioxidant, antibacterial and anti-inflammatory activity.[15]

However, the research data is very limited about the effect of glycyrrhizin and *C. sappan* extract to treat an oral mucosal ulcer. Therefore, this study was

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designed to investigate single and combination treatment's role and histologic effect in experimentally chemically-induced oral mucosal ulcer using rats.

MATERIALS AND METHODS

Materials

Glycyrrhizin 98% (GL) in fine powdery form was purchased from Shaanxi Jintai Biological Engineering Co., Ltd. (Shaanxi, China, Batch: JT190819); Sappan wood (*Caesalpinia sappan*) dried powdered extract (CSE) was purchased from Hunan Greenplant Resource Development Co., Ltd. (Hunan, China, Batch: GR-20200916-1); Triamcinolone acetonide (TCA) was obtained from Dexa Medica (Palembang, Indonesia, Batch: 408011658); and analytical grade solvents like ethyl acetate, n-hexane and acetic acid (Merck).

Thin layer chromatography (TLC)

The presence of brazilin in the sappan wood extract was identified through Thin Layer Chromatography (TLC) silica gel 60 F_{254} (Merck). The mobile phase was ethyl acetate:n-hexane (6:4 v/v). The TLC plate was visualized by ultraviolet (UV) light at 254 and 366 nm.[16] The major compound of the extract was confirmed with brazilin (Sigma Aldrich, USA) as a reference standard.

Animals

Male Sprague-Dawley rats aged 7 weeks (200-300 g) were used after one-week acclimatization. The animals were maintained on a 12 h light/dark cycle (06.00 to 18.00 lights on) and housed in a temperature and humidity-controlled room (22–25 °C and 40–60%) with food pellets and water provided ad libitum. The protocol for animal study (No. 20-09-1195) was approved by The Ethics Committee of the Faculty of Medicine, University of Indonesia. All animals were treated by following the procedures and guidelines according to the institutional and national guideline for the care and use of animals. All efforts were made to minimize animal suffering.

Oral mucosal ulcer model

Animals were anesthetized intraperitoneally (i.p.) with ketamine (50 mg/kg; Agrovet Market S.A., Canada) and xylazine (10 mg/kg; Interchemie, Netherlands). A round filter paper (d: 5 mm, Whatman No. 1) was soaked in 15 µL of 50% acetic acid and was used to create round ulcers. This acid-soaked paper was applied onto the oral mucosal region for 30 s[17]. This chemical stimulation would induce tissue necrosis and created an obvious ulcer form 3 days later (±5 mm in diameter). Complete wound healing of oral ulceration normally occurs up to 14 days.[18]

Experimental design and treatment

Animals were randomly selected and divided into seven groups (n = 7 in each group). Group 1 received no treatment and their measurements served as the control. Group 2 was administered with 0.1% TCA. Group 3 and 4 were administered with a single dose each of 3% GL and 3% CSE. Then, groups 5 – 7 served as 3% GL + CSE groups wherein animals received various combinations between GL and CSE (1:1, 1:2 and 2:1 respectively). All samples were diluted with distilled water and treated once a day into the oral ulceration started after ulcer initiation (day 0).

The total observation period was set as 14 days and the examination was performed every day including measurement of ulcer size and body weight. Ulcer size was measured along its longest diameter using a sterile vernier caliper. In each group, the blood sample was collected on days 0, 4, 9 and 14 for leukocyte examination. The clinical healing was defined by the decrease in the ulcer size and the leukocyte number.

Histological study

Tissue specimens of the oral mucosal were excised and fixed in 10% neutral buffered formalin (Bio-Optica) on the fourteenth day. Those specimens were embedded in paraffin blocks for histological processing. Tissue sections (4 µm thick) obtained from each paraffin block were stained with hematoxylin-eosin (HE) and evaluated histologically. The healthy oral epithelium was used for comparison.

Statistical analysis

Statistical descriptive were performed. All data were expressed as mean ± standard error of the mean (SEM). The difference of the mean ulcer size between the groups on each observation day was analyzed using one-way ANOVA. For the comparison, t test was used to perform a comparison between group and observation days. The significant difference was considered when the p value <0.05. Many literatures were used to determine and compare the result of histological examination.

RESULTS AND DISCUSSION

TLC profiles of reference standard and CSE are shown in Figure 1. The extract presented a chromatographic band corresponding to the brazilin standard. The *C. sappan* extract has known to contain various types of phenolic compounds, such as brazilin, protosappanin, chalcone, xanthone, and homoisoflavone.[8, 15] In CSE there is a very thin chromatographic band. According to the previous study,[16] the very thin chromatographic band was protosappanin A with a similar TLC profile. In addition, brazilin appeared as a brown spot observed under visible light and the spot has similar color and Rf value with the brazilin standard.

In this study, the oral mucosal ulcer was used to determine the effectiveness of GL and CSE on ulcer closure during healing process. We presented an animal model with a uniform oral ulcer and identical position on the oral mucosal region using acetic acid. Ulcer models also can be produced by various agents, such as mechanical, radiation

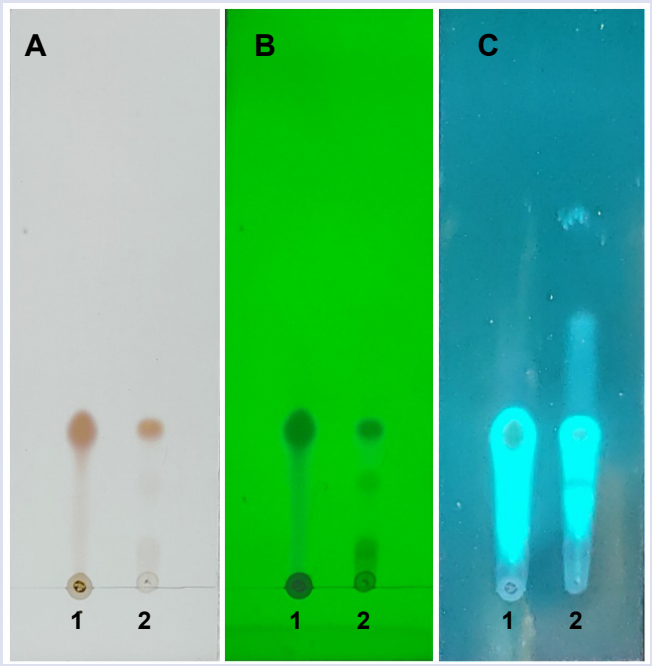

Figure 1: TLC profile of brazilin standard (1) and CSE (2). The stationary phase was TLC silica gel 60 F_{254} and the mobile phase was ethyl acetate:n-hexane (6:4 v/v). Detection: A = visible light, B = UV 254 nm, C = UV 366 nm.

and thermal trauma.[19, 20] In previous study, a white rabbit model of oral ulceration with the use of 15 µL of 50% acetic acid applied to the maxillary labial mucosa was reported in the formation of uniform circular ulcers.[21] The initial treatment was started after ulcer initiation (day 0, Figure 2C, black circled) when the round ulcer formed around 5 mm in diameter with a filter paper disc (d: 5 mm) as the calibration of the ulcer size.

The ulcer usually presents on the movable or nonkeratinized oral mucosa with the buccal and labial mucosa being affected frequently and heal without scarring up to 14 days.[2] According to this, a 14 days healing period was used to obtain a complete ulcer healing. The ulcer size of the samples on days 0, 4, 7 and 9 are presented in Table 1. The largest ulcer sizes were observed on day 0 and there is no significant difference between the ulcer initiation on day 0 (p = 0.16). Although all sites showed a healing progression, a significant difference could be observed. After 4 days of treatment, there are three groups that showed a highly significant decrease in ulcer size from 0.1% TCA group (0.43 ± 0.16 mm), GL:CSE (1:2) group (2.20 ± 0.11 mm), and GL:CSE (2:1) group (0.55 ± 0.20 mm) compared to control (p<0.0001). But, only from GL:CSE (2:1) group showed no significant difference compared to the 0.1% TCA group. The mean percentage of ulcer closure showed >80% of ulcer closure for both 0.1% TCA and GL:CSE (2:1) groups attaining 91.53 ± 3.20% and 89.24 ± 3.90% respectively (Figure 3) and there is no significant difference. Otherwise, reduction of the ulcer diameter was up to 9 days of treatment at the control group (Figure 2D-F) with the percentage of ulcer closure only 47.53 ± 1.91 (<50% healing).

Oral ulcer is an inflammatory condition with a lesion, disintegration, and ulceration of epithelial tissue. This ulceration with mixed inflammatory cell infiltrates is caused by dysregulation of the immune system in the oral mucosa.[22] In this study, we found that total leukocyte number increase significantly (>14000 /µL) in response to the oral ulcer (Figure 4A). On day 4, the number of leukocyte started to decrease as long as ulcer closure started to increase. The treated group of GL:CSE (2:1) showed a significant decrease compared to control (p = 0.0006) with the number of leukocyte 9650 ± 170.8 /µL that achieve the normal values (4000–10000 /µL)[23] since day 4, while the number of leukocyte from 0.1% TCA group is 10475 ± 332.6 (p = 0.001). At the end of the study, all sites showed a decrease in the number of leukocyte that indicates complete ulcer healing. All animal groups also experienced body weight loss on the day 0–4 and started to increase after day 4 of

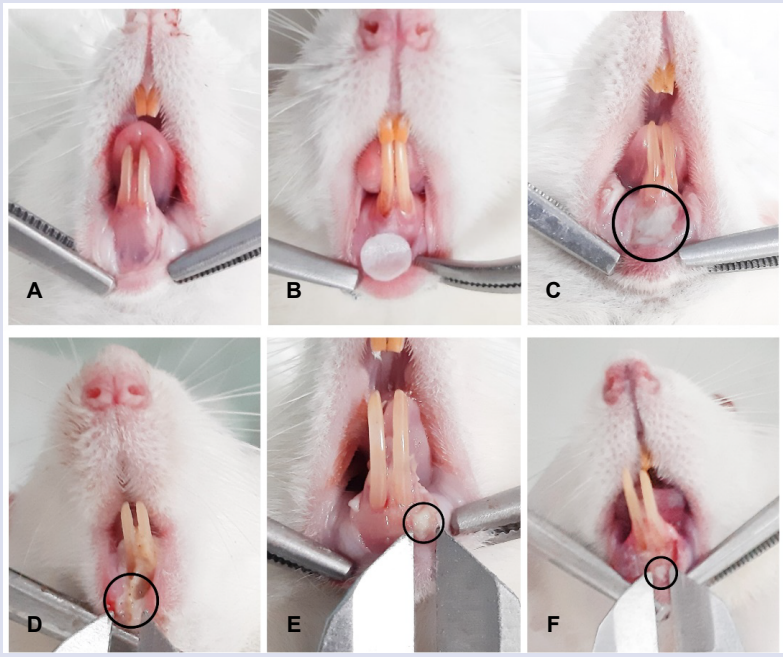

Figure 2: (A) Healthy rats oral mucosa. (B) Acid-soaked filter paper disc (d: 5 mm) application. (C) Ulcer initiation on day 0 (d: 5 mm). Measurement of the (D) 4 days ulcer, (E) 7 days ulcer and (F) 9 days ulcer from the control group.

Table 1: Mean ulcer diameter on day 0, 4, 7 and 9

Group	Ulcer diameter (mm)			
	Day 0	Day 4	Day 7	Day 9
Control	5.02 ± 0.03	4.18 ± 0.06	3.41 ± 0.12	2.63 ± 0.08
0.1% TCA	5.04 ± 0.02	0.43 ± 0.16*	0.00 ± 0.00*	0.00 ± 0.00*
3% GL	5.06 ± 0.02	3.42 ± 0.32	2.23 ± 0.19	1.20 ± 0.04*
3% CSE	5.06 ± 0.03	3.82 ± 0.22	2.27 ± 0.22	1.16 ± 0.05*
GL:CSE (1:1)	4.98 ± 0.04	2.97 ± 0.26	1.37 ± 0.09*b	0.78 ± 0.21*
GL:CSE (1:2)	5.07 ± 0.04	2.20 ± 0.11*b	0.91 ± 0.16*	0.00 ± 0.00*
GL:CSE (2:1)	5.10 ± 0.04	0.55 ± 0.20*a	0.00 ± 0.00*	0.00 ± 0.00*
p (one-way ANOVA)	0.16	<0.0001	<0.0001	<0.0001

n=7; *p<0.0001 compared to control; [a]not significant and [b]p<0.0001 compared to 0.1% TCA group (t-test)

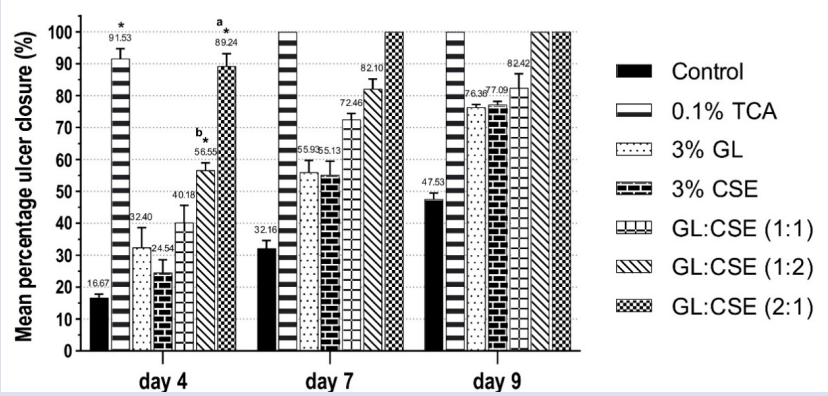

Figure 3: Mean percentage of ulcer closure (%). *p<0.0001 compared to control; [a]not significant and [b]*p*<0.0001 compared to 0.1% TCA group (t-test).

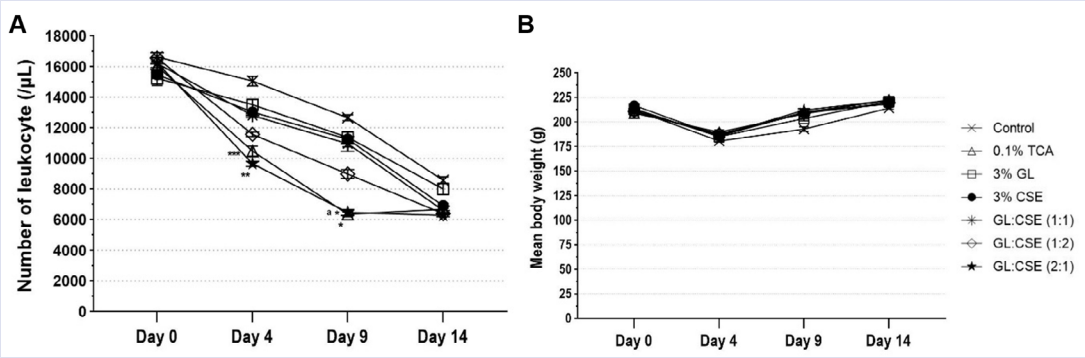

Figure 4: (A) The number of leukocyte (/µL) and (B) Mean body weight (g) measurement on day 0, 4, 9, and 14. *p<0.0001; **p<0.001; ***p<0.01 compared to control; [a]not significant compared to 0.1% TCA group (t-test).

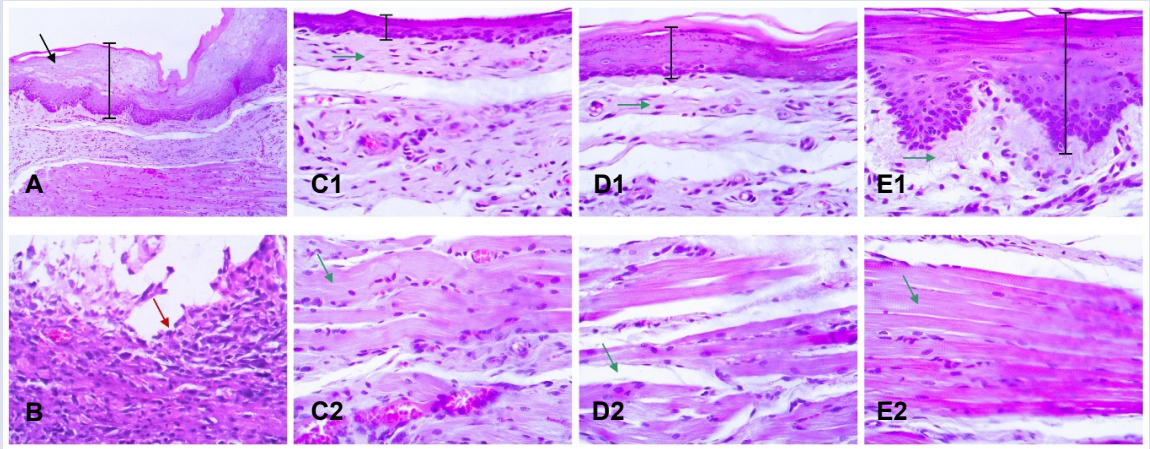

Figure 5: Photomicrograph of histological sections of (A) the healthy oral mucosa showing normal epithelium without any inflammatory cell infiltration and thickened epithelial cells (HE, 10X). (B) day 0 of ulcer initiation showing epithelial disruption and inflammatory cell infiltration (HE, 40X). Tissue remodeling after day 14 treatment of (C_{1-2}) untreated group, (D_{1-2}) 0.1% TCA and (E_{1-2}) GL:CSE (2:1) (HE, 40x). Black arrow demonstrates normal epithelium of oral mucosa; red arrow demonstrates inflammatory cell infiltration; green arrows demonstrate tissue remodeling; the black lines demonstrate epithelial thickness.

treatment (Figure 4B). The loss of body weight indicates that the ulcer symptoms may affect the food intake and causing pain during eating or swallowing.[24]

Decreasing ulcer size is amongst the determinants of healing but the resolution of pain is the most important issue that can be assessed from the epithelialization of the oral mucosa.[25,26] Based on the data analysis, group 2 (0.1% TCA) and 7 (GL:CSE = 2:1) showed the highly significant both for the percentage of ulcer closure and decreasing the leukocyte number since day 4 of treatment. So, the histological assessment of both groups was compared to the healthy oral mucosa and untreated group at the end of the treatment. On the initiation day of ulcer showed severe damage of the epithelial layer accompanied by granulation tissue and

inflammatory cell infiltration (Figure 5B) compared to healthy mucosa (Figure 5A). The chemically injured epithelial cells became necrotic and no longer adhered to one another.[27] The healthy mucosa showed intact epithelial layers and an absence of inflammatory cell infiltration. After the treatment, all epithelial layers had completely closed and showed the remodeling connective tissue. In the normal oral mucosa, the epithelium was identified as the keratinized stratified squamous type and a thick layer beneath the mucosa layer.[27,28] In this study, tissue profile for GL:CSE (2:1) treatment had been completely regenerated (Figure 5E), showed thickened epithelial cells and resembles the healthy epithelium.

The combination treatment of GL:CSE (2:1) showed a significant result on oral mucosal healing. As the major active compound of the licorice, GL has been reported in many studies to have strong anti-inflammatory activity.[11, 29] In the previous study indicates that GL is not a reactive oxygen species (ROS) scavenger but has the anti-inflammatory action that was reported by inhibiting the generation of ROS by neutrophils that is the most potent mediator of inflammation at the site of inflammation.[30] Another study showed that GL also suppressed inflammatory activities in chronic periodontitis rats.[31] From the study, we found that the effect of GL increases when given in combination with CSE. For some constituents, there are synergistic effects when they are given in combination. The use of licorice in Traditional Chinese Medicine (TCM) is more commonly in combination with other prescriptions. It has a unique effect on moderating and complementing the characteristics of other herbs in low dosage.[32] The reduction of the oral ulcer may be caused by many factors. Combination with CSE may provide strong antioxidant and antibacterial properties and accelerate complete healing. A comparison study between brazilin rich extract (BRE) and pure brazilin showed that BRE possessed antioxidant, antibacterial and anti-inflammatory activity almost equal to that pure of brazilin.[15] Those findings confirm the results of the present study that the combination between GL and CSE (2:1) was almost equally effective with the 0.1% TCA and the histological examination was more similar with the healthy oral mucosa.

CONCLUSION

Combination treatment of glycyrrhizin (GL) and *C. sappan* extract (CSE) with the ratio of 2:1 in a concentration total of 3% enhanced the closure of oral mucosal ulcer and demonstrate complete tissue regeneration. These results support positive evidence that GL and CSE can be used as effective in the treatment of oral ulcer. This study suggests that it could be developed as an alternative therapeutic agent for oral ulcer disease.

ACKNOWLEDGEMENTS

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ABBREVIATION

BRE: Brazilin rich extract; CSE: *Caesalpinia sappan* dried extract; GL: Glycyrrhizin; HE : hematoxylin-eosin; ROS: Reactive Oxygen Species; SEM: Standard Error of the Mean; TCA: Triamcinolone acetonide; TCM: Traditional Chinese Medicine; TLC: Thin Layer Chromatography.

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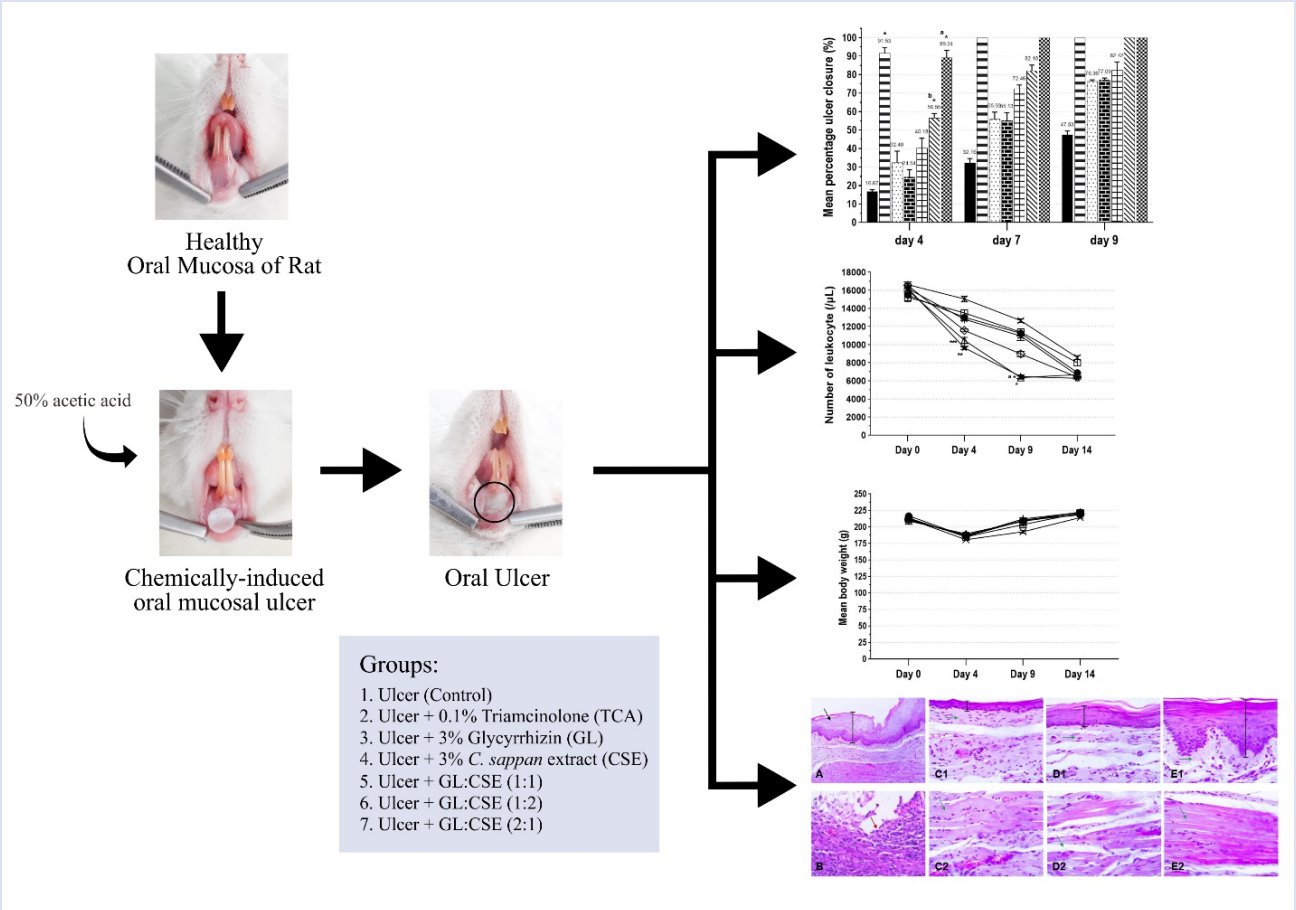
SUMMARY

This study was conducted to evaluate the effect of glycyrrhizin (GL) and *C. sappan* extract (CSE) in experimentally chemically-induced oral mucosal ulcers using rats. The oral ulcer was induced by 15 µL of 50% acetic acid to the seven groups of male Sprague-Dawley rats. The rats with oral ulcers were treated by various treatments. Combination treatment of GL:CSE (2:1) enhanced the closure of oral mucosal ulcer (>80%) and decreased leukocyte number since day 4, also demonstrate complete tissue regeneration similar to the healthy oral mucosa.

GRAPHICAL ABSTRACT



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Ariiq Azmi Rofiqi Sulkhan is a student of the Graduate Program of Herbal Medicine, Faculty of Pharmacy, Universitas Indonesia. Currently, the research focuses on herbal drug development and pharmaceutical technology.

Abdul Mun'im is a Professor at the Department of Pharmacognosy-Phytochemistry, Faculty of Pharmacy, Universitas Indonesia. Currently, the research focuses on herbal drug discovery, extraction technology, separation, isolation, analysis, structure elucidation of bioactive compounds, and *in vitro* and *in vivo* bioassay of medicinal plants and their biomarker.

Sutriyo is a lecturer at the Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Indonesia. Currently, the research focuses on pharmaceutical technology and nanoparticle drug delivery system.

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