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Review Article

OPTIMIZING ORAL DROP FORMULATIONS OF SAND SEA CUCUMBER (*HOLOTHURIA SCABRA*) AND RED DRAGON FRUIT PEEL (*HYLOCEREUS POLYRHIZUS*) FOR IMPROVED POSTOPERATIVE HEALING IN TONGUE CANCER PATIENTS (PRELIMINARY STUDY)

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ABSTRACT

This study analyzed the effectiveness of oral drops containing *Holothuria scabra* extract and *Hylocereus polyrhizus* peel at concentrations of 25%, 30%, and 35% for accelerating healing of post-excision stage 1 tongue cancer wounds in the tongue of rats with pre-clinical observations of wound closure and histopathology by observing fibroblast cells, neutrophils, and neocapillarization. A total of 45 male Wistar rats were divided into five groups, receiving either the test oral drops (25%, 30%, or 35%), a positive control (tetrachlorodecaoxide), or a negative control (no treatment). All rats were induced with a 2% DMBA compound on the lateral tongue to induce tongue cancer over 4 w. After tumour formation, the excised lesions were treated with oral drops and wound healing was monitored on days 3, 7, and 14 post-treatment, using macroscopic observation and histopathological examination. Data were analyzed using the Kruskal-Wallis and Mann-Whitney post hoc tests, with significance set at $p<0.05$. Oral drops containing combined extracts of *Holothuria scabra* and *Hylocereus polyrhizus* peel at 25%, 30%, and 35% showed similar effectiveness to the positive control in promoting wound healing, according to the reduction of length, width, and depth of the wound and histopathologically. Combining *Holothuria scabra* extract and *Hylocereus polyrhizus* peel in oral drop formulations at 25%, 30%, and 35% concentrations effectively accelerates healing of post-excision tongue cancer wounds. Among these, the 25% concentration is recommended for its efficacy and potential cost-effectiveness balance.

Keywords: *H. scabra*, *H. polyrhizus* peel, Oral drops, Tongue cancer, Wound healing

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INTRODUCTION

The most commonly found type of oral cancer is Oral Squamous Cell Carcinoma (OSCC), which is the sixth most common cancer worldwide [1]. The primary location for OSCC is the tongue [2]. According to data from the Global Cancer Observatory (GLOBOCAN), in 2020, oral cancer cases in Indonesia reached 5,780 cases [3]. OSCC has a relatively poor prognosis, with patients surviving an average of 2-3 y [4]. Several effects experienced by OSCC patients include speech disorders, swelling in certain areas, nausea, vomiting, and weakness due to reduced bodily function [5].

The clinical diagnosis of early-stage tongue cancer (T2N0Mx) is determined according to the TNM (Tumor, Nodes, Metastasis) classification system of the Union for International Cancer Control. The classification of tongue cancer size based on the TNM system ranges from stage 0 to stage 4. Stage 1 or early-stage tongue cancer is usually characterized by the appearance of a lump ≤ 2 cm on the tongue. The lump may be painful, and an ulcer with exophytic edges persists for 2 w. Therefore, surgery is the primary action that can be taken [6, 7]. If the diameter of the tongue cancer is less than 2 cm (early stage), excision will be performed transorally in an elliptical shape with a minimum margin of 2 cm [6].

A wound will form on the tongue post-surgery, with an average healing time of 20 d or longer [8]. The length of wound healing in post-operative patients depends on the presence or absence of complications and intrinsic and extrinsic factors, including infections [9]. The dentist will prescribe anti-infection medication to accelerate wound healing after OSCC surgery [10].

The wound-healing process increases the number of neutrophils and macrophages, as well as the oxygen consumption in tissues, which produces Reactive Oxygen Species (ROS). In normal amounts, ROS plays a role in killing microorganisms. However, when present in excessive amounts, tissues experience oxidative stress, slowing wound healing [11]. Antioxidants like flavonoids, alkaloids, tannins, steroids, phenols, and saponins are needed to accelerate wound healing [12]. Moreover, natural substances containing antioxidants can enhance the human immune system and prevent oxidative stress [13].

Wound healing is greatly influenced by collagen formation. Collagen synthesis requires active substances capable of stimulating fibroblast proliferation, leading to the formation of collagen that will constrict the wound [14]. Natural sources of collagen can be obtained from marine animals, one of which is sand sea cucumbers (*Holothuria scabra*) [15]. Indonesia has 10% of the approximately 650 species of sea cucumber (Holuthuria) found worldwide. Along the West Coast of Natal, in the Natal District, Mandailing Natal Regency, North Sumatra, three types of sea cucumbers can be found: *Holothuria atra* (black sea cucumber), *Holothuria leucospilota* (black slime sea cucumber), and *Holothuria scabra* (sand sea cucumber) [16].

H. scabra contains the highest collagen content in the waters of North Sumatra, around 80-86% [17, 18]. The extract of *H. scabra* can accelerate wound healing within an average of 10-18 d whereas previously healing occurred in 20 d or more, this because due to its bioactive compounds, such as flavonoids as antioxidants, saponins as anticancer agents, triterpenoids to stimulate collagen formation, and collagen to aid in tissue regeneration during wound healing [15]. The bioactive compounds also function as antibacterial agents capable of inhibiting the growth of several pathogenic bacteria [19]. Abdulkadir *et al.* (2021) investigated the effects of a 30% extract of *H. scabra* on incision wound healing in male rats, finding that wound closure or white scar tissue appeared within 9-10 days [20].

This study will combine a drop formulation derived from *H. scabra* with red dragon fruit peel extract (*Hylocereus polyrhizus*) to accelerate healing in post-surgical tongue cancer in rats. The *H.*

polyrhizus peel extract is used as a natural dye because it contains anthocyanins, which helps patients detect the extent of medication coverage on the wound area [21]. The *H. polyrhizus* peel extract has antioxidants such as vitamin C, flavonoids, tannins, alkaloids, steroids, and saponins that could accelerate wound healing and boost the immune system, especially in cancer patients [22]. The choice of a drop formulation in this study is due to better drug absorption, which increases the drug's effectiveness.

This research aims to analyze the effectiveness of oral drops containing *H. scabra* extract and *H. polyrhizus* peel at concentrations of 25%, 30%, and 35% for accelerating healing of post-excision stage 1 tongue cancer wounds in the tongue of rats with pre-clinical observations of wound closure and histopathology by observing fibroblast cells, neutrophils, and neocapillarization.

MATERIALS AND METHODS

This experimental laboratory study uses a post-test only control group design. The extraction of *H. scabra* was conducted at the Phytochemistry Laboratory of Pharmacy USU, the Cendikia Laboratory, and the Integrated Laboratory of USU. The formulation and examination of the oral drops were carried out at the Pharmaceutics Laboratory of Pharmacy USU, the testing of the oral drop preparation on animals was conducted at the Pharmacology and Toxicology Laboratory of Pharmacy USU, and the histopathological examination was performed at Adam Malik Hospital. This study has received Ethical Clearance from the Health Research Ethics Committee of Universitas Sumatera Utara with number: 808/KEPK/USU/2023.

The sample size was calculated using Federer's formula, dividing the rats into 5 groups, resulting in a total of 45 male Wistar rats. The first group was treated with a combination of *H. scabra* and peel of *H. polyrhizus* oral drops at a 25% concentration, whereby 3 rats were euthanized on the third day, 3 on the seventh day, and 3 on the fourteenth day for histopathological examination. The remaining four groups were treated with *H. scabra* and peel of *H. polyrhizus* oral drops at concentrations of 30%, 35%, a positive control (tetrachlorodecaoxide), and a negative control (no treatment), with each group also consisting of 9 rats.

The inclusion criteria for this study were male white Wistar rats aged 2-3 mo, with a body weight of 150-200 g, and active movement. The exclusion criteria were if the rats experienced a weight loss of more than 10% or weighed less than 150 g. The sampling technique used in this study was simple random sampling.

Extraction of *H. scabra*

5 kg of *H. scabra* was cleaned and diced into 1 cm cubes, then dried in an oven at 40 °C for 48 h, and ground using a blender. The *H. scabra* was then macerated using 96% ethanol at a 1:10 ratio for 3 d, stirred once a day. The macerate was filtered using Whatman No.1 filter paper and re-macerated with the same type and solvent volume. The filtrate was then concentrated using a vacuum rotary evaporator at 50 °C, and the extract was thickened using a water bath at 100 °C [20, 23].

The procedure for extracting collagen from *H. scabra* was 200 g of *H. scabra* simplisia, which was macerated at a 1:10 (w/v) ratio using a 0.1 M NaOH solution for 24 h. It was then homogenized in an orbital shaker-incubator at a speed of 150 rpm at 4 °C for 24 h, followed by centrifugation at 10 000 × g at 4 °C for 10 min. The resulting pellet was washed with distilled water until a neutral pH was achieved, then soaked in a 0.1 M CH3COOH solution at a 1:10 (w/v) ratio. Afterwards, it was homogenized in the orbital shaker incubator for 24 h at 4 °C at a speed of 150 rpm. The filtrate was centrifuged at 4 °C for 10 min at 10 000 × g, and the collagen solution was thickened using a water bath [24].

Extraction of *H. polyrhizus* peel

H. polyrhizus peel was cleaned and cut into small pieces, dried at 40 °C for 48 h in the oven, and blended until it became a powder. Subsequently, the simplisia was extracted using the microwave-assisted extraction (MAE) method. This entailed dissolving the simplisia with 70% ethanol in a 1:1 ratio, then stirring gently until the solvent and simplisia were thoroughly mixed. Simplisia was then heated at 450 watts for 8 min in the microwave and filtered with Whatman paper no.1. The same process was repeated twice. Finally, the extract was thickened using a water bath at 100 °C [25].

Preparation of oral drops

The 25% concentration was formulated by combining 25 ml (25%) of *H. scabra* extract solution with 0.2 g (10%) of citric acid as a dispersant (buffer), sodium benzoate as a preservative as much as 0.5 g (0.5%), glycerin as a viscosity increaser and sweetener as much as 4 g (4%), 3 drops (0.25%) of *H. polyrhizus* peel extract were added as a colourant. Subsequently, distilled water was added up to 100 ml (60.25%), and the mixture was homogenized using a mortar and pestle. Furthermore, the process was repeated for 30% and 35% concentrations [26].

Oral drops preparation test

An organoleptic test was used to observe the physical condition (clarity, odour, taste and colour) [27], and the density determination test was done using a pycnometer. The pycnometer was washed with aquadest, rinsed using alcohol, dried in the oven at 100 °C, cooled in a desiccator, and weighed the empty pycnometer containing the preparation. The viscosity test was also determined with a 12 Rpm, 30 Rpm, and 60 Rpm speed viscometer [28]. In addition, a pH test was done using a pH meter by dipping the electrode into the oral drops preparation [27]. The physical stability test (organoleptic) used the cycling test method by storing the preparation at 4 °C±2 °C for 24 h and then storing it at 40 °C±2 °C for 24 h; the time spent at two different temperatures was considered as one cycle and was continued for 12 d. The physical condition of the oral drops was compared during the experiment with the previous preparation [29].

In vivo test

Anesthesia was administered to all groups of rats using ketamine until the rats showed symptoms of unconsciousness. Then, a 2% DMBA (7,12-Dimethylbenz[a]anthracene) compound was injected to induce cancer with as much as 17.5 mg/kgBW on the lateral tongue [30]. The injection technique was done submucosally once and then observed for 4 w until cancerous protrusions appeared [31]. The tumors that developed on the rats' tongues after 4 w were varied in size; however, the excisions were standardized to a size of 5 × 2 mm with a depth of 2 mm (the tumor size did not exceed the wound dimensions). Tumor excision wounds were created using a No. 11 surgical blade and surgical scissors. The cancerous tissue protruding from the tongue was carefully removed using forceps.

Groups 1, 2, and 3 were given an oral drops preparation of *H. scabra* extract combined with *H. polyrhizus* peel at a concentration of 25%, 30%, and 35% daily, twice a day, as much as 2 drops within 14 d. Group 4 was given tetrachlorodecaoxide twice a day within 14 d, and group 5 was treated without medication.

Preclinical observation of rats and histopathology

Wound healing was observed daily, and measurements were taken on days 3, 7, and 14 after drug application to excision wounds. Preclinical observations included the length, width, and depth measuring of wounds using a probe, while local infection, allergic reactions, and wound closure were assessed visually based on a modified scoring system by Nagaoka *et al.* (2000) [32].

Histopathological observations were conducted on days 3, 7, and 14 after three rats were anaesthetized with ketamine, followed by neck dislocation and tongue tissue collection using a blade. Preparations were made with Hematoxylin and Eosin (HE) staining and examined under a microscope at magnification of 40x, 100x, and 400x. The wound healing process was histopathologically assessed by observing changes such as proliferation processes with fibroblasts, neutrophils, and neovascularization.

Histopathological values were scored from 0 to 3, with 0 indicating normal epithelium and connective tissue, no vasodilation, minimal inflammatory infiltration, and no bleeding, ulceration, or abscess. Score 1 indicated mild vascular hyperemia, reepithelialization, unclear inflammatory infiltrate, and no bleeding, ulceration, or

abscess. Score 2 indicated moderate vascular hyperemia, hydropic epithelial degeneration, inflammatory infiltrate with neutrophil prevalence, and possible bleeding, oedema, and occasional ulceration without abscess. Score 3 indicated severe vascular hyperemia with vasodilation, inflammatory infiltrate with neutrophil prevalence, and extensive areas of bleeding, oedema, ulcers, and abscesses.

Statistical analysis

Data analysis for comparing oral drop characteristics among three concentrations was performed using *One-way ANOVA*. Data analysis for comparing drop concentrations of 25%, 30%, 35%, positive control, and negative control based on preclinical and histopathological observation scores was conducted using the *Kruskal-Wallis test* with *post hoc Mann-Whitney* analysis. Data analysis using the Shapiro–Wilk and Levene's tests indicated that the data were not normally distributed; therefore, nonparametric statistical methods were employed. Significance was set at $p<0.05$.

RESULTS

Results of identification and screening tests of *H. scabra* and *H. polyrhizus*

The morphological identification of the *H. scabra* was performed at the Animal Systematics Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. Based on the letter number 179/UN5.2.1.11/KRK/2023, it was confirmed that the tested sample was indeed *H. scabra*. Active compound identification in the preparation was conducted at the Natural Product Organic Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. The results of the qualitative test for active compounds in *H. scabra* indicated the presence of alkaloids (+), flavonoids (+), terpenoids (+), saponins (+), while steroids (-) and tannins (-) were absent. In contrast, the qualitative test results for the skin of *H. polyrhizus* revealed the presence of alkaloids (+), flavonoids (+), terpenoids (+), saponins (+), and tannins (+), while steroids (-) were absent.

Results of the characteristic test of oral drop preparation

An organoleptic evaluation using a cycling test was carried out, with observations recorded at cycle 0 (initial observation) showing that the preparation's colour, odour, taste, and physical form corresponded to the data presented in table 1. Upon conducting the cycling test in cycle 1 (2 x 24 h), no changes were observed in colour, odour oral drops', taste, or physical form. This lack of change persisted throughout subsequent cycles (2, 3, 4, 5, and 6), with no observable organoleptic alterations [33]. The evaluation of the oral drop formulations at concentrations of 25%, 30%, and 35% demonstrated significant differences in adhesion, pH, viscosity, and spreadability, as summarized in table 1.

Table 1: Organoleptic observations and characteristic test results of oral drop preparations with three concentrations

Macroscopic observations	*H. scabra* and *H. polyrhizus* oral drops formulation			
	F1 (25%)	F2 (30%)	F3 (35%)	
a. Organoleptic observations				
Color	Yellow	Light Brown	Dark Brown	
Odor	Characteristic of Sea cucumber	Characteristic of Sea cucumber	Characteristic of Sea cucumber	
Taste	Tasteless	Tasteless	Tasteless	
Physical Form	Liquid	Liquid	Liquid	
b. Formulation evaluation				
	$\bar{x}$+SD	$\bar{x}$+SD	$\bar{x}$+SD	p
Adhesion (s)	0.18+0.01	0.29+0.00	0.34+0.02	*0.01
pH	7.22+0.03	7.16+0.02	7.04+0.02	*0.01
Viscosity (cPa's)	1.95+0.06	2.4+0.01	2.55+0.06	*0.03
Spreadability (cm)	11.77+0.25	10.97+0.15	10.4+0.26	*0.01
Homogenity	Homogeneous	Homogeneous	Homogeneous	

*Significant difference with *ANOVA* test

Preclinical observation results

Preclinical observations were conducted on days 3, 7, and 14 by measuring three parameters: wound length, width, and depth, macroscopically for each test group. The results are presented in table 2. All treatment groups, including the oral drops and the positive control, showed complete healing by day 14, with a score of 0 (indicating normal epithelium and connective tissue, no vasodilation, minimal inflammatory infiltration, and absence of bleeding, ulceration, or abscess). In contrast, the negative control group still exhibited unhealed wounds.

Table 2: Preclinical observation of wound healing based on the average length, width, and depth of wounds on days 3, 7, and 14

Wound healing indicator	Group 25% (a)	Group 30% (b)	Group 35% (c)	Positive control group (d)	Negative control group (e)	p
	Mean+SD	Mean+SD	Mean+SD	Mean+SD	Mean+SD	
a. Average wound length (mm)						
Day 3	4.00+0.00	3.83+0.28	3.5+0.50	3.67+0.29	4.83+0.29	0.045*
Day 7	0.33+0.58	1.00+1.00	0.67+0.58	0.83+0.58	2.50+0.50	0.105
Day 14	0	0	0	0	1.67+1.15	0.008*
b. Average wound width (mm)						
Day 3	1.33+0.29	1.67+0.29	1.67+0.29	1.67+0.29	2.00+0.00	0.14
Day 7	0.33+0.58	0.67+0.58	0.67+0.58	0.33+0.58	1.33+0.58	0.077
Day 14	0	0	0	0	1.17+0.29	0.008*
c. Average wound depth (mm)						
Day 3	1.83+0.29	1.5+0.00	1.5+0.00	1.83+0.29	2.00+0.00	0.061
Day 7	0.33+0.58	0.5+0.5	0.33+0.29	0.83+0.29	1.33+0.29	0.064
Day 14	0	0	0	0	1.17+0.29	0.008*

*Significant using *kruskal-wallis* test

Post hoc analysis with *Mann Whitney test* on day 3 for wound length obtained: a vs e = 0.034; b vs e = 0.043; c vs d = 0.046, d vs e = 0.043, while in other groups, there was no significant difference. On day 14, the comparison for wound length, width, and depth between the *H. scabra* and *H. polyrhizus* peel oral drops groups and the positive group showed a significant difference with the negative group p<0.05. This means the test groups of *H. scabra* and *H. polyrhizus* peel concentrations of 25%, 30%, and 35% had the same statistical effectiveness as the positive control in wound closure. All test groups except the negative group had closed the wound on the 14th day of observation. The negative group still had wound length of 1.67±1.15 mm, wound width of 1.17±0.29 mm and wound depth of 1.17±0.29 mm (table 2). Observations for local infection, allergic reactions, and wound closure time in each group according to the Modified Nagaoka *et al.* (2000) scoring score can be seen in (table 3) [32].

Table 3: Observation of infection, allergic reaction and wound closure time based on Nagaoka *et al.* assessment score (2000) [32]

Wound healing indicator	Group 25% (a)	Group 30% (b)	Group 35% (c)	Positive control group (d)	Negative control group (e)	p
	Mean+SD	Mean+SD	Mean+SD	Mean+SD	Mean+SD	
a. Localized infection						
Day 3	3.00+0.00	2.67+0.58	2.67+0.58	2.67+0.58	1.00+0.00	0.053*
Day 7	3.00+0.00	3.00+0.00	3.00+0.00	2.33+0.58	2.33+0.58	0.106
Day 14	3.00+0.00	3.00+0.00	3.00+0.00	2.33+0.58	1.00+0.00	0.015*
b. Allergic reactions						
Day 3	3.00+0.00	3.00+0.00	3.00+0.00	3.00+0.00	3.00+0.00	1.00
Day 7	3.00+0.00	3.00+0.00	3.00+0.00	3.00+0.00	3.00+0.00	1.00
Day 14	3.00+0.00	3.00+0.00	3.00+0.00	3.00+0.00	3.00+0.00	1.00
c. Wound healing time (Observation group day 14)						
<7 day	0	0	0	0	0	
7-13 day	3	3	3	3	0	0.007*
>14 day	0	0	0	0	3	

*Significant using kruskal-wallis test

Post hoc analysis using *the Mann-Whitney test* on day 3 for local infection obtained: a vs e=0.025; b vs e= 0.03; c vs d=0.034; d vs e= 0.034, while other group comparisons did not differ significantly. *Post hoc* analysis on day 14 for local infection obtained: a vs e=0.025; b vs e= 0.025; c vs d=0.025; d vs e= 0.034. *Post hoc* results explained a difference in the incidence of local infection between the test drops group and the positive control against the negative control group. 2 test animals died in the negative control group on observation day 3 and 14 due to post-excision infection.

The results showed that the 25% concentration treatment group did not find local infection in all rats. The 30% and 35% concentration treatment group and positive control showed that some rats had local infection without pus at different times. Good antibacterial ingredients, such as flavonoids, alkaloids, and saponins in the drops can overcome the infection that occurs.

No allergic reactions were found in all three concentrations of drops. Wound closure time with the test drops in the 25% group averaged 10 d; the 30% group averaged 9 d, the 35% group averaged 9.7 d, and the positive group averaged 8.7 d (p=0.09); this means that the drops accelerate wound healing after excision, which is usually around 20 d or more [8].

Histopathology observation results

The histopathological observations showed differences in the amount of fibroblast cells, neutrophil cells, and neocapillaries formed in each group. Histopathological observations of excisional wound healing on days 3 and 7 after administration of concentrations of 25%, 30%, and 35%, positive control, and negative control showed neucapillary cells, fibroblasts, and neutrophils (fig. 1 and table 4).

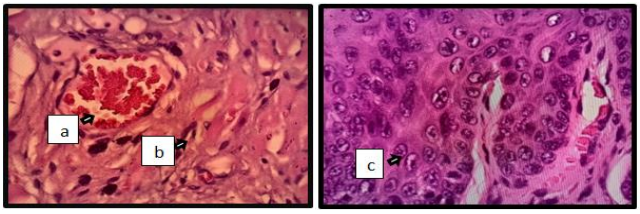

Fig. 1: **Histopathological observations oral drop concentration 25% on days 3 and 7 (a) neocapillary cells in wound healing, (b) fibroblast cells in wound healing, and (c) neutrophil cells in wound healing**

Table 4: Histopathological observation of wound healing based on neutrophil count, neocapillarization count, and fibroblast count on days 3, 7 and 14

Wound healing indicators	Group 25% $\bar{x}$+SD	Group 30% $\bar{x}$+SD	Group 35% $\bar{x}$+SD	Positive control $\bar{x}$+SD	Control negative $\bar{x}$+SD	p
a. mean neutrophil count						
Day 3	11+1	11.3+1.5	11.6+1.5	12.6+1.5	14+1.4	0.25
Day 7	7.3+0.5	6+1	7.6+1.5	8+1	15.3+1.5	0.04*
Day 14	3.3+1.5	2.6+1.1	2+1	2.3+0.5	16+1.4	0.16
b. mean number of neocapillarizations						
Day 3	5+1	6+1	6.3+0.5	4.3+2.1	1+0.0	0.08
Day 7	7,3+0.5	8+1	8.6+0.5	8+1	3.3+0.5	0.05
Day 14	8+1	8+1	8.6+0.5	7.6+0.5	3.5+0.71	0.13
c. mean number of fibroblasts						
Day 3	15.6+1.1	16.6+0.5	16+2.6	16.6+2.5	9+1,4	0.23
Day 7	26.6+2.0	26+1.7	27.3+0.5	24.3+4.5	16.6+1.5	0.09
Day 14	36+5.2	39.6+3.2	39+2.6	38.3+5.1	24.5+0.7	0.24

*Significant with Kruskal-Wallis test

The post hoc results in this study with the Mann-Whitney test for neutrophil mean were found a vs e = p 0.04; b vs e = p = 0.05; c vs e = p = 0.05; d vs e = p = 0.05. The results of this study showed that on day 7, the total number of neutrophils in the negative control group had the highest number among the other groups, and this difference was statistically significant.

DISCUSSION

One of the common treatments for tongue cancer is surgery. The research found that the average post-surgical wound healing time for cancer is 20 d, but it can be longer [8]. The wound-healing process is strongly influenced by collagen formation. Collagen content plays a role in accelerating the formation of granulation tissue on the wound surface [15]. Triterpenoids contained in Holothuria stimulate collagen production by stimulating fibroblast migration and proliferation and stimulating the biosynthesis of collagen types I and III, which occurs by activating the Smad pathway and inducing collagen synthesis by triggering the Smad kinase pathway without growth factor beta receptor I (TβRI) or Kinase-Independent Smad pathway [34].

The results of the analysis of postoperative wound healing of stage 1 tongue cancer showed that the administration of drops containing *H. scabra* and *H. polyrhizus* peel had the same effect as the positive control, namely, topical tetrachlorodecaoxide in wound closure based on the reduction of wound length, wound width, and wound depth. Statistically, there was no difference in effectiveness between the three concentrations of the test drops.

However, subtly, it was seen that wound closure at 25% concentration was better than 30%, 35% concentration and the positive control. This can be seen in reducing the wound's length, width, and depth, which is 25% higher than other concentrations. This result is in accordance with the characteristics of the 25% concentration of drops, which has a higher spreadability than the 30% and 35% concentrations of drops. The higher spreadability at 25% concentration can be easily applied to the tongue's surface, and penetration into the wound area is also better [33, 35].

Based on this post hoc analysis, there is a difference in the incidence of local infection between the test drops group and the positive control group compared to the negative control group. 2 test animals died in the negative control group on the 3rd and 14th day of observation, which was because this group was not given medication, making it easier for post-excision infection to occur. This was characterized by the discovery of infection accompanied by abscesses in rats.

Immunity of the test animals decreased due to the presence of compounds that induce cancer in the animal's body [36], causing the infection to spread and causing death in rats. In this condition, compounds such as flavonoids are needed to increase the body's immunity. Flavonoid compounds, including phenolic components contained in Holothuria, can increase the patient's immune system by inducing increased secretion of cytokines involved in CD4+cell activation; both compounds trigger up-regulation of T helper cells by increasing IL-2 cytokine production [37].

The results also showed that the 25% concentration treatment group did not have local infection in all rats. The 30% and 35% concentration treatment group and positive control showed that some rats had local infection without pus at different times. Although local infection was found, the oral drops contain good antibacterials such as flavonoids, which disrupt bacterial cell wall function through complex formation with extracellular proteins and inhibiting bacterial motility.

The destruction of the bacterial cell wall, which consists of lipids and amino acids, will react with the alcohol group of the flavonoid compound, causing the permeation of the compound into the bacterial cell nucleus. Then, the DNA contained in the bacterial cell nucleus will react with flavonoid compounds through the difference in polarity between alcohol groups and lipids that make up DNA, causing bacterial cell nuclei to lysis [38, 39, 40]. In addition, this oral drop test solution also contains saponin compounds that act as antibacterial and anticancer agents.

Saponins possess hydrophilic and lipophilic molecules, which reduce cell surface tension and compromise membrane permeability. This disruption of the cell wall surface tension facilitates the penetration of antibacterial agents into the cell, ultimately resulting in cell death. Furthermore, the damage to membrane permeability may lead to impaired bacterial survival. These active compounds can also induce leakage of proteins and enzymes from bacterial cells [41, 42].

The anticancer activity of saponins enhances the therapeutic potential of this solution by preventing cancer recurrence following surgery. As anticancer agents, saponins inhibit the overexpression of Bcl-2, induce the expression of caspase-3 (which is typically under-expressed), increase p53 expression, and may trigger G1 cell cycle arrest [43]. The tannin compounds in the peel of *H. polyrhizus* also exhibit anticancer properties by activating apoptosis pathways in cancer cells. Tannins inhibit cancer cell proliferation, partly through the inhibition of protein kinase activity, thereby disrupting the signal transduction pathways from the cell membrane to the nucleus.

Moreover, tannins inhibit the activity of tyrosine kinase receptors, which play a critical role in the malignant progression of cancer cells [43]. Alkaloid compounds in this oral drop test solution also function as antibacterial agents by disrupting the peptidoglycan layer in bacterial cell walls, a structure essential for bacterial survival in hypotonic environments. When the peptidoglycan layer is damaged, bacterial cell walls become rigid, leading to cell death [44]. The average wound closure time achieved with this oral drop test solution it was ranged from 7 to 13 d. Specifically, wounds treated with the 25% concentration of the solution closed within an average of 10 d, those treated with the 30% concentration closed within 9 d, and wounds treated with the 35% concentration closed within 9.7 d.

In contrast, the positive control group achieved wound closure within an average of 8.7 d. These findings suggest combining *H. scabra* extract and *H. polyrhizus* peel accelerates post-excision wound healing, typically taking 20 d or longer [8]. These results are consistent with the study conducted by Abdulkadir *et al.* (2021), which reported that a 30% methanolic extract of *H. scabra* healed incision wounds on the backs of male rats (*Mus musculus*) within 9-10 d [8, 20].

Histopathological examination revealed the presence of fibroblast cells, neutrophils, and newly formed capillaries, with varying quantities across the different treatment groups. Histopathological analysis of excision wound healing on days 3 and 7, following treatment with 25%, 30%, and 35% concentrations and positive and negative controls, showed evidence of neocapillarization, fibroblasts, and neutrophils.

These observations are consistent with the established understanding that granulation tissue formation, marked by the emergence of new capillaries and infiltration of inflammatory cells such as neutrophils, is vital to the wound healing process. Granulation tissue forms part of the wound healing process following the inflammatory phase. Initially, vasodilation and increased permeability of blood vessels surrounding the wound allow immune cells and plasma proteins to infiltrate the wound site. New capillaries subsequently form through angiogenesis, providing essential oxygen and nutrients to the healing tissue. Inflammatory cells such as neutrophils and macrophages migrate to the wound area to clear debris and pathogens while stimulating tissue repair. Fibroblasts then produce collagen and extracellular matrix components, forming loose connective tissue rich in new capillaries called granulation tissue. This process is regulated by various growth factors and cytokines that coordinate cellular activities involved in healing. Eventually, granulation tissue is replaced by denser scar tissue as the wound progresses to the next phase of healing [45, 46].

The saponin compounds present in the drops are capable of increasing collagen production in mucosal fibroblast cells through the phosphorylation of Smad 2 protein, thereby helping to accelerate the regeneration of the extracellular matrix (ECM) that is damaged due to injury. [47] A study demonstrated that the ethyl acetate fraction of H. scabra extracts (EAHS) effectively inhibited the synthesis of pro-inflammatory cytokines at both transcriptional and translational levels, specifically targeting nitric oxide (NO), inducible nitric oxide

synthase (iNOS), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and prostaglandin E2 (PGE2). Moreover, EAHS was shown to downregulate the expression of IκB/NF-κB and JNK, effects that are likely attributable to its high content of phenolic compounds and triterpene glycosides. Consequently, EAHS presents significant potential for development as a natural anti-inflammatory agent [48].

Based on the post hoc test results with the *Mann-Whitney* test for the mean number of cells that day 7, the number of neutrophils in the negative control group had the highest number among the other groups, and this difference was statistically significant. Neutrophil cells are one of the first immune cells to migrate to the site of inflammation or infection. The high number of neutrophil cells on day 7 in the negative control group indicates that inflammation still occurs in that group. In contrast, the other groups found a decrease in neutrophil cells.

An increase in neutrophil cells will inhibit the wound healing process, and new epithelial repair will take a long time; the healing process will take place quickly if there are few neutrophil cells. An increase in neutrophil cells in a wound can inhibit healing because these cells, although essential for fighting infection, can damage healthy tissue with the enzymes and free radicals they produce. This damage slows down epithelial repair and granulation tissue formation.

In contrast, with lower neutrophil counts and a controlled inflammatory response, wound healing becomes faster and more efficient, allowing for more optimal epithelial regeneration and new tissue formation. In addition to clearing pathogens, neutrophils also regulate inflammation and generate growth factors and cytokines to induce wound healing. In the wound environment, neutrophils have exhibited the ability to upregulate gene expression of chemokines that are key recruiters of macrophages, T cells, and additional neutrophils [49]. This is also supported by more neocapillary and fibroblast cells in the drops test group and positive control compared to the negative control.

An increase in the number of new blood vessels means that the wound-healing process is underway. An increase in new blood vessels, or angiogenesis, indicates that the wound-healing process is active. These new capillaries provide oxygen and nutrients that are essential for tissue repair. In addition, fibroblast cells, which increase in number, play a key role in healing by producing collagen and extracellular matrix components that form granulation tissue. Fibroblast cells also contribute to wound contraction, the process by which the wound edges close together, accelerating wound closure. With angiogenesis and fibroblast proliferation, wound healing can occur more quickly and efficiently, leading to more optimal repair and better recovery of tissue function. More fibroblast cells will accelerate wound contraction and healing [50-53].

The cyclic temperature stress test includes the medication preparation stability test method with the 6-cycle cycling test. The advantage of this test is the short test time, but it is good enough to test the preparation. The homogeneity check showed that the three oral drips preparation formulations were homogeneous, there were no solid particles, and there was no clumping, indicating that the components in the three formulations were evenly dispersed and stable. The adhesion test results of the three formulations showed less than 1 second, indicating that the drops adhere faster and the active substances can work optimally.

The pH test results show that all formulations are in the normal pH range, so the body can accept them. The spreadability is inversely proportional to the viscosity value shown in each formula. The greater the spreadability, the wider the tongue's surface area in contact with the oral drops will be, and the active substance will be well distributed. A good medication preparation has a significant spreadability value so that it can be applied easily on the tongue's surface without excessive pressure. The highest spreadability of the three concentrations of oral drops is 25% concentration, meaning that this concentration is the best in terms of the drug characteristics test.

The ability of *H. scabra* extract and *H. polyrhizus* peel to accelerate the wound healing process is influenced by the activity of active substances in the oral drops, such as high collagen and good antibacterial and antioxidant properties. Selain itu keberhasilan penyembuhan luka suatu obat dapat dipengaruhi oleh sifat obat, lokasi spesifik mukosa, dan metode pemberian (misalnya obat tetes). Umumnya obat yang diberikan melalui rute mukosa dapat diserap ke dalam aliran darah, baik secara langsung maupun setelah metabolisme lintas pertama jika tertelan [54]. Oleh karena itu perlu dilakukan penelitian lebih lanjut mengenai farmakokinetik dari obat tetes ini. Therefore, the results of this study have great potential to be developed as a wound-healing medication, especially in patients with postoperative tongue cancer. However, this study has certain limitations, as it is a preliminary investigation. Therefore, the sample size was not yet optimal for drawing more robust conclusions. Further studies are planned to expand and refine the findings through continued research and development. In addition, it is necessary to conduct toxicity testing of H. scabra drops, including a second phase involving animal testing and a third phase involving *in vivo* studies in humans to assess the safety of the drops. Further research is also needed to investigate the potential synergistic effects between H. scabra and H. polyrhizus extracts through factorial design experiments to determine whether the combination of bioactive compounds can enhance wound healing outcomes.

CONCLUSION

It can be concluded that the combination of *Holothuria scabra* extract and *Hylocereus polyrhizus* peel in oral drop formulations at 25%, 30%, and 35% concentrations effectively accelerates healing of post-excision tongue cancer wounds. Among these, the 25% concentration is recommended for its balance of efficacy and potential cost-effectiveness.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

No conflicts of interest were declared concerning the publication of this article.

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