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Antibacterial Activities of Purple Miana Leaves Extract Inhibiting *Aggregatibacter actinomycetemcomitans* on Rats

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Abstract— *A.actinomycetemcomitans* are pathogenic bacteria that cause damage to periodontal tissue so that it is clinically seen as a periodontal disease (periodontitis). There are no studies that report bacterial load after the use of purple miana leaves extract (PMLE) in Periodontitis. This research objective was to determine the effect of purple miana leaves extract on bacterial load in wistar rats that have been induced by *A.actinomycetemcomitans.* Rats were divided into three groups, purple miana leaves extract (PMLE) 510 mg/ kgbw, negative control (aquadest), antibiotic levofloxacin 500 mg/kgbw as positive control. Samples of *A.actinomycetemcomitans* bacteria were taken seven days after induction of $3x10^8$ cfu/ml *A.actinomycetemcomitans* in gingival sulcus of the anterior mandibular teeth of the rat/after periodontitis (D8) and seven days after intervention (D15). Bacterial load is measured by calculating colony forming units. The results obtained are processed using SPSS. Bacterial load on D8 to D15 had a different pattern between the PMLE group, the aquades group (negative control) and the levofloxacin group (positive control). In negative controls, an increase in bacterial load but not significantly different ($p>0.05$). In PMLE and positive control, there was a decrease in bacterial load and significantly different ($p<0.05$). Purple miana leaves extract (PMLE) 510 mg/kgbw and levofloxacin 500 mg/kgbw successfully suppressed the growth of *A.actinomycetemcomitans* (bacterial load) colonies for 7 days of use.This study represents that PMLE can be an alternative drug in patients with *A.actinomycetemcomitans* infection, especially in the case of periodontitis.

Keywords— miana leaves, *A.actinomycetemcomitans*, periodontitis, antibacterial, bacterial load.

1. Introduction

Periodontitis is an infectious disease that affects the tooth supporting tissues and is initiated by increased concentration of some Gram-negative bacteria in dental plaque[1]. Periodontal disease is a pandemic disease

that is characterized as an inflammatory reaction to bacterial infections [2], has been reported as a potential source of infection for risk factors for cardiovascular disease, cerebrovascular disease, peripheral arterial disease, respiratory disease, low birth weight and cases of pyogenic liver abscess caused by periodontal bacteria [3]. Periodontal disease is a significant global public health concern and is probably the most common chronic infectious disease of humans [4], as a potential risk for increased morbidity and mortality for diabetes, insulin resistance, rheumatoid arthritis, obesity, osteoporosis, and pregnancy complications [5].

A.actinomycetemcomitans is a gram negative coccobacillus, this bacterium finds major ecological in the oral mucosa, dental plaque and periodontal pockets[6]. *A.actinomycetemcomitans* is strongly implicated in the pathogenesis of periodontitis [7,8] and in extra-oral infections, including infective endocarditis, bacterial arthritis, pregnancy associated septicemia, cerebral abscesses and osteomyelitis [9]. *A.actinomycetemcomitans* have all demonstrated the ability to invade human oral epithelial cells in cell culture [10]. The prevalence of this bacterium shows great variation depending on the geographical origin, age and life style of the examined population [11].

Antibiotics are one of the most valuable tools in the treatment of periodontitis, some studies have shown good results with the adjunctive use of fluoroquinolones [12], Levofloxacin was found to be successful in patients suffering from invasive *A.actinomycetemcomitans* infection[13]. However, it has severe side effects [14]. Therefore, many scientific researchers seek plant-derived extracts in herbal medicine and related metabolites due to their efficacy effects [15,16,17]. This is the background of researchers to examine herbal plants which can be a substitute therapy for periodontitis cases; one of the plants that has been widely used empirically in Indonesia is Miana. Miana is one of the medicinal plants of the family Lamiaceae which contains flavonoids, tannins and alkaloids, essential oils. Flavonoids are synthesized by plants in response to microbial infection. [18]

2. Methods

2.1. Design

This research was conducted at the Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, South Sulawesi, Indonesia. This study was a purely experimental pre and post test design with a control group using the spread plate method by calculating bacterial load on agar after a series of dilution and bacterial culture of *A.actinomycetemcomitans*. The aim is to see the effectiveness of PMLE in inhibiting the growth of *A.actinomycetemcomitans* bacteria in periodontitis in the gingival sulcus of mandibular anterior teeth on rats.

2.2. Preparation and adaptation of experimental animals

Male wistar rats (200-300 grams) were obtained from the Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, South Sulawesi, Indonesia. Rats are placed in cages covered with iron nets with a cage area of 30 cm × 50 cm × 15 cm. Rats are placed in cages containing five tails each. Ratio standard feed is given to rats as much as 300 g/hour/tail and given enough drink, and the cage is cleaned every day. To maintain a stable environment, rats were placed in a room with adequate air circulation and maintained in accordance with room temperature at standard conditions (28 ± 2°C) with 50 ± 10% humidity and cycle room lights 12 hours on and 12 hours extinguished. This procedure is carried out for 1 week. Furthermore, rats were randomized [19].

2.3. Purple Miana Leaves Extract (PMLE)

Purple miana leaves were taken from Soppeng district, South Sulawesi, Indonesia and extraction was carried

out at the Phytochemical Laboratory, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia. A total of 500 grams of purple miana leaves simplicia powder was put into a jar, then soaked with a 96% ethanol solution, covered with aluminum foil and left for 3 days while occasionally stirring. After 3 days, the samples soaked were filtered using filter paper to produce filtrate, then evaporated using a rotary evaporator, so that a thick extract of Miana leaves was obtained. The resulting thick extract is left at room temperature until all the ethanol solvents evaporate. The extract was weighed and stored in a closed glass container before being used for testing. The PMLE dose used in this study was 510 mg/kgbw, dissolved with 15 b/v aquadest [20].

2.4. Preparation of A.actinomycetemcomitans

Taking 1-2 ose of bacterial colonies of *A.actinomycetemcomitans* in the microbiology laboratory, Faculty of Medicine, Hasanuddin University and suspended in 0.9% NaCl solution as much as 5 ml. The bacterial suspension was then vortexed until homogeneous until the turbidity was obtained according to Mc Farland 1 standard (3 x 10^8 cfu/ml). then the rats were anesthetized using ketamine HCl as much as 80 mg/kgbw or equal to 0.22 ml injected intramuscularly on the thigh muscle, about 10-15 minutes after the rats were seen to start limp & slowing movement, a gingival sulcus cleft for placement of silk ligature on mandibular anterior teeth, silk ligature tightly linked in the cervical area of the tooth by wrapping the anterior teeth so that they do not come loose. Silk ligature is inserted and pushed into the gingival sulcus with the help of a sonde. Furthermore, the induction of *A.actinomycetemcomitans* 3 x 10^8 cfu/ml as much as 0.25 ml was carried into the gingival sulcus of the mandibular anterior teeth using 0.5 cc of B-Braun dispo on 15 rats.

2.5. Administration of PMLE

We use PMLE obtained by extraction of maceration methods in the form of thick PMLE, which is then diluted using a dilution method by adding BR-2 aquadest. PMLE is given orally. PMLE therapy will begin 7 days after infection with *A.actinomycetemcomitans* (rats already diagnosed with periodontitis). The prescribed dosage is PMLE 510 mg/200 grams body weight rats administered orally using Frekat Tube brand nasogastric sonde silicone belly tube Fr 12 size 12 silicone every 24 hours for 7 days.

2.6. Administration of Aquades (negative control) and Levofloxacin (positive control)

We used 9 mg dose of levofloxacin/200 grams body weight rat and 0,3 cc aquadest. Levofloxacin and aquadest were administered orally using a nasogastric sonde silicone belly tube Fr 12 tube size 12 silicone 12 times every 24 hours for 7 days.

2.7. Bacterial sampling, culture and suspension of A.actinomycetemcommitans

2-3 sterile paper points number 15 are inserted into the gingival sulcus and wait for 10 seconds. The sample is then put into a sterile vacuum tube that contains a transport fluid. Then the vacuum tube is put in the refrigerator. Samples that have been taken are then cultured on AaGM media so that by taking a sample on the vacuum tube using an ose needle, then etched on the surface of AaGM by the T. streak technique. The cup is divided into 3 parts using markers. Bacteria are inoculated with zig-zag streak. After that the petri dish is tightly closed and put into a candle jar. Then incubation was carried out for 48 hours at 37^0C. 1-2 ose bacterial colonies of *A.actinomycetemcomitans* culture results are suspended in 0.9% NaCl solution by 5 ml. The bacterial suspension was then vortexed until homogeneous until the turbidity was obtained according to Mc Farland 1 standard (3 x 10^8 cfu/ml) [21].

2.8. A Bacterial Dilution of A.actinomycetemcommitans

Dilution with serial dilution method. 1: 9 ratio is used for the sample and first dilution. Then prepare 7 tubes filled with 9 mL NaCl. Then from a tube that has been measured according to Mc Farland 1 standard (3 x 10^8 cfu/ml), 1 ml of *A.actinomycetemcomitans* suspension is taken and then mixed with a dilution tube 1 (10^{-1})

and homogenized. Then from tube 1 taken 1 ml with an eppendorf pipette then transferred to tube 2 (10^{-2}) and homogenized. From tube 2, 1 ml is taken with an eppendorf pipette then transferred to dilution tube 3 (10^{-3}) and then homogenized. And so, on so that the last tube of the dilution series [22,23].

2.9. Calculation of bacterial load

0.1 ml of *A.actinomycetemcomitans* suspension was taken using an eppendorf pipette from the last dilution tube and then dripped on a petri dish containing AaGM media by the spread plate method. Then incubated for 2-3 days at 37^{o}C in anaerobic atmosphere. Observations were made after 2-3 days by calculating bacterial load of *A.actinomycetemcomitans* on petri dishes on condition that the number of colonies growing on the media was 30-300 cfu/ml[22,23].

3. Result

***Table* 1:** Data distribution of *A.actinomycetemcomitans* calculation before and after intervention

Sample	Bacterial load(cfu/ml) PMLE	Bacterial load(cfu/ml) Aquadest	Bacterial load(cfu/ml) Levofloxacin
Bacterial Load	$185x10^7$	$175x10^7$	$99x10^7$
Before Intervention (D8)	$177x10^7$	$197x10^7$	$106x10^7$
	$191x10^7$	$189x10^7$	$199x10^7$
	$173x10^7$	$169x10^7$	$232x10^7$
	$164x10^7$	$154x10^7$	$220x10^7$
After Intervention (D15)	$104x10^7$	$120x10^7$	$36x10^7$
	$81x10^7$	$270x10^7$	$83x10^7$
	$54x10^7$	$245x10^7$	$22x10^7$
	$23x10^7$	$261x10^7$	$12x10^7$

PMLE: Purple miana leaves extract, **cfu:** colony forming unit, **D8:** 8th days (seven days after induction of $3x10^8$ cfu/ml *A.actinomycetemcomitans* in gingival sulcus of the anterior mandibular teeth of the rat/after periodontitis, **D15 :** 15th days (seven days after intervention)

In Table 1 shows the data distribution from the bacterial load of *A.actinomycetemcomitans* in periodontitis before and after the intervention. Bacterial load was calculated from bacterial sampling in gingival sulcus of mandibular anterior teeth from rats.

Table 2: Average results of *A.actinomycetemcomitans* calculation in all groups

Sample	Bacterial load (cfu/ml) PMLE	Bacterial load (cfu/ml) Aquadest	Bacterial load (cfu/ml) Levofloxacin
Bacterial load before intervention (D8)	$178x10^7$	$176.80x10^7$	$171.20x10^7$
Bacterial load after intervention (D15)	$68x10^7$	$199.80x10^7$	$46.80x10^7$

Table 2 shows the calculation results of the average bacterial load of *A.actinomycetemcomitans* in periodontitis in three groups showing different patterns, the PMLE group 510 mg/kg body weight and levofloxacin 500 mg/kg body weight showed significant results in decreasing bacterial load of *A.actinomycetemcomitans* on the 15th day (7 days after intervention), whereas the aquadest group showed an increase in bacterial load of *A.actinomycetemcomitans*.

Figure 1. Calculation results of *A.actinomycetemcomitans* on 7 days after induction

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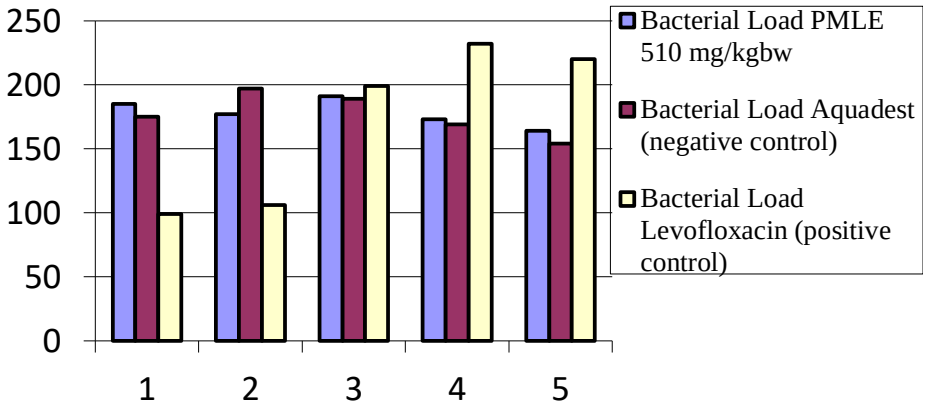

Figure 1 shows the calculation results of bacterial load of *A.actinomycetemcomitans* 7 days after induction from three groups with different numbers.

Figure 2. Calculation results of *A.actinomycetemcomitans* on 7 days after intervention in all groups

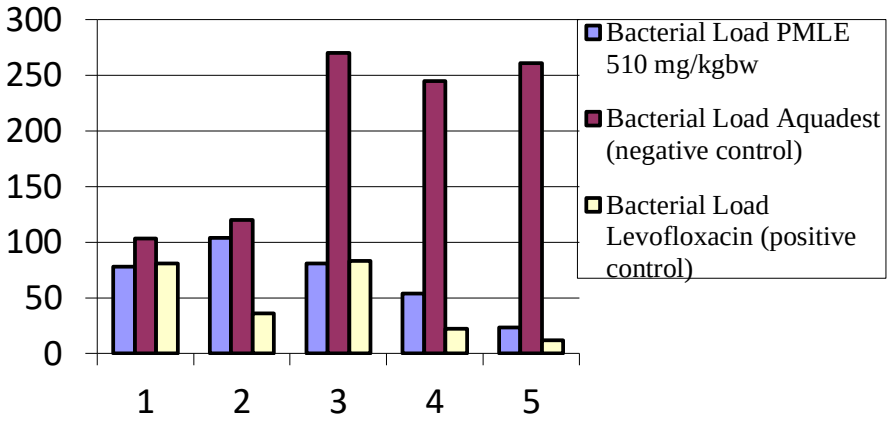

Figure 2 shows the calculation results of bacterial load of *A.actinomycetemcomitans* on the 15th day (7 days after the intervention), found a significant decrease in the bacterial load of *A.actinomycetemcomitans* in PMLE and levofloxacin group.

Figure 3. Bacterial Load of *A.actinomycetemcomitans* before and after intervention in the three groups

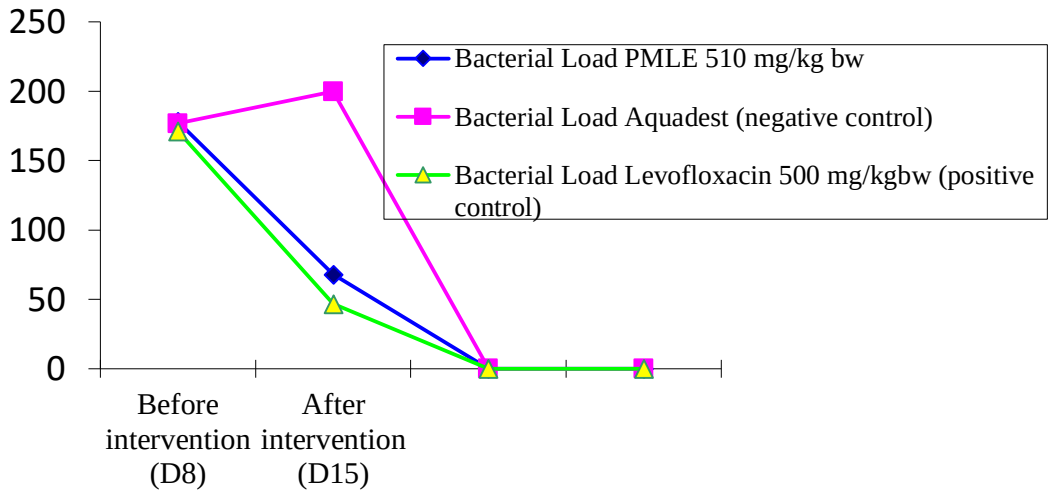

Figure 3 shows the calculation results of the average bacterial load of *A.actinomycetemcomitans* in the periodontitis of three groups, a decrease in *A.actinomycetemcomitans* on the 15th day (7 days after the intervention) significantly in the PMLE and levofloxacin groups; this means that PMLE has the ability to inhibit *A.actinomycetemcomitans* in periodontitis which is similar to the ability of the antibiotic levofloxacin, whereas for the aquadest group there is an increase in *A.actinomycetemcomitans*.

Table 3. Analysis of differences in bacterial load 7 days after induction of *A.actinomycetemcomitans* (D8) and 7 days after intervention (D15)

Group	**Bacterial Load (cfu/ml)**			
	D8	**D15**	**Mean differfence**	**p**
	Mean±SD	**Mean±SD**	**(95%CI)**	
PMLE	178.00x10^7±10.49	68.00 x10^7±30.77	110.00(79.46-140.54)	0.001
Akuades	176.80 x10^7±16.89	199.80 x10^7±81.32	1.33(99.69-102.36)	0.974
Levofloxacin	171.20 x10^7±63.86	46.80 x10^7±33.25	121.50(41.69-201.31)	0.011

p: paired sample t-test, **SD:** Standard deviation, **PMLE:** Purple Miana Leaves Extract, **D8:** 8th days (seven days after induction), **D15:** 15th days (seven days after intervention), not significantly different if p> 0.05 and significantly different if p <0.05

Table 3 shows the results of the paired sample t-test statistical analysis of the three groups. Paired sample t-test was used to see if there was a significant difference in the decrease in bacterial load of *A.actinomycetemcomitans* in the two observations, 8th day and 15th day of the three intervention groups. Paired sample t-test is used for normally distributed data. The results obtained are: Observation of the 8th day to the 15th day showed a significant decrease in the PMLE group 510 mg/kgbw with a value of p = 0.001 (p <0.05), a significant decrease in the levofloxacin 500 mg/kgbw group with a value p = 0.011 (p <0.05), and an increase in bacterial load of *A.actinomycetemcomitans* in the aquadest group but not significant with p = 0.974 (p> 0.05).

Table 4. One way anova analysis results in all three groups after intervention

Kelompok	Mean	Mean Difference	p**	p*
PMLE	68.00x10^7	131,80	0,042	
Aquadest	199.80x10^7			
PMLE	68.00x10^7	21,20	0,571	0,001
Levofloxacin	46.80x10^7			
Aquadest	199.80x10^7	153,00	0,023	
Levofloxacin	46.80x10^7			

p*: one way anova test, **p** :** uji post hoc Games-Howell, not significantly different if p> 0.05 and significantly different if p <0.05

Table 4 shows the results of the one way ANOVA statistical analysis test from three groups. One way ANOVA was used to look at the average differences of the three groups and find out which groups showed significant results, the ANOVA test was used for normally distributed data. The results obtained were a significant difference between the PMLE group 510 mg/kgbw with the aquadest group with p = 0.042, the Aquadest group with the Levofloxacin group with p = 0.023, while the PMLE group with the Levofloxacin group was no significant difference with p = 0.571. Thus it can be concluded that the effects of PMLE in suppressing

A.actinomycetemcomitans were the same as Levofloxacin.

4. Discussion

At D8, which is 7 days after induction of *A.actinomycetemcomitans* in gingival sulcus of mandibular anterior teeth of rats, rats undergo periodontitis and based on the results of bacterial load examination showed the formation of a number of *A.actinomycetemcomitans* colonies in all groups; the PMLE group was $178x10^7$ cfu/ml, the aquadest group ($176.80x10^7$ cfu/ml) and the levofloxacin group ($171.20x10^7$ cfu/ml) (**Figure 1**). *A.actinomycetemcomitans* has a number of virulence factors involved in the pathogenesis of periodontal disease. *A.actinomycetemcomitans* produces numerous extracellular outer membrane vesicles which are shed from the surface of the bacteria. The vesicles contain leukotoxin and LPS, their small size permits them to cross epithelial barriers such as the pocket epithelium, producing epitheliotoxins that can damage epithelial cells and facilitate bacterial penetration of epithelial junctions and pockets, produces proteolytic enzymes that can reduce the local effectiveness of antibodies against these bacteria. *A.actinomycetemcomitans* has Cdt (Cytholethal distending toxin) which is responsible for the inhibition of proliferation of human periodontal ligament cells and gingival fibroblasts. This seriously affects the physiology of the periodontium and exacerbation disease [4].

Bacterial load on the 15th day (7 days after the intervention), there was a significant decrease ($p<0.05$) in 2 intervention groups, decrease in the levofloxacin 500 mg/kg body weight with bacterial load of $46.8x10^7$ cfu/ml; PMLE group 510 mg/kg body weight at $68x10^7$ cfu/ml; whereas in the aquadest group an increase in bacterial load was $199.8x10^7$ cfu/ml (**Figure 2**). Purple miana leaves extract or levofloxacin succeeded in suppressing the increase in bacterial load. The findings of our study prove that the intervention of purple miana leaves extract can inhibit the increase in bacterial load when administering a dose of 510 mg / kg bw, perhaps purple miana leaves extract can act as an anti-bacterial drug. High bacterial load up to 199.8x107 cfu/ml on the 15th day in the negative control group (aquadest) indicates that the induction of *A.actinomycetemcomitans* affects the increase in bacterial load of wistar rats that are not treated with either purple miana leaves extract or levofloxacin so it fails to control infection.

The change dynamics of bacterial load in the PMLE group on D8 to D15 decreased **(Figure 3).** This shows that there is an effect of purple miana leaves extract on *A.actinomycetemcomitans*. The chemical content of purple miana leaves extract which is antibacterial affects the decrease in bacterial load. Previous studies have shown that PMLE contains active substances such as polyphenols, flavonoids, alkaloids, saponins, tannins [18]. Polyphenols such as gallic acid act possibly by binding to bacterial dihydrofolatreductase enzymes, inducing topoisomerase IV enzyme-mediated DNA cleavage and bacterial growth stasis, mediation of solute transport inhibition in membranes and affect the phospholipid membranes of bacterial cell wall [24]. Flavonoids are synthesized by plants in response to microbial infection. Their antimicrobial activity is probably due to their potensial to form complexes with extracellular and soluble proteins as well as the complexation with bacterial cell walls, thereby inducing microbial cell membrane disruptions [25]. Saponins might confer by revamping the permeability of cell walls and consequently exert toxicity on all organized tissues and by integrating with cell membranes to obtain cell morphology changes leading to cell lysis [26]. Tannin antimicrobial activity include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth, direct action on microbial metabolism through inhibition of oxidative phosphorylation, metal ions deprivation or formation of complexes with the cell membrane of bacteria causing morhological changes of the cell wall and increasing membran permeability [27]. Alkaloids can inhibit the bacterial growth by changing the nature of cell proteins (denaturation), thus increasing the permeability of cell membranes, either by increasing the permeability of the cell membrane of the bacteria. The cell membrane causes loss or leakage of the contents of a cell of bacteria to the outside or through a direct

link membrane of cell bacteria,causing the demise of polar membrane of bacteria, which lead to the death of a cell bacteria gradually [28]

The ability of purple miana leaves extract to inhibit the growth of *A.actinomycetemcomitans* is also influenced by outer membrane vesicle (OMV), OMV naturally shed by most Gram-negative bacteria, OMV could deliver biologically active CDT and additional virulence factors into susceptible cells of the periodontium. CDT toxicity may play a part in the early pathogenesis of periodontitis. This would be consistent with OMV promoting damage in the sulcular/junctional epithelium. OMVcan deliver toxins and other virulence factors to the host at relatively high concentration without requiring close contact between the bacterial and target human cells and they believed to represent a key factor in effecting an inflammatory response in the host toward bacterial pathogens [29].

Previous studies conducted by Amsyah, et al (2019) showed that male wistar rats model induced by *A.actinomycetemcomitans* $3x10^8$ cfu/ml in sulcus gingiva of mandibular anterior teeth in seven days could induce periodontitis and the accumulation of *A.actinomycetemcomitans* could stimulate or reduce the expression of interleukin 10 (anti-inflammatory cytokines) through Real Time Polymerase chain Reaction (RT-PCR) examination. This shows that *A.actinomycetemcomitans* had a virulence factor that can affect the host's inflammatory response to bacterial pathogens [30].

Infections caused by gram-negative bacteria are difficult to treat because they are intrinsically resistant to many antibiotics. The double membrane structure of gram-negative bacteria and intrinsic production of efflux pumps allows antibiotics to be exported thereby reducing intracellular concentrations. Gram-negative bacteria have many efflux pumps on their membranes that will transport various molecules of bacterial cells, some of these pumps can transport antibiotics out of bacteria thereby reducing the concentration of drugs in cells and allowing bacteria to survive at higher levels of external concentration of antimicrobial drugs, which leads to survival and resistance [31].

5. Conclusion

Purple miana leaves extract 510 mg/kgbw and levofloxacin 500 mg/kgbw successfully suppressed the growth of *A.actinomycetemcomitans* (bacterial load) colonies for 7 days of use. This research represents that PMLE can be an alternative drug in patients with *A.actinomycetemcomitans* infection, especially in the case of periodontitis.

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