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Research Article

Sauropus androgynus Effect on Periodontal Tissue Inflammatory Cells with Orthodontic-Separators

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Abstract

Orthodontic treatment aims to correct the improper arrangement of teeth. Tooth movement caused by orthodontic treatment causes tension and compression areas in the tooth's supporting tissues. The periodontal ligament will respond to this stimulus by triggering the inflammatory process. Flavonoids in *Sauropus androgynus* act as anti-inflammatories by inhibiting lipoxygenase and cyclooxygenase. This experimental research was conducted on 32 guinea pigs, which were grouped into 2 groups: the control group had a rubber separator applied for 14 days, and the experimental group had a rubber separator applied also given ethanol extract of *Sauropus androgynus* 78.3 mg/kg BW. Samples were taken on days 0, 3, 7 and 14 and statistically analyzed using an independent T-test and Mann-Whitney test. The results showed that the ethanol extract of *Sauropus androgynus* affected the number of inflammatory cells. Based on statistical results, there were significant differences between the control group and the treatment group in neutrophil cells (p=0.000), eosinophils (p=0.000), lymphocytes (p=0.002), and monocytes (p=0.000). In contrast to macrophage cells, statistical results showed no significant difference between the control and experimental groups with p=0.064. The observed effects of the ethanol extract of *Sauropus androgynus* on periodontal ligament inflammatory cells (neutrophils, eosinophils, lymphocytes, and monocytes) suggested its potential as an anti-inflammatory agent.

Keywords: flavonoids; inflammation; Katuk leaves; orthodontic

INTRODUCTION

Occlusion in dentistry is the contact between the teeth or the relationship between the maxillary and mandibular teeth at rest or during movement. Abnormalities in the alignment of teeth or dental arches that do not conform to normal conditions are known as malocclusions. The prevalence of malocclusion ranks third in dental and oral health problems worldwide. One factor that influences the length of orthodontic treatment is the type of malocclusion. The type of malocclusion can be distinguished by the Angle classification, which is divided into 3 classes of malocclusion.[1-3]

One of the treatments in dentistry is orthodontic treatment. Orthodontic treatment aims to correct the improper arrangement and position of the teeth so that a stable occlusion relationship is obtained to restore masticatory function, muscle balance and facial aesthetics. Orthodontic treatment requires the movement of teeth that will occur when the teeth are put under pressure. The pressure will be transmitted to the supporting tissues of the teeth and will cause pressure and strain area.[4-6]

Bone remodelling in the tissue around the tooth's root due to the applied force causes tooth movement. An orthodontic separator is used between

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adjacent teeth to force the teeth and cause the initial phase of tooth movement. Tooth movement in orthodontic treatment is a biological process caused by mechanical stress so that the supporting tissues of the teeth would adapt. When orthodontic forces are applied to the teeth, the periodontal ligament responds to mechanical forces and causes alveolar bone apposition with the help of fibroblasts, osteoblasts, osteoclasts, and cementoblasts. The pressure side will experience a narrowing of the periodontal ligament and vascular constriction, followed by a decrease in cell replication and production of collagen fibres, so osteoclast activity results in alveolar bone resorption. The function of osteoclasts is to resorb bone during the remodelling process. Meanwhile, the activity of osteoblasts causes bone apposition on the reverse side. The periodontal space on the strain side will widen so that vascularization, cell replication, and collagen fibre production increase, resulting in bone apposition by osteoblasts. The function of osteoblasts is the secretion of the bone organic matrix that helps mineralise the uncalcified matrix.[4,6,7]
A complex process in orthodontic tooth movement involves many factors. Tissue trauma, periodontal ligament compression and bone deformation are things that will occur due to the orthodontic forces applied to the teeth. It is followed by a change from mechanical forces into the form of molecular events called signal transduction and involves prostaglandins. Prostaglandins are released when cell membranes are deformed, which results from arachidonic acid metabolism and plays a role in bone metabolism and tooth movement. The mechanically deformed periodontal ligament cells will cause the release of the first messenger. The first messenger then stimulates the release of the second messenger. The release of the second messenger involves cAMP and intracellular calcium, which will activate osteoclasts. In addition, RANKL formation is triggered by prostaglandin that bonds to the EP4 receptor on the surface of osteoblast cells. When it bonds to the RANK on the surface of osteoclast progenitor cells, osteoclast differentiation, which will resorb the bone, is initiated.[4]

Flavonoids are a class of phenolic compounds found in *Sauropus androgynus*. Isoflavones are part of the flavonoid group. Genistein in isoflavones functions to inhibit IL-1β, IL-6, and TNF-α. Flavonols such as kaempferol, quercetin, and myricetin can act as lipoxygenase inhibitors. Besides that, quercetin, together with apigenin, can also inhibit the production of nitric oxide (NO).[6,8,9]

Flavonoid compounds are thought to provide anti-inflammatory activity by inhibiting the release of serotonin and histamine to the site of inflammation and inhibition of lipoxygenase and cyclooxygenase (COOX) in the synthesis of prostaglandins and arachidonic acid.[10,11]

Based on the explanation above, this study aims to determine the effect of oral *Sauropus androgynus* leaf ethanol extract on the number of inflammatory cells in guinea pig teeth driven by orthodontic separators.

MATERIALS AND METHODS

This research is a laboratory experimental research with Post Test Only Control Group Design research using 2 groups of guinea pigs taken using a simple random sampling technique. The research was conducted at the Laboratory of Pharmacology and Therapy, Faculty of Medicine, Padjadjaran University and the Laboratory of Cytohistotechnology, Faculty of Health Sciences and Technology, Universitas Jenderal Achmad Yani, from October to December 2021. This research has received ethics from the Medical Research Ethics Commission, Faculty of Medicine, Padjadjaran University, with a letter of ethics number 964/UN6.KEP/EC/2021.

Samples and the subject of the research

Guinea pigs (*Cavia cobaya*) were obtained from the Cililin Farm and have adapted to

the Pharmacology and Therapy Laboratory of the Faculty of Medicine, Padjadjaran University, for 7 days. The inclusion criteria in this study included guinea pigs with good tooth contact, 2-4 months of age, 300-400 grams of body weight, physically healthy (characterized by active movement), had a good appetite, came from the same breeding site, all groups guinea pigs are fed the same. Exclusion criteria in this study were sick guinea pigs (marked by decreased body weight >10%), guinea pigs that died during the research process, guinea pigs that did not want to eat, and guinea pigs with diastema central incisors. According to the Federer formula calculation, the number of guinea pig samples used was 32. The guinea pigs used in this study were grouped into 2 groups based on the treatment. The 2 groups consisted of two groups: the control group included the guinea pig, which only had an orthodontic separator applied, and the experimental group was the guinea pig with a rubber separator applied and the ethanol extract of *Sauropus androgynus* at a dose of 78.3 mg/kgBW. Samples were taken on days 0, 3, 7 and 14, with 1 sample on day 0, 3, 7 and 13 samples on day 14. Limiting the number of samples at earlier time points (days 0, 3, and 7) was a strategy to minimize the use of animals in research, aligning with ethical considerations. It might be assumed that significant changes in inflammatory cell counts might not occur until later.

Research and tool materials

This study included materials for manufacturing ethanol extract of Sauropus androgynus and materials for treatment. Identification and determination of katuk plants were done at the Jatinangor Herbarium, Plant Taxonomy Laboratory, Department of Biology, Padjadjaran University, with identification number 14/HB/09/2020. *Sauropus androgynus* obtained from the Pharmacy Medicine Garden of Universitas Jenderal Achmad Yani. The ethanol extract of Sauropus androgynus was manufactured at the Biochemistry Laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani. The ingredients for the treatment were orthodontic separator rubber, which was applied to the teeth of guinea pigs and ethanol extract of *Sauropus androgynus.* Tissue preparations were made using hematoxylin and eosin.

The tools used in this study consisted of extraction tools, guinea pig-rearing tools, maintenance tools, and tissue preparation tools. The extract-making equipment included a rotary evaporator, Erlenmeyer tube, oven, analytical balance, water bath, and pH meter. A guinea pig cage with a tub made of braided wire, digital scales for guinea pigs, and food and drink containers were tools for raising guinea pigs. The separator and scalpel pliers were included in the treatment tool. Minor surgical instruments, microtome, object glass and deck glass were tools for making tissue preparations.

Procedures

Preparation of *Sauropus androgynus* ethanol extract: 1 kg *Sauropus androgynous* and washed with clean water. Maceration was carried out with 70% ethanol for 24 hours. The maceration results were filtered using 5 mm of filter paper, and the maceration was repeated several times until the colour of the solvent disappeared. The maceration process was carried out again approximately 5-6 times. After the extraction results were collected, a Buchi rotary evaporator was used to obtain a more concentrated consistency. It was done until there was no remaining alcohol solvent dripping.

Treatment of experimental animals: The guinea pig was adapted for 7 days at the Pharmacology and Therapeutic Laboratory, Faculty of Medicine, University of Padjadjaran. The guinea pig was anaesthetized by an intraperitoneal technique using ketamine 20 mg/kgBW in the posterior 2/3 of the right abdomen. Separator rubber was applied to the guinea

pig's mandibular incisors using separator pliers. The separator rubber was replaced on the 8th day because the rubber separator easily lost force, especially the first 24-48 hours after use, and the force would continue to decrease over time. The guinea pigs in the experimental group were given ethanol extract of *Sauropus androgynus* at a dose of 78.3 mg/kgBW orally once a day for 14 days with an oral sonde every morning at 7 am.

Figure 1. Rubber separator applied to guinea pig teeth

Euthanasia was performed on days 0, 3, 7 and 14 with ketamine 30 mg/kgBW anaesthetic. Tissue was taken from the longitudinal direction of the mandibular bone and both guinea pig incisors. The tissue observed was in the distal area of the periodontal ligament of the incisor, which was applied with a rubber separator. The tissue was fixed and preserved for 24 hours using a 10% formalin solution. Histological preparations used the Hematoxylin-Eosin staining method. The first step was decalcification of the tissue with 8% formic acid for ten days. The next step was dehydration, clearing, impregnation, embedding, and cutting.

Observations used a light microscope on a high power field (HPF) of view with 400x magnification to observe the number of inflammatory cells in the periodontal ligament, and at least 2 people observed.

Data analysis

The histological observations of the number of inflammation cells were statistically analyzed using the independent T-test for normally distributed data and the Whitney test for data that were not normally distributed. The Statistical Product and Service Solution (SPSS) program was used to analyze the data with a 95% confidence level of 0.05 significance level (p-value 0.05).

RESULT

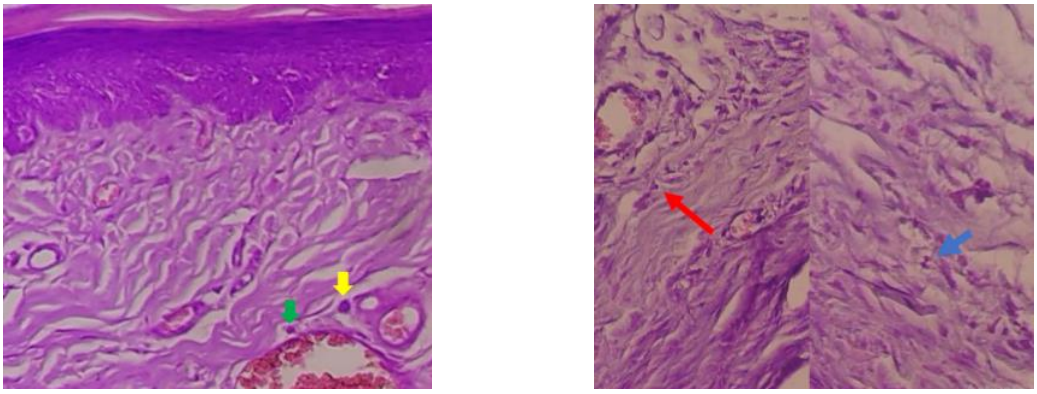
Calculation of the number of inflammatory cells in the periodontal tissue of guinea pig incisors was carried out by histological observation using a light microscope with 400x magnification on 5 fields of view.

Table 1. Inflammatory cells number with an orthodontic separator on day 0, 3rd, 7th, and 14th

Cells	Day 0		Day 3rd		Day 7th		Day 14th	
	C	E	C	E	C	E	C	E
Neutrophils	0.4	0.2	2.6	4	7.6	18	16.29	5.66
Eosinophils	1.4	0.6	2	3	1.4	3.4	2.03	0.72
Lymphocytes	0.6	0	3.2	3.2	4.4	3.2	14	8.20
Monocytes	0.2	0.6	1.2	1.6	2.2	4.4	3.29	0.72
Macrophages	0	0	0.8	0.6	1.2	1.2	1.03	0.60

Remark C: Control group, E: Experiment group

Table 1 on day 0 shows that in neutrophils, the control group was 0.4 and the experiment group was 0.2; on eosinophils, the control group was 1.4 and the experiment group was 0.6; on lymphocytes, the control group was 0.6 and not present in the experiment group; on monocytes, the control group was 0.2 and the experiment group was 0.6; there were no macrophages in the control or experiment groups. Inflammatory cells can be seen in Figure 1.

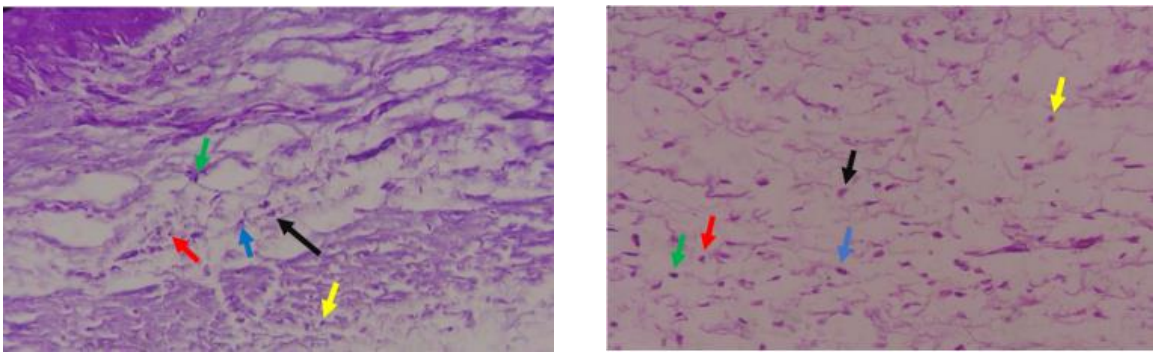


(a) **(b)**

Figure 2 Microscopic image of the number of periodontal tissue inflammatory cells on day 0 magnification 400x; (a) control group (b) experiment group.

Description: Blue: neutrophils; red: eosinophils; green: lymphocytes; yellow: monocytes.

Table 1 on day 3 shows that in neutrophils, the control group was 2.6 and the experiment group was 4; on eosinophils, the control group was 2 and the experiment group was 3; on lymphocytes, the control group was 3.2 and the experiment group was 3.2; on monocytes, the control group was 1.2 and the experiment group was 1.6; on macrophages, the control group was 0.8 and the experiment group was 0.6. Inflammatory cells can be seen in Figure 2.

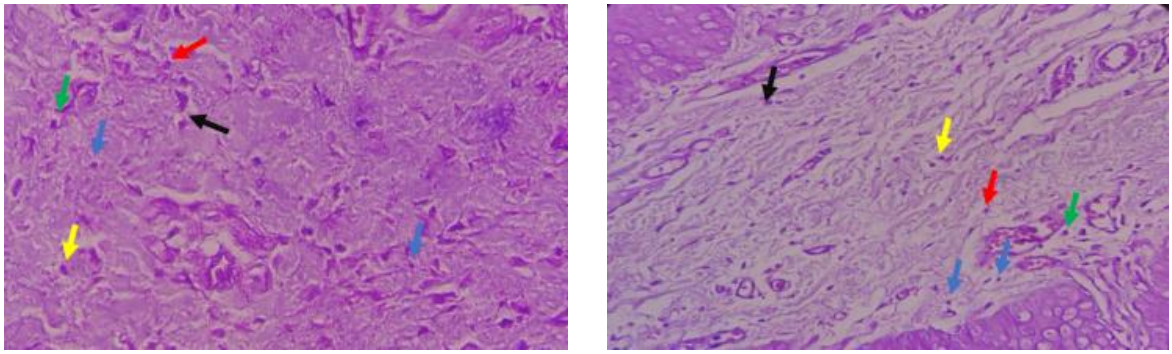


(a) **(b)**

Figure 3 Microscopic image of the number of periodontal tissue inflammatory cells on day 0 magnification 400x; (a) control group (b) experiment group.

Description: Blue: neutrophils; red: eosinophils; green: lymphocytes; yellow: monocytes; black: macrophages.

Table 1 on day 7 shows that in neutrophils, the control group was 7.6 and the experiment group was 18; on eosinophils, the control group was 1.4 and the experiment group was 3.4; on lymphocytes, the control group was 4.4 and experiment group was 3.2; on monocytes, the control group was 2.2 and the experiment group was 4.4; on macrophages, the control group was 1.2 and the experiment group was 1.2. Inflammatory cells can be seen in Figure 3.

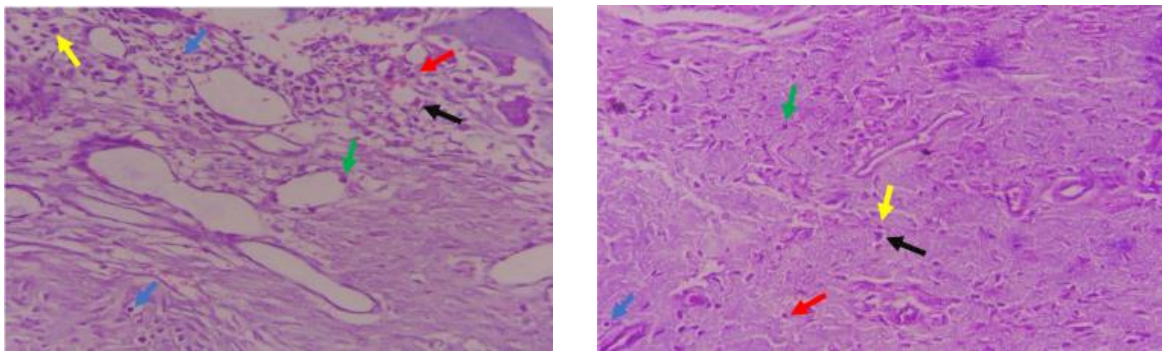


(a) **(b)**

Figure 4. Microscopic image of the number of periodontal tissue inflammatory cells on day 0 magnification 400x; (a) control group (b) experiment group.

Description: Blue: neutrophils; red: eosinophils; green: lymphocytes; yellow: monocytes; black: macrophages.

Table 1 on day 14 shows that in neutrophils, the control group had a mean value of 16.29 and the experiment group was 5.66; on eosinophils, the control group had a mean value of 2.03 and the experiment group of 0.72; on lymphocytes, the control group had a mean value of 14 and experiment of 8.20; on monocytes, the control group had a mean value of 3.29 and experiment of 0.72; on macrophages, the control group had a mean value of 1.03 and the experiment group of 0.60. Inflammatory cells can be seen in Figure 4.



(a) **(b)**

Figure 5. Microscopic image of the number of inflammatory cells on day 0 magnification 400x; (a) control group (b) experiment group.

Description: Blue: neutrophils; red: eosinophils; green: lymphocytes; yellow: monocytes; black: macrophages.

The data were normally distributed for neutrophil cells ($p<0.05$), so the statistical test used was the T-independent test. In contrast to eosinophils, monocytes, and macrophages, the data were not normally distributed ($p>0.05$), so the statistic used was the Mann-Whitney test.

Table 2. Effect of Ethanol Extract of *Sauropus androgynus* on the number of inflammatory cells

Cells	Mean ± SD		Differences	P-value*
	Control	Experiment		
Neutrophils	16.29 ± 7.13	5.66 ± 2.14	10.63	0.000[a]
Eosinophils	2.03 ± 1.15	0.72 ± 0.24	1.31	0.000[b]
Lymphocytes	14.00 ± 4.72	8.20 ± 4.58	5.8	0.002[b]
Monocytes	3.29 ± 2.19	0.72 ± 0.30	2.57	0.000[b]
Macrophages	1.03 ± 0.59	0.60 ± 0.29	0.43	0.064[b]

*) The value is declared significant if $p \leq 0.05$

a) independent t-test

b) Mann-Whitney test

The statistical test between the control group and the experiment group was explained as follows: in neutrophil, eosinophil, lymphocyte, and monocyte cells, there was a significant difference between the control and experiment groups with the p-value for neutrophils was 0.000; eosinophils is 0.000; lymphocytes is 0.002; and monocytes is 0.000. Statistical tests on macrophages showed no significant difference between the control and experiment groups, with a p-value of 0.064.

DISCUSSION

Orthodontic tooth movement includes several phases, namely the initial, lag, and post-lag phases. The initial phase occurs 24-48 hours after applying orthodontic force using a separator. This initial phase is characterized by direct tooth movement when the orthodontic appliance is first placed. This phase affects several cells in the body, including osteoblast progenitor cells, osteoclast progenitor cells, and inflammatory cells.[12-14]

Orthodontic forces applied to the teeth will cause areas of pressure and strain on the periodontal ligament. The area under pressure will experience hypoxia, which induces an aseptic inflammatory response. Orthodontic forces also stimulate the nerve endings of the periodontal ligament, which is closely associated with blood vessels. Nerve endings that are distorted due to orthodontic forces will release vasoactive neurotransmitters. Vasoactive neurotransmitters interact with vascular endothelial cells, resulting in blood vessel vasodilation and increased blood vessel wall permeability with blood plasma leakage. Active endothelial cells will recruit circulating leukocytes and macrophages to the periodontal ligament so that the acute inflammatory process begins to appear.[15]

On day 0 of applying the orthodontic separator, an initial phase occurs, stimulating inflammatory cells. Inflammatory cells often found at the beginning of acute inflammation are neutrophil cells followed by monocytes. Neutrophil cells are very abundant in the blood. These cells will respond first when there is a stimulus. The cell infiltrate is dominated by neutrophils in acute inflammation, which accumulate for several days before being replaced by macrophages.[13,14,16]

Eosinophils were more numerous than neutrophils in guinea pigs on day 0 of the separator application in this study. It can occur due to a hypersensitive reaction due to the treatment received by the guinea pig, such as an allergy to extracts, rubber separators, or metal oral probes. Eosinophils are the main cells that respond when a hypersensitivity reaction occurs.[14]

On day 3, the treatment group should ideally be less than the control group. However, in this study, the number of inflammatory cells on day 3 was higher in the treatment group than in the control group. Neutrophils usually persist for the first 2 days and are the first cells to respond to injury, but neutrophil infiltration may persist for days depending on the type of injury and the body's response. The force used in this study is very small as it only uses a rubber separator. It affects the magnitude and influence of the inflammatory response that occurs. The length of time an inflammation occurs is also caused by several other factors. One of the factors that can occur in guinea pigs is the stress factor. Stress can cause immunological changes in periodontal tissues by influencing cortisol and epinephrine levels. Cortisol and epinephrine levels can interfere with hemostasis. Cortisol can lose the ability to inhibit inflammation so that inflammation can continue to occur in the periodontal tissue and inhibit the healing process.[14,17]

The phase of tooth movement after the initial phase is the lag phase. The lag phase is characterized by hyalinized areas in the pressure area so that there is no tooth movement in this phase. This phase lasts for 20-30 days. Lymphocytes and macrophages that are responsible for chronic

inflammation begin to dominate. The lag phase involves macrophages and osteoclasts that eliminate necrotic tissue in the pressure area. Tooth movement may reoccur once the necrotic tissue has been removed.[13,18]

The number of inflammatory cells in the treatment group on the seventh day was more than the control group. The number of inflammatory cells in the treatment group should be less than the control group. It could be due to the guinea pig being injured during the orthodontic separator application. The presence of injury to the supporting tissue of the tooth can cause a greater inflammatory response so that the number of cells in the injured area increases. Stress factors can also trigger increased inflammation.[14,17]

Some aspects of this study did not align with the usual inflammation theory. It occurs because the inflammation in orthodontic tooth movement differs from normal inflammation. Inflammation in orthodontic tooth movement can last longer as long as there is still an orthodontic force that induces the release of inflammatory cells. The orthodontic force in this study also tends to be small as it only uses a rubber separator, so it does not recruit many inflammatory cells.[18]

Flavonoids in *Sauropus androgynous* can inhibit the release of serotonin and histamine to the site of inflammation and inhibit lipoxygenase and cyclooxygenase in the synthesis of prostaglandins and arachidonic acid. Isoflavones that are part of the flavonoid group have Genistein, which inhibits IL-1β, IL-6, and TNF-α. Flavonols such as kaempferol, quercetin, and myricetin can act as lipoxygenase inhibitors. Besides, quercetin and apigenin can also inhibit the production of nitric oxide (NO) production. The lipoxygenase and cyclooxygenase pathways in the inflammatory process are inhibited, causing proinflammatory cytokines to be inhibited so that the release and activity of inflammatory cells will decrease.[6,8,9]

The results of this study indicated the effect of the ethanol extract of *Sauropus androgynus* on the number of neutrophils, eosinophils, lymphocytes, and monocytes. Neutrophils can break down elastin and extracellular matrix with neutrophil proteases elastase and proteinase- 3. This ability will decrease if neutrophil infiltration is inhibited, resulting in reduced inflammation and faster wound healing. In 2014, a study by Kusumastuti et al. revealed that Sauropus androgynus contained flavonoids that act as antioxidants. These antioxidants can inhibit the synthesis of IL-1 and TNF-α, affecting the decrease in neutrophil infiltration into tissues.[19]

Research by Adelgrit in 2017 proved that the flavonoid content in the ethanol extract of Dayak onion bulbs orally affected the decrease in eosinophil cells. Flavonoids reduce the number of eosinophils in the body by inhibiting the activation of IL-5.[20]

The results of the research conducted by Aria in 2020 revealed a decrease in the number of stem neutrophil cells, monocytes and lymphocytes in the administration of ethanol extract from piladang leaves containing flavonoids.[21] Mulyadi in 2020 reported that topical application of curd has a significant effect on the healing process of rat gingival inflammation because the antioxidants and probiotic contents can overcome inflammation.[21]

Orthodontic movements on day 14 were still in the lag phase. Based on the results of this study, there were fewer macrophages in the treatment group than in the control group, although the effect of the ethanol extract of *Sauropus androgynous* was not statistically proven. The possible factor that caused it was that the force used in this study was very small because it only used a rubber separator, so the inflammatory response was not too large. The emerging macrophages were not so dominant that their effect was not visible. Lymphocytes have started to dominate today, and macrophages may dominate this

phase in the future. Macrophages are the cells responsible for eliminating hyalinized areas during the lag phase and began appearing on days 7 to 14 in this study.[12-14]

CONCLUSION

The orthodontic separator-induced stress initiated an inflammatory cascade in the periodontal ligament, and the ethanol extract of *Sauropus androgynus* potentially modulated this inflammation by affecting various inflammatory cell types, except for macrophages, possibly through its flavonoid constituents.

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