Research article

Utilization of Rhizosphere Earthworm Extracts to Support the Immune System

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Abstract.
Periodontitis is an inflammatory condition caused by periodontal bacteria that are recognized by Toll-like receptors on neutrophils, macrophages, and other immune cells. As a result of this interaction, immune cells produce inflammatory molecules like cytokines and chemokines. In addition to the pathogenic effects of periodontitis, dysregulation of neutrophil activity can occur. The neutrophil acts as a double-edged sword in periodontitis, mobilizing defence mediators and tissue repair mechanisms while also causing further tissue damage. The goal of this study was to determine how *Lumbricus luberus* worm extract (EEW) affected the number of neutrophil cells in rats with periodontitis. A post-test design was used. Five Wistar rats, each infected with *P. gingivalis*, were divided into groups: a control group (no earthworm extract), an oral EEW (200 mg/kg/body weight) group, and a topical EEW gel group (20%). The number of neutrophil cells was measured on days 3, 7, 14, and 21. The study was carried out at the Udayana University Analytical Laboratory and the Veterinary Medicine Laboratory. The differences between control, oral, and topical *Lumbricus rubellus* extract administration on days 3 and 7 were significant ($p < 0.05$). On days 14 and 21, there was a significant difference ($p < 0.05$) between the control vs. oral administration groups and between the control vs. earthworm extract gel groups. However, on days 14 and 21, there was no significant difference between the oral administration and *L. rubellus* gel extract groups ($p > 0.05$). The number of neutrophils was significantly lower following the oral administration of *L. rubellus* earthworm extracts.

Keywords: Wistar periodontitis rats, Lumbricus rubellus earthworm extract, neutrophil

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1. Introduction

Microorganisms that cause damage to the supporting tissues and bones that support teeth[1]. Research by Nazir *et al*. (2020) analyzed data from 27 low- to high-income countries ranging from adolescents, adults and the elderly. Belarus has the highest prevalence of periodontal disease in adolescents because there are no adolescents without periodontal disease. Then 1% of adolescents without periodontal disease were

owned by Norway, followed by Germany as much as 2%, while in Iran (30%) and Taiwan (14%) there were adolescents with periodontitis. In China and India almost all adults with periodontal disease, as well as in Belarus. While in Germany and Taiwan only 1% of adults without periodontal disease[2].

Chronic periodontitis occurs due to the induction of Gram-negative anaerobic bacteria such as *Aggregatibacter actinomycetemcomitans* (27.6%), *Treponema denticola* (33.6%), *Tannarela forsythia* (41.4%), *Porphyromonas gingivalis* (46.6%). *Phrophyromonas gingivalis* as the main cause of chronic periodontitis, this bacterium triggers immune and inflammatory responses[1]. Neutrophils and macrophages are immune cells that play a role in the inflammatory process. Neutrophils are non-specific inflammatory cells that first appear in large numbers in the exudate in the first hour of inflammation. There are approximately 5000 neutrophils per cubic millimeter of blood circulating at any time[3,19]. Neutrophils migrate into the blood circulation, infiltrate into tissues, and are phagocytosed. Neutrophils are a hallmark of acute inflammation, but can also be found in chronic inflammation, such as bacterial infection or tissue necrosis. Strict control of neutrophil survival after bacterial clearance through induction of apoptosis provides a protective effect on host tissue and prevents tissue damage after inflammation[4]. Failure to properly regulate the excess and turnover of neutrophils directly contributes to the pathogenesis of periodontitis. In addition to periodontal pathogenic effects, bacteria, dysregulation of neutrophil activity also play a role in periodontitis. Neutrophils function as a double-edged sword in periodontitis, causing not only the mobilization of defense mediators and tissue repair mechanisms but also further tissue damage[5]. Historically, neutrophils were an acute inflammatory response and are considered to be short-lived non-recirculating antibacterial effector cells. However, recent evidence suggests that neutrophils are quite flexible and perform previously unexpected functions, including transmigration and the ability to cross-talk with and regulate leukocytes in innate and adaptive immunity[6].

Giving antibiotics and anti-inflammatory for periodontitis therapy is needed to overcome inflammation, repair tissue damage, and eliminate the causative bacteria. However, the use of antibiotics such as Metronidazole often has side effects such as abdominal pain, headache, nausea and changes in taste[7]. For this reason, it is necessary to look for natural ingredients as an alternative therapy in periodontitis which has few side effects, namely the extract of the earthworm *Lumbricus* rubellus[8]. Research by Mihara *et al.* (1991) found an enzyme from the digestive tract of earthworms, which consists of six serine protease izoenzymes called Lumbrikinase. Lumbrokinase is thought to have antiplatelet and anti-inflammatory effects with a mechanism similar to aspirin, by inhibiting

the action of the cyclooxygenase (Cox) enzyme[9]. Earthworms have antioxidant activity that can increase the content of glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). The polyphenol content in earthworms has the potential as anti-inflammatory, antipyretic and anticarcinogenic, while the coelemic fluid in earthworms functions as an anti-bacterial. The publication of Dharmawati (2019) proves that there is anti-bacterial activity in the extract of the earthworm Lumbricus rubellus which has the ability to inhibit the growth of the bacterium Phorphyromonas gingivalis[10]. This study aimed to determine the ability of Lumbricus rubellus worm extract to reduce the number of neutrophil cells in Wistar rats with periodontitis.

2. Methodology

This is an experimental study with a posttest only control group design on Wistar white rats, with 3 treatment groups: oral administration of EEW (200m/kg/BW), topical gel EEW (20%), control (not given gel, mechanical treatment only). The research was conducted at the Analytical Laboratory of Udayana University, Bali, Indonesia and obtained ethical clearance from the Faculty of Veterinary Medicine, Udayana University. The sample size was calculated by Federer's formula – each 5 mice in the treatment group[10]. Wistar male rats aged 8 -10 weeks, body weight 250g – 300g.

2.1. Procedure steps

Steps 1. Making EEW *Lumbricus rubellus*

With the matseration method, the extraction of 1 kg of earthworm flour was dissolved in 3500 ml of ethanol, then stirred and allowed to stand for 24 hours. Furthermore, the filtrate and soaking residue will be obtained. The filtrate was separated from the residue by filtering it using Wattman filter paper and it was obtained as much as 1500 ml. By using a rotary evaporator, the filtrate was evaporated to obtain a thick consistency of 15.570 grams of earthworm extract[10].

Steps 2. Preparation of EEW *Lumbricus rubellus* 20% gel

With a basic mixture of glycerin, 0.18% methyl paraben as a preservative, 0.05% propyl paraben, and 3% CMC Na as the main ingredient, 15% glycerin and 100% aquadest are added as basic ingredients. Weigh and measure all ingredients, then make a mixture by dissolving methyl and propyl paraben in the amount of glycerin available and stirring until homogeneous (Mixture I). Develop CMC Na in some available water by stirring

using an overhead stirrer for 30 minutes (Mixture II). Add mixture II little by little to mixture I while stirring using an overhead stirrer until homogeneous[10].

Step 3. Periodontitis rat manufacture.

Rats were anesthetized using ketamine Hcl injection i.m in the hamstrings at a dose of 0.2 ml/250 g bw, then a periodontal silk ligature was placed on the anterior mandible in the sub gingival area. Then, Porphyromonas gingivalis was induced. Induction of Porphyromonas gingivalis with the amount of 3x108 Mac Farland, as much as 0.25ml once in the buccal area. Installation of the silk ligature for 7 days, after the ligature was removed, for 3 days no debridement was carried out, with the aim that the bacteria in the plaque persisted until chronic periodontitis occurred on the 11th day[11].

Step 4. Oral administration of EEW *Lumbricus rubellus*:

The earthworm extract was administered orally using a syringe. The rat was held against the scalp so that its mouth was facing upwards. Then slowly insert the probe into the mouth until it reaches the stomach. Then, earthworm (*Lumbricus rubellus*) extract was sprayed. Extract dilution using distilled water can be done when the extract will be used. The extract was given at a dose of 200 mg/kg/day with 0.3 ml of distilled water. at 7 a.m. to 7 p.m.). Giving as much as 2x a day for 21 days [10].

Step 5. Topical administration of EEW *Lumbricus rubellus* 20%:

Topical administration of 20% earthworm *Lumbricus rubellus* extract gel which was applied with the help of a slow speed 1 ml syringe that could be inserted into the bottom of the pocket as much as 0.3 ml. Giving 3 times a day at 07.00: 13.00: 19.00 Wita, for 21 days [10].

Step 6. Decafutation was carried out on days 3, 7, 14 and 21 to see the number of neutrophil cells, using a micrometer under a light microscope with 400x magnification, with HE staining. The data obtained were tested for normality with *Kolmogorov Smirnov*, homogeneity test with *Levene test*, followed by treatment test with *One Way Anova*.

3. Result and Discussion

The normality test results of EEW *Lumbricus rubellus* both orally and topically gel and control, on days 3,7,14 and 21 showed data were normally distributed ($P > 0.05$).

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 1. The mean reduction in the number of neutrofil of the 3rd day in each treatment group were significant ($p < 0.05$).

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 1: Mean number of neutrofil on the 3rd day in each treatment group.

Group Criteria:		n	Mean ± SD	*p*
	Control	5	144±8,59	0,000
	Oral EEW	5	116±2,74	0,000
	Topical EEW	5	103±3,27	0,000

Table 2: Mean number of neutrophils on day 3 between treatment groups.

Group Criteria:		Mean diff.	*p*
	Control-Oral	27.800	0.000
	Control-Topical	41.000	0.000
	Oral -Topical	13.200	0.003

Table 2. Differences in mean number of neutrofil between controls, oral and topical administration, were significant ($p < 0.05$).

Table 3: Mean number of neutrofil on the 7th day in each group.

Group Criteria:		n	Mean±SD	*p*
	Control	5	143±12.64	0.000
	Oral EEW	5	73±2.07	0.000
	Topical EEW	5	39±2.59	0.000

Note: Analysis by the *One Way Anova* Test, * significant at $p < 0.05$

Table 3. The mean number of neutrofil of the 7th day in each treatment group were significant ($p < 0.05$).

Table 4: Mean number of neutrofil on the 7th day between treatment groups.

Group Criteria:		Mean diff.	*p*
	Control - Oral	69.200	0,000
	Control - Topical	103.800	0,000
	Oral - Topical	34.600	0,000

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 4. Differences in mean number of neutrofil between controls, oral and topical administration, were significant ($p < 0.05$).

Table 5: Mean number of neutrofil on the 14th day in each group.

Group Criteria		n	Mean±SD	*p*
	Control	5	135±12.49	0.000
	Oral EEW	5	33,40±2.30	0.000
	Topical EEW	5	26,40±2.07	0.000

Note: Analysis by the *One Way Anova Test*, * significant at $p < 0.05$

Table 5. The mean number of neutrofil on the 14th day in each treatment group were significant differences (p <0.05).

Table 6: Mean number of neutrofil on the 14th day between treatment groups.

Group Criteria		Mean diff.	*p*
	Control - Oral	101.600	0.000
	Control - Topical	108.600	0.000
	Oral - Topical	7.000	0.162

Note: Analysis by the *One Way Anova Test*, * significant at p <0.05

Table 6. Difference in mean decrease in the number of neutrofil between control and oral, control with topical were significant (p <0.05), whereas oral and topical was not significant (p> 0.05)

Table 7: The mean number of neutrofil of the 21st day in each group.

Group Criteria		n	Mean±SD	*p*
	Control	5	138±4.04	0.000
	Oral EEW	5	39±2.59	0.000
	Topical EEW	5	35±2.30	0.000

Note: Analysis by the *One Way Anova Test*, * significant at p <0.05

Table 7. The mean number of neutrofil of the 21st day in each treatment group showed a significant difference (p <0.05).

Table 8: Mean differences in the number of neutrofil of the 21st day between treatment groups.

Group Criteria		Mean diff.	*p*
	Control - Oral	98.800	0.000
	Control - Topical	104.000	0.000
	Oral - Topical	5.200	0.020

Note: Analysis by the *One Way Anova Test*, * significant at p <0.05

Table 8. Differences in mean number of neutrofil of the 21st day between the control and oral groups and between controls and topicals were significant (p <0.05). The difference between oral and topical groups was significant (p < 0.05).

This research proves of giving Lumbricus rubellus worm extract to the number of neutrophil cells given orally, topically compared to controls there was significant difference in number of neutrophil cells on days 3, 7, 14 and 21 which was shown with a P value <0.05. This proves that different treatments and methods of giving earthworm extracts gave different results on the number of neutrophil cells. With the administration of earthworm extract, the number of neutrophils was much smaller than the control, which only carried out basic treatment in the form of scaling and root planning.

Observations on number of neutrophil cells on day 3 showed a significant difference between control with oral and topical administration of earthworm extract, this was indicated by $P < 0.05$. The oral and topical administration of earthworm extract also showed a significant difference ($P < 0.03$). Likewise for observations on the 7th day there was a significant difference between the control group, oral and topical administration of earthworm extract, as indicated by $P < 0.05$. Descriptively, seen from the mean and standard deviation values, it showed that topical administration of earthworm extract proved the least number of neutrophil cells (38 ± 2.59).

Observations on the 14^{th} day of the number of neutrophil cells between the control with oral and topical administration of earthworm extract showed a significant difference where the P value <0.05. However, between oral and topical earthworm extracts, there was no significant difference with $P > 0.05$. Similarly, on 21^{st} day, between control and administration of earthworm extract orally and topically, there was a significant difference where $P < 0.05$. Meanwhile, there was no significant difference between the oral and topical groups ($P > 0.05$). This shows that the administration of earthworm extract orally and topically gave almost the same effect on number of neutrophil cells on the 14^{th} and 21^{st} days.

Periodontitis is an inflammatory condition and periodontopathic bacteria (*Phorphyromonas gingivalis*) are recognized by neutrophils, macrophages and other immune cells with the help of Toll-Like receptors which recognize the molecular patterns of pathogenic microbes (PAMPs-lipopolysaccharides) and this interaction triggers the production of inflammatory molecules such as cytokines and chemokines by cell immunity[5]. Historically, Neutrophils are a response to acute inflammation as short-lived non-recirculating antibacterial effector cells. However, recent evidence suggests that neutrophils are cells that have multiple functions, including transmigration and cross-interaction capabilities to regulate leukocyte innate and adaptive immunity[12]. In addition, the half-life of circulating neutrophils is now thought to be longer than previously thought (a few days compared to hours). Complement triggers through various cascades (classical, lectin, alternative) will activate C3, this mechanism triggers the formation of effector molecules that activate neutrophils. This complement system synergizes with TLRs to activate neutrophils and innate immunity. This proves that neutrophils are not only involved in acute inflammation but also in chronic inflammation[6].

Phorphyromonas gingivalis is the main bacteria in periodontitis capable of manipulating complement and TLR signals, inducing bacterial persistence. TLR2/TLR4 plays an important role in eliminating bacteria through activation of the MyD88 adapter protein[12,8]. Toll-like receptor 4 specifically recognizes the lipopolysaccharide of the

Gram-negative bacterium *Phorphyromonas* gingivalis[13]. Activation of the TLR4 pathway signal activates the MyD88 adapter protein to bind to IRAK4, which in turn activates *transforming growth factor-B activated kinase* (TAK1), further activating NFKB inducing kinase (NIK) to degrade phosphorylation of IKK to IKB for binding to P50 and P65 proteins (NFKB). to ubiquitin as an intermediary for proteosome degradation[14,15].

This research proves a decrease in the number of neutrophil cells from days 3, 7, 14 and 21. This was because the extract of the earthworm Lumbricus rubellus contained the enzyme lumbrokinase in the digestive tract which consisted of serine protease izoenzymes. Lumbrokinase is a proteolytic bioactive enzyme that has a molecular weight between 25 kDa to 32 kDa. Lumbrokinase is a plasminogen and plasmin activator, similar to tissue plasminogen activator (t-PA) found in other species[16,17]. Earthworm extract also contains polyphenol compounds as anti-oxidants and anti-inflammatory14 and in the body cavity segment of earthworms contains coelemic, 18 amino acids, fatty acids, micro elements, lumbritin, lumbrofebrin, tertrolum brolysin, purines, choline, cholesterol and vitamins[16,17].

Research results Wang *et all*. (2016) proved Lumbrokinase in *Lumbricus rubellus* can inhibit the activation of TLR4 which is dominant in periodontal tissue. Research has shown that lumbrokinase can inhibit phosphorylation or activation of JNK and cFos expression, which are signaling regulatory pathways responsible for injury[13]. The ability of Lumbrokinase to inhibit the TLR4 signaling pathway, resulted in decreased activation of IRAK4 phosphorylation, JNK phosphorylation, IKB phosphorylation, ERK1/2 phosphorylation and P38 phosphorylation. Inhibitors of the NFKB and MAPK pathways, which are pathways for stimulating inflammation, result in a decrease in pro-inflammatory cytokines[16,19].

Earthworm extract also has the ability to inhibit the Cyclooxygenase enzyme, resulting in a decrease in oedema, this is evidenced in studies conducted by Mathur *et al*., (2011) and Shofianingsih (2016) where *Lumbricus rubellus* earthworm extract has almost the same effect as aspirin[18,19]. Lumbrokinase of earthworm extract can suppress the expression of Cox2 and inos. Cyclooxygenase 2 and inos are inflammatory proteins that have a fundamental role in pathophysiological conditions[15]. Dharmawati's research (2019) also proves that earthworm extract can physically reduce the depth of the periodontal pocket in periodontitis rats[20]

4. Conclusion

This research proves that *Lumbricus rubellus* worm extract can be used as an anti-inflammatory with its ability to reduce the number of leukocytes that play a role in inflammation of the periodontal tissue (periodontitis).

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