ORIGINAL ARTICLE

The Potential of Bromelain Enzyme Extract from Pineapple Weevil Cayenne for Healing Gingivitis in Wistar Rats (*In vivo*)

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ABSTRACT

Introduction: Gingivitis is an inflammation that involves the soft tissue around the teeth. Neutrophils are the first cells to migrate from the blood vessels during inflammation and act as a defense against the body to phagocytize incoming microorganisms. The treatment of potential natural materials, such as pineapple fruit, is very likely to be developed to reduce the severity of tissue damage. Bromelain found in pineapple, especially pineapple weevil, can act as an anti-inflammatory agent by inhibiting inflammatory mediators. This study aimed to determine the number of neutrophils in the gingiva of rats with induced inflammation after administration of bromelain enzyme extract to pineapple weevils. **Materials and methods:** This research was a true experiment with post-test and control group design. The samples used in this study were 27 samples of gingival tissue from the mandibular incisor teeth of male Wistar rats (*Rattus norvegicus*) which were divided into four treatments comprised of negative and positive controlled treated with bromelain 20 %, 40%, and 60%. Samples were induced using ligature wire, following the inclusion and exclusion criteria. Inflammation in rats is characterized by redness and swelling of the gums where the ligature wire is applied. **Results:** Based on the Kruskal-Wallis test, there was a significant difference in the number of neutrophils ($p < 0.05$) between the treatment groups. A significant decrease was also observed in the treatment group with a 60% concentration on day 7 using the Mann-Whitney test. **Conclusion:** Bromelain enzyme extract in pineapple weevils reduced the number of neutrophils in the gingiva of rats that experienced inflammation.

Keywords: Inflammation, Gingivitis, Neutrophil, Pineapple Weevil, Bromelain

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INTRODUCTION

The gingiva is a soft tissue composed of epithelial tissue in the oral cavity that surrounds the teeth, and functions as a protective and chewing pressure barrier. The gingiva is prone to inflammation, both in oral care measures and when performing its functions. Inflammation of the gingiva can be caused by several factors, including periodontal disease, trauma and surgery (1, 2). Gingivitis is an inflammation that involves the soft tissue around the teeth, namely the gingival tissue. The clinical picture of gingivitis includes the appearance of a reddish color at the gingival margin, enlargement of the gingival contour, and easy bleeding. Gingival inflammation can be treated by maintaining oral hygiene, mechanical therapy (scaling and root planing), and medicinal treatment with anti-inflammatory drugs and antibiotics (3, 4).

The inflammatory mechanism begins when there is tissue damage; in this case, a sharp object tears the gingiva, resulting in a wound. Inflammation causes substances to be released endogenously and is known to be an inflammatory mediator. Cycloogenase-1 (COX-2) plays a role in normal function, namely, maintaining body homeostasis, whereas cyclooxygenase-2 (COX-2) is an enzyme whose presence is influenced by stimuli in the tissue. These stimuli include cytokines, bacterial lipopolysaccharides, inflammation, and other pathological conditions. Inflammation results in the accumulation of white blood cells, predominantly neutrophils and monocytes, at the site of injury to eliminate or limit the causative agent of injury. (5).

Neutrophils are the first cells to migrate from blood vessels during inflammation and act as a defense against the body to phagocytize incoming microorganisms. Wound healing can be successful if each healing phase consists of hemostasis, inflammation, proliferation, and remodeling phases well passed. The inflammatory phase of wound healing is essential because neutrophil infiltration occurs towards the wound, eliminating microorganisms and preventing microorganism contamination from colonizing and subsequently becoming an infection (6, 7). During the inflammatory process, the most important aspect is accumulating white blood cells at the injury site, predominantly neutrophils and monocytes. Some white blood cell products are activators of the inflammatory reaction and, in certain instances, cause significant

tissue damage. Chemotaxis is the process of white blood cell migration to the injured tissue due to chemical influences that can diffuse. Almost all types of white blood cells are affected to varying degrees by chemotaxis factors. Neutrophils and monocytes are most reactive to chemotactic stimuli. Neutrophils are one of the earliest and most important proinflammatory agents in the induction and control of acute-phase protein synthesis in trauma, infection, surgery, and burns. Neutrophils and mast cells release leukotrienes and prostaglandins during inflammation. Prostaglandins are generated by activating the cyclooxygenase pathway of arachidonic acid metabolism (8).Treatment with potential natural ingredients is still very likely to be developed to minimize these side effects; one of these ingredients is pineapple fruit. Pineapple is a leading commodity in Indonesia. Pineapple (Ananas comosus L. Merr.) contains antioxidants, vitamins, calcium, phosphorus, magnesium, potassium, iron, dextrose, sucrose, and bromelain. The bromelain content in pineapple can act as an antibacterial and anti-inflammatory agent and reduce the synthesis of PGE2, which plays a role in inflammation. Frikinda DA et al., in their research on the anti-inflammatory effects of pineapple juice and green apples in male rats, showed results where the concentration of pineapple fruit extract 60% was effective as an anti-inflammatory in reducing the volume of inflammation of the feet in rats. This is thought to be due to the content of the enzyme bromelain in pineapple and flavonoid compounds, whose mechanism of action is the same as that of diclofenac sodium, which inhibits the enzyme cyclooxygenase so that no pain mediators are formed that cause inflammation. This has been proven by the decrease in the volume of rat feet in both treatments, which was the same at the 4th hour (9).

According to Kusmatuti et al., the neutrophils could be seen around day 1 until day 4 after inflammation it is because the infiltration process of neutrophils occurs 24 hours to 3 days after inflammation occurs, then continues with the proliferation phase from day 3 to day 7. (5) But, in several research the neutrophils still could be found in one until two weeks after injury. According to Fajriani et al., wound healing decreased neutrophils count on day 4 of the acute inflammatory phase. It keeps getting worse until the 8th day of the healing process. Previous research has also demonstrated that, despite a significant drop in quantity, neutrophil cells can still be detected on day 15. This means that neutrophils were still found on day 8 and day 15 even though in small numbers because they had decreased (10). This study aimed to prove the effect of giving bromelain enzyme extract at 20%, 40%, and 60% concentration on the number of neutrophils in gingivitis rats on days 7 and 14.

MATERIALS AND METHODS

This research is a pure experimental (True experimental) with a post-test and Control Group Design. The samples used in this study were gingival tissue in the mandibular incisor teeth of 24 male Wistar rats (Rattus norvegicus). The ethical approval was sought and granted from Universitas Sumatera Utara (Animal Research Ethics Committees) with No.0156/KEPH-FMIPA/2023.

Preparation of pineapple weevil extract

Cayenne pineapple was taken from Jambu Rea Village, Siempat Rube District, High West Pakpak Regency. Pineapple stumps are sliced thinly then put into a blender and add 70% ethanol to taste, then blended until evenly distributed, put into a closed container, and then add the remaining ethanol. Calculation of the amount of ethanol: the amount of simplisia from 1 kilogram of sample is 200 grams so the amount of ethanol needed is 2000 ml (1 gram of simplisia: 10 ml of 70% ethanol). Macerate for 24 h, filtered using cotton and filter paper to obtain the macerate. Macerate was collected and evaporated using a rotary evaporator to obtain the filtrate. Subsequently, concentrations of 20, 40, and 60% were prepared.

Separation of bromelain enzyme in pineapple weevil

Prepare the pineapple weevil extract, centrifuge, and cuvette. The extract in each cuvette was centrifuged. The speed and duration of centrifugation were set at 5000 rpm for 15 min. After centrifugation, the cuvette was removed, and the supernatant liquid was separated from the sediment. The supernatant obtained from centrifugation is a bromelain enzyme crude extract solution, which showed a dark red color.

Inflammation induction and bromelain enzyme extract treatment in rats

The rats were acclimated for a week and fed two times a day. Rats were anesthetized using ketamine (0.2 ml/injection) intramuscularly using a 1 ml syringe on the thigh of the rat, waiting for 3-4 minutes for the anesthetic to take effect. The ligature wire was carefully attached to the cervical incisor of one mandible using the needle holder method. The ligature wire was pressed down such that it was right on the cervical tooth. The ligature wire was positioned above the gingival sulcus to prevent irritation, and the rat gingiva was adapted (Figure 1A). The rats were observed for about seven days until the gingiva of the rat became inflamed with clinical signs such as redness, swelling, and periodontal pockets, which were examined by visual and dental probing. After that, remove the ligature wire (Figure 1B). The bromelain enzyme extract from pineapple weevil 20%, 40%, and 60% as a positive control was dripped around

the gingiva as much as 0.5 ml on the labial side of the mandibular incisor twice a day in the morning and evening. In contrast, the negative control groups were not given any treatment (Figure 1C). Each sample group had a different sampling time. Group one was observed for seven days, and group 2 was observed for 14 days.

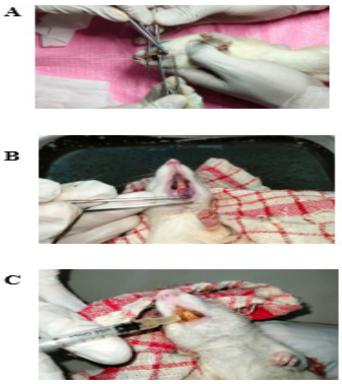

Figure 1: Inflamation induction (A) Gingivitis (B) and bromelain enzyme extract treatment in rats (C). A) Rat induction was carried out using a wire ligature on the mandibular incisor gingiva. B) Gingivitis formed after 7 days. C) Bromelain treatment was given to rats.

Rat mandible segment separation

The samples were taken by dislocating the neck quickly. The neck of the rat was cut and arranged on the container. Unnecessary tissue was removed using a blade and scalpel. Samples were washed with running water to clean it from blood and bacterial contamination. Samples were put into a containing formalin to maintain the integrity of the samples for 24 hours.

Preparation of tissues and histological staining

Each sample is soaked using a decalcification agent which aims to soften the sample. The rat's mandibular incisor gingiva was taken using a blade and scalpel (Figure 2A) and the rat's mandibular incisor gingiva sample was inserted into a tube. The fixation, dehydration, clearing and embedding processes are carried out by dipping the tissue on the tube into the solution and processing for 12 hours (Figure 2B). Making paraffin blocks is done by infiltrating liquid paraffin at a temperature of 57°C-59°C into a paraffin box to fill the cavity in the tissue that is occupied by water to form a paraffin block. The paraffin block is cooled briefly in the freezer so that it is not too soft, then the sections were sliced using a microtome with a thickness of 6-10 microns, and placed in a water bath at a temperature of around 46°C. Incubate on a hot plate at a temperature of 58°C-60°C for 15-30 minutes to evaporate the water in the tissue. The preparations were soaked in xylol with different concentrations to remove residual paraffin. The preparations were rehydrated with alcohol to remove residual xylol. The remaining alcohol was removed by washing the preparation under running water and taken to be applied with Hematoxylin Eosin staining. Hematoxylin Eosin which gives a blue color to the cell nuclei followed by rinsing under running water to remove remaining paint, and eosin as a counterstain material which gives a red color as a contrast. The preparation is dipped in water, after that the preparation is rehydrated using graded alcohol to remove water.

Figure 2: Rat Mandible Segment Separation (A) Soaking the Rat Mandible Segment (B). A) Rat Mandible Segment after surgery. B) Soaking the rat mandible segment with 10% formaldehyde solution.

Observation and calculation of neutrophil cell count container

Research data were obtained using a binocular microscope with observation of histological preparations. Staining of preparation was done using the Hematoxylin Eosin (HE) method and then counted under a microscope with 400x magnification for three fields of view. Calculation of the number of neutrophil cells in each preparation systematically starts from the left corner, then shifted to the right and pulled up so that all fields of view can be read. Then, calculate the average number of neutrophil cells per sample by calculating the average number of neutrophil cells from the three fields of view (Figure 3).

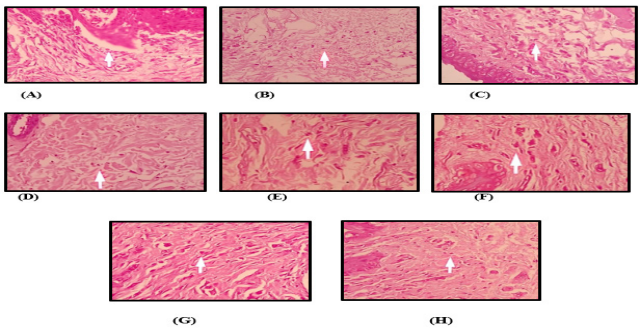

Figure 3: Histopathological picture of neutrophils, the white arrow shows the neutrophil cell (A) Control Group on Day 7 (B) Bromelain Group 20% on Day 7 (C) Bromelain Group 40% on Day 7 (D) Bromelain Group 60% on Day 7 (E) Control Group on Day 14 (F) Bromelain Group 20% on Day 14 (G) Bromelain Group 40% on Day 14 (H) Bromelain Group 60% on Day 14.

Statistical Analysis

The data obtained were analyzed using IBM SPSS software version 25. The results of the study were tested for normality with Shapiro-Wilk to determine whether the data were normally distributed or not. Then to see significant groups between treatments, the Mann-Whitney test was conducted.

RESULTS

After inducing inflammation in the gingiva using a ligature wire, to see the success of the inflammation, the Gingival Index measurement is used which aims

to determine the severity of the gingival inflammation. These parameters can be seen from the gingival color, gingival contour, and bleeding in the rat's gingiva. Table I shows the results of calculations using the Loe and Silnes Gingival Index; it is known that inflammation has occurred in the gingiva of rats with a score range of 1.5-2, which is classified as moderate. Table II shows that each group had a decrease in the number of neutrophils based on the results of the Kruskal-Wallis test. In the test, there was a significant difference in the number of neutrophils ($p<0.05$) between the treatment groups. Table III shows the results of this test when the Mann-Whitney test was conducted to compare time groups between day seven and day 14. The results of this test showed significant differences. In the 40% bromelain group (day 7) with 40% bromelain (day 14), p=0.05, and in the 60% bromelain (day 7) with 60% bromelain (day 14), p=0.046. As for the control group (day 7) with control, (day 14) p=0.376 and the 20% bromelain group (day 7) with 20% bromelain (day 14) p=0.127 which means $p>0.05$ indicates that there is no significant difference in the decrease in neutrophil counts in the two groups.

Table I: Gingival Index measurement data on the sample

No Sample	Gingival Index	No Sample	Gingival Index
K1U1	2	K5U1	2
K1U2	2	K5U2	2
K1U3	1,5	K5U3	2
K2U1	2	K6U1	2
K2U2	1,5	K6U2	1.5
K2U3	2	K6U3	2
K3U1	2	K7U1	2
K3U2	2	K7U2	1,5
K3U3	1,5	K7U3	1,5
K4U1	2	K8U1	2
K4U2	2	K8U2	2
K4U3	1,5	K8U3	2

Notes:
K1: Control (Day-7) U1: Sample 1
K2: Control (Day-14) U2: Sample 2
K3: Bromelain 20% (Day-7) U3: Sample 3
K4: Bromelain 20% (Day-14)
K5: Bromelain 40% (Day-7)
K6: Bromelain 40% (Day-14)
K7: Bromelain 60% (Day-7)
K8: Bromelain 60 % (Day-14)

Table II: Difference in mean neutrophil count at day 7 and 14

No.	Observation	Group (N = 3)	Mean ± SD	P- *Value*
1.		Control	6,13 ± 1,33	
2.		Bromelain 20%	2,73 ± 0,61	
3.	Day 7	Bromelain 40%	3,67 ± 0,41	P = 0,033*
4.		Bromelain 60%	2,6 ± 0,2	
5.		Control	5,47 ± 0,76	
6.		Bromelain 20%	1,6 ± 0,2	
7.	Day 14	Bromelain 40%	2,6 ± 0,87	P = 0,021*
8.		Bromelain 60%	1,4 ± 0,2	

Tabel III: Comparison of neutrophil counts of each group on days 7 and 14

Observation	Group Comparison	Sig
Day 7	Control – Bromelain 20%	1
	Control – Bromelain 40%	0,127
	Control – Bromelain 60%	0,05*
	Bromelian 20% - Bromelain 40%	0,05*
	Bromelain 20% - Bromelain 60%	0,05*
	Bromelain 40% - Bromelain 60%	0,045*
Day 14	Control – Bromelain 20%	0,05*
	Control – Bromelain 40%	0,261
	Control – Bromelain 60%	0,05*
	Bromelian 20% - Bromelain 40%	0,05*
	Bromelain 20% - Bromelain 60%	0,05*
	Bromelain 40% - Bromelain 60%	0,47*

The results of the Mann-Whitney test show a significant difference in value. In the 40% bromelain group (7th day) with 40% bromelain (14th day) p=0.05 and in the 60% bromelain group (7th day) with 60% bromelain (14th day) p=0.046. Meanwhile, for the control group (7th day) with control (14th day) p=0.376 and the 20% bromelain group (7th day) with 20% bromelain (14th day) p=0.127 which means $p>0.05$ indicates there is no significant difference in the decrease in the number of neutrophils in the two groups. (Table IV).

Table IV: Comparison of neutrophil counts of each group on days 7 and 14

Time Group Comparison	Sig
Control (day 7) – Control (day14)	0,376
Bromelain 20% (day 7) – Bromelain 20% (day 14)	0,127
Bromelain 40% (day 7) – Bromelain 40% (day 14)	0,05*
Bromelain 60% (day 7) – Bromelain 60% (day 14)	0,046*

Notes: Mann-Whitney Test
*p significant p<0,05

DISCUSSION

Changes that occur in the gingiva of rats where before being given the induction of rat gingiva is still in a normal state, after ligature wire induction the gingiva undergoes changes that are quite clearly visible, namely the color of the gingiva which is dark red and there is swelling. This is due to the decreased function of the gingival tissue due to ligature wire and plaque buildup on the gingiva which increases causing microorganisms to enter and damage the gingival tissue of rats.

Lucaciu Ondine et al, about periodontal disease induced in Wistar rats, stated the results after being given ligature induction the first changes occurred three days after ligature installation when the gingival tissue began to lose its normal aspect and structure. The color of the gingiva changed from pink to intense red (11). Peiya Lin et al, in a study of the application of ligature induction of periodontitis to the molecular mechanism of periodontal

disease, said after induction of periodontitis using ligature when inflammation occurs neutrophils were found to increase in the gingiva and spinal cord. This shows the potential of using ligature to have an impact on gingival inflammation and cause an increase in the number of neutrophils (12).

Differences in mean neutrophil counts after administration of bromelain enzyme extract at concentrations of 20%, 40% and 60% on day 7 and day 14. The results showed a significant effect on the number of neutrophils after administration of bromelain enzyme extract at concentrations of 20%, 40% and 60% both on day 7 and day 14. The results of the Kruskal Wallis Test conducted showed that on day 7 the p-value = 0.033 and on day 14 the p-value = 0.21 which means there is a significant effect ($p < 0.05$) after treatment. This is caused by the content of bromelain enzyme crude extract in pineapple fruit which plays a role in reducing the severity of gingival inflammation in patients with gingivitis. The content of bromelain contained in pineapple fruit has the potential as antibacterial and anti-inflammatory so that it can kill bacteria that cause gingivitis and reduce inflammatory conditions in the gingiva (13).

Research conducted by Widyawati et al stated that 0.5% pineapple fruit extract was effective in reducing leukocyte levels in catfish (*Clarias batrachus*) infected with *Aeromonas hydrophila* bacteria and 0.7% was effective in reducing neutrophil levels in infected catfish *(Clarias batrachus). Aeromonas hydrophila* bacteria. This is because pineapple contains antibacterial compounds, namely flavonoids. The active flavonoid compounds in Pineapple Fruit Extract can form complexes with bacterial cell proteins through hydrogen bonds. The structure of the bacterial cell wall and cytoplasmic membrane, which contains protein, becomes unstable because the protein structure of the bacterial cell becomes damaged due to hydrogen bonds with flavonoids, so the bacterial cell protein loses its biological effectiveness, as a result, the permeability function of the bacterial cell is disrupted and causes bacterial lysis and death cells (14).

This condition will affect the movement of neutrophils. The decrease in the number of neutrophils in the gingival sulcus fluid is due to a reduction in the severity of inflammation in the gingiva. According to the statement of Leoni et al., neutrophils are the initial response that infiltrates the wound area in the first 12 hours and, on day 3, will be phagocytosed by macrophages. Although neutrophils play an essential role in preventing infection in inflammation, the prolonged presence of neutrophils in the inflammatory area can cause tissue damage. Neutrophils have a dual role in healing, in addition to playing a role in the phagocytosis of pathogenic microorganisms. Still, the proteases and antimicrobial substances produced by neutrophils are not specific to certain pathogens, so the prolonged presence of neutrophils will damage tissues and hinder healing (13).

More clearly, Sudjarwo Sri's research on the signal transduction of bromelain as an anti-inflammatory in rat foot edema said that the mechanism of action of bromelain as an anti-inflammatory is due to its ability to inhibit the production of PGE2 through its inhibition, especially on COX-2 activity rather than COX-1. COX-1 enzyme plays a role in maintaining the body's homeostasis function, while COX-2 is an enzyme whose activity increases during the inflammatory process. Inhibition of these two enzymes by anti-inflammatories can inhibit prostaglandin formation, reducing and resolving inflammatory conditions. Thus, bromelain was shown to downregulate COX-2 expression rather than COX-1 in inflammation and suggests that the anti-inflammatory effect of bromelain is due to its ability to inhibit PGE2 production through its inhibition of COX-2 activity rather than COX-1 (15).

In this study, the group with the administration of bromelain enzyme crude extract at concentrations of 20%, 40%, and 60%, the number of neutrophils was significantly lower than the control group, namely without the administration of bromelain enzyme extract, which showed that the inflammatory healing process in the gingiva of rats using bromelain enzyme crude extract had a significant decrease in the number of neutrophils in the gingiva of rats that experienced inflammation.

Comparison of neutrophil count results of each group on day seven and day 14. This test showed a significant difference in value; the comparison between the 40% bromelain group with 60% bromelain had a $p=0.045$ on day seven and a $p=0.047$ value on day 14, which means there was a significant decrease between the two concentrations. In comparing the 20% bromelain group with 40% bromelain, there was a decrease with a value of $p = 0.05$, which means that the value decreased significantly. Still, when viewed in comparison between the control group and 40% bromelain with a value of $p = 0.127$ on day seven and a value of $p = 0.261$ on day 14, it means that there is no significant decrease between the control group and the 40% bromelain group. When compared, these data show a difference between the control group and the treated group. So, in the table, the more effective concentration is the bromelain treatment group with a concentration of 60% because this concentration, when compared to the control group and the treatment group, still experienced a significant decrease.

This study is in line with the research of Febrina Rasia et al. on the effect of gargling pineapple fruit extract solution on reducing the number of neutrophils in the gingival sulcus fluid of patients with mild gingivitis where the results showed that after gargling a 50% pineapple fruit extract solution was shown to significantly reduce the number of neutrophils in patients with mild gingivitis. The study showed that the effectiveness was close to where the concentration in the research conducted

was 60% (16). So, from this study, the higher the concentration, the effect of pineapple fruit extract also has more significant potential in reducing the number of neutrophils (16). Comparison of the results of neutrophil counts on day seven and day 14. The Mann-Whitney test results significantly differed between the day seven and 14 groups. Bromelain 60% showed a vital value, meaning there was a significant decrease in neutrophil counts on days seven and 14. Bromelain, with 40%, also effectively reduced the number of neutrophils. The control group and the 20% bromelain treatment group did not experience a significant decrease on day seven and day 14.

In this study, the results of index measurements on day 7 and day 14 showed that the gingival index of the mice was moderate in all groups. This is also a possible reason that in this study, the neutrophils were still found on day 7 and day 14 but in small numbers because there had been a decrease. This study is in line with the research of Fajriani et al that showed neutrophils were still found on day 8 and day 15 even though in the lowest numbers because they had decreased (10).

The inflammatory phase causes leukocyte migration to phagocytose incoming microorganisms, including neutrophils and macrophages. Neutrophils are the first cells released after inflammation. Neutrophils provide an immune response to protect tissues by digesting foreign particles and killing incoming bacteria. An increase in the number of neutrophils causes symptoms of swelling, as seen in the clinical picture of the rat when the ligature wire is removed. The process of neutrophil infiltration occurs 24 hours to 3 days after inflammation is continued by the proliferation phase from day 3 to day 7, and the remodeling phase happens from day 7 to day 14 (5, 17). This shows that the control group and the 20% bromelain group, although not experiencing a significant decrease in neutrophil counts, basically the healing process has occurred after day three, where the role of neutrophils in the proliferation phase has been replaced by macrophages so that the average number of neutrophils in each control and treatment group has decreased. When viewed from the results of the Mann-Whitney test, the group with a significant decrease is the treatment group with a concentration of 60%, which means that the bromelain enzyme crude extract at that concentration has more significant potential in reducing the number of neutrophils compared to other concentrations. The effective time group is on day seven because the number of neutrophils decreases over time due to being replaced by the proliferation phase.

CONCLUSION

There is a significant difference in the administration of bromelain enzyme extract in pineapple weevils on the number of neutrophil cells in the gingiva of rats that experience inflammation on days 7 and 14, especially in 60% and 40% concentrations. From these results, it can be concluded that bromelain enzyme extract in pineapple weevils could reduce the number of neutrophils in the gingiva of rats that experienced inflammation.

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