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Analysis of sea bidara leaf (*Ziziphus mauritiana*) ethanol extract biorespon on hydrophobicity and phospholipase of *Streptococcus pyogenes* cell surface in tonsillitis isolate

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ABSTRACT

Background: Tonsillitis is an inflammation of the palatine tonsil that is often found in otorhinolaryngology and is one of the world's socio-economic health problems. Streptococcus pyogenes is the most common bacterial cause of tonsillitis, with hydrophobicity and phospholipase activity on the cell surface that has a major effect on tonsillitis pathogenicity. Ethanol extract of the leaves of sea bidara (Ziziphus mauritiana) has an antibacterial impact containing neophytadiene, hexadecanoic acid, linolenic acid, octadecanoic acid, squalene, and vitamin E.

Objective: To determine the ability of sea bidara leaf (Ziziphus mauritiana) ethanol extract inhibiting the hydrophobicity and phospholipase activity of the Streptococcus pyogenes cell surface in tonsillitis isolate.

Methods: Laboratory experimental design with a post-test-only control group.

Result: Ethanol extract of Ziziphus mauritiana leaf could inhibit the surface hydrophobicity activity of Streptococcus pyogenes cell with different quantity and quality at each concentration and incubation time. Analysis of independent test (p>0.05;0.965) and (p>0.05;0.683) proved that there was no significant difference in incubation time inhibiting hydrophobicity and phospholipase activity of Streptococcus pyogenes cell surface. Kruskal-Wallis analysis (p>0.05;0.689) and (p>0.05;0.162) proved that there was no significant difference in inhibiting the hydrophobicity and phospholipase activity of Streptococcus pyogenes cell surface.

Conclusion: Ethanol extract of Ziziphus mauritiana leaf can inhibit the hydrophobicity and phospholipase activity of Streptococcus pyogenes cell surface in isolate tonsillitis.

Keywords: Hydrophobicity, Phospholipase, *Streptococcus pyogenes*, Tonsillitis, *Ziziphus mauritiana*.

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INTRODUCTION

Tonsillitis is an inflammation of the palatine tonsils is one of the most common diseases found in the ENT department and becomes one of the world's health problems in the world because of the frequency, recurrence, and effect on the socio-economy.[1] Tonsillitis is a common disease that occurs in children and adolescents.[2] The case of tonsillitis at Luxembourg Ear Nose Throat Head and Neck Surgery department Children's Hospital in 2018 totaled 315 people with 60.5% acute tonsillitis, 24.5% chronic tonsillitis, 5% adenotonsillitis, and 19.05% complications with an average age of 14.25 years.[1] Based on ENT disease epidemiology data in seven provinces of Indonesia in September 2012, the incidence of tonsillitis is the highest after acute nasopharyngitis by 3.8%.[3] Based on data collected at dr. Zainoel Abidin Hospital Banda Aceh in 2018, the number of tonsillitis cases were found as many as 96 people.

The most common etiology of tonsillitis is *Streptococcus pyogenes*.[4] The cell surface of *Streptococcus pyogenes* has hydrophobicity ability as a determinant of pathogenicity of bacteria to the host cell.[5] In addition, *Streptococcus pyogenes* also have phospholipase enzyme activity.[6] Fatty acids mediate the binding of LTA to host receptors and inhibit the adhesion of *Streptococcus pyogenes* on the host.[7] *Streptococcus pyogenes* can be isolated on the tonsillar surface from exudate swab culture up to 60% of cases can be found in the tonsilar crypt.[4] The Rapid Strep Test of tonsillar swabs is very specific (95%) but less sensitive (60-100%) than culture. Throat culture with a swab of the pharyngeal area and posterior tonsil can be done when the body temperature is> 38.3°C or when the patient presents only with a sore throat, or the Rapid Strep Test is negative in highly suspected cases.[5]

Penicillin and amoxicillin are the initial treatment options in the majority of tonsillitis cases.[5] Initial antibiotics can eradicate bacteria and carrier conditions. However, prolonged use of penicillin and amoxicillin can cause side effects in the form of hypersensitivity reactions, skin rashes, resulting in cross-reactions and sensitivity to degradation products by alkaline hydrolysis, diarrhea, vaginal candidiasis,

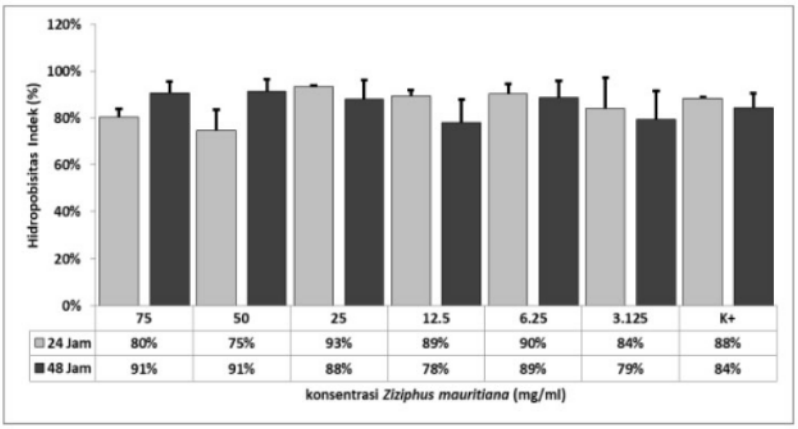
hematological reactions, anaphylactic allergic reactions.[4,8] Increasing antibiotic resistance has resulted in global socio-economic problems. Thus attention is paid to the use of plant extracts and biologically active compounds isolated from herbal plants.[9,10] The World Health Organization (WHO) reports that nearly 80% of the world's population, especially developing countries, trusts medicines derived from plants. The dangerous side effects of prolonged use of antibiotics have also resulted in treatment using natural plants believed to be an option.[11] One of them is *Ziziphus mauritiana* (sea bidara leaf), the plant parts such as leaves, fruit, and bark contain in vitro antibacterial and anti-cancer properties.[12] Based on the results of the Jakarta Regional Health Laboratory examination in 2019, the sea bidara leaf (*Ziziphus mauritiana*) is one of the plants that have potential as an alternative treatment because the ethanol extract is antibacterial, which contains neophytadiene compounds, hexadecanoic acid, linolenic acid, octadecanoic acid, squalene, and vitamin E.[9] The ethanol extract of sea bidara leaves (*Ziziphus mauritiana*) has antibacterial effects against *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Aspergillus niger,* and *Candida albicans*. *Streptococcus pyogenes* is the most common cause of tonsillitis and is the most susceptible to ethanol extract of sea bidara leaves (*Ziziphus mauritiana*).[13]

Based on this information, we chose the sea bidara leaves (*Ziziphus mauritiana*) as the plant to be studied, which could be used as a test material to prevent hydrophobicity activity and the enzyme phospholipase surface of Streptococcus pyogenes cells. It is hoped that the results obtained can be developed and become a reference for further research to find out how the in vivo bio-response of the ethanol extract of sea bidara leaves (*Ziziphus mauritiana*) may be a choice of therapy in tonsillitis besides antibiotics.

METHOD

This is an experimental laboratory study with a post-test-only control group design. The sampling is conducted from June to August 2020 at ENT Polyclinic of RSUDZA Banda Aceh. Afterwards, the production of *Ziziphus mauritiana* ethanol extract was carried out at the Chemistry Laboratory of the Faculty of Mathematics and Nature Sciences, Syiah Kuala University, identification of *Streptococcus pyogenes* and hydrophobicity and phospholipase activity tests on the surface of *Streptococcus pyogenes* cells were carried out at the Research Laboratory of Veterinary Medicine Faculty, Syiah Kuala University. The sample size was one *Streptococcus pyogenes* from the study population in the form of a swab of tonsillitis patients who went to the ENT Polyclinic of dr. Zainoel Abidin Hospital, Banda Aceh. The sample size in this study was one *Streptococcus pyogenes* based on the results of tonsillar swabs of tonsillitis patients who went to the ENT Polyclinic of RSUDZA Banda Aceh from June to August 2020 and the test material for the ethanol extract of sea bidara leaves (*Ziziphus mauritiana*) came from the Lamnyong area, Banda Aceh.

The variables in this study consisted of independent variables (ethanol extract of leaves of *Ziziphus mauritiana* 75%, 50%, 25%, 12.5%, 6.25%, 3.125%, and *Streptococcus pyogenes*) and dependent (formation of hydrophobicity and phospholipase on the surface of *Streptococcus pyogenes* cells). The research procedure began with sterilization of



	75	50	25	12.5	6.25	3.125	K+
24 Jam	80%	75%	93%	89%	90%	84%	88%
48 Jam	91%	91%	88%	78%	89%	79%	84%

Figure 1. Hydrophobicity index of Ziziphus mauritiana leaf ethanol extracts against *Streptococcus pyogenes.* The hydrophobicity index of the 24-hour incubation time was better than 48 hours (p> 0.05: 0.965), Bar (hydrophobicity index), and Bar error (Standard Deviation) in 6 repetitions.

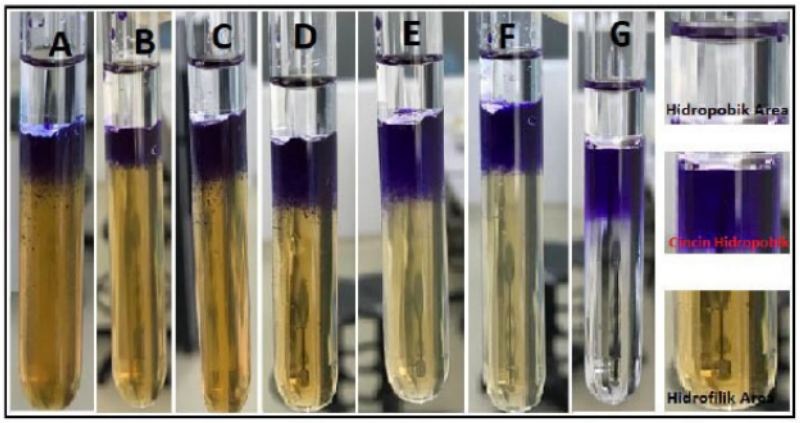


Figure 2. Hydrophobicity activity profile of *Ziziphus mauritiana* leaves ethanol extract against Streptococcus pyogenes cell surface.

Table 1. **Hydrophobicity index distribution of *Ziziphus mauritiana* leaves ethanol extract against *Streptococcus pyogenes***

Concentration	24 Hours					48 Hours					P-value
	N	OD	SDV	Scale	H-Index	N	OD	SD	Scale	H-Index	
75	7	0.152	0.036	Strong	80%	7	0.106	0.048	Strong	91%	Incubation TimeP>0.05 (0.965)
50	7	0.196	0.089	Moderate	75%	7	0.100	0.053	Strong	91%	
25	7	0.053	0.006	Strong	93%	7	0.138	0.080	Strong	88%	
12.5	7	0.083	0.026	Strong	89%	7	0.253	0.098	Strong	78%	ConcentrationP>0.05 (0.689)
6.25	7	0.076	0.044	Strong	90%	7	0.131	0.074	Strong	89%	
3.125	7	0.125	0.133	Strong	84%	7	0.238	0.123	Moderate	79%	
K+	7	0.089	0.005	Strong	88%	7	0.181	0.062	Strong	84%	

Note: Hidrofobisitas index: 80-100(%)= strong; 50-79(%)= moderate; 10-49 (%)= low; and1-10 (%)=no activity: OD (Optical Density)540 nm

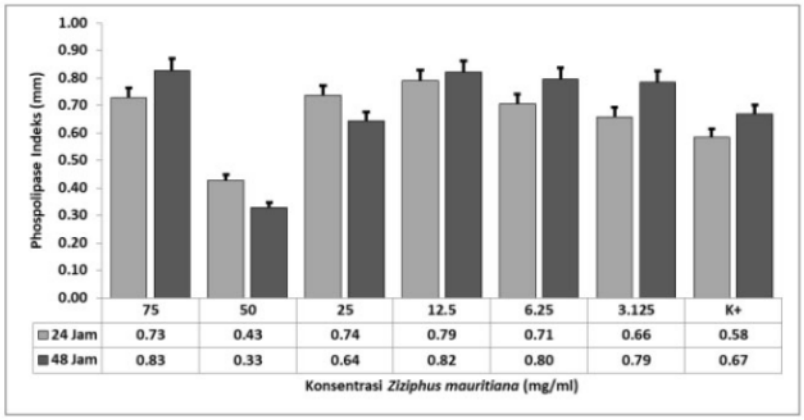


Figure 3. *Streptococcus pyogenes* phospholipase enzyme Inhibition after being interacted with *Ziziphus mauritiana* leaves ethanol extract. The 50% concentration has a lower inhibitory ability than other concentrations. Bar (phospholipase index) and Bar error (error bar with percentage).

tools, production of *Ziziphus Mauritiana* leaf extract, *Ziziphus Mauritiana* ethanol extract concentration variable, tonsil swab, bacterial isolation, identification of *Streptococcus pyogenes*, *Streptococcus pyogenes* suspension, hydrophobicity and phospholipase test of *Streptococcus pyogenes* cell surfaces, and data analysis.

The research results that have been obtained are collected, the obtained data is tabulated. The data results were analyzed using the Statistical Package for the Social Science (SPSS) software for the independent sample T test and ANOVA. Previously, all numerical data was assessed for distribution using the Shapiro Wilk test. If the data is not normally distributed, it would be analyzed using the Kruskal Wallis test as an alternative.

RESULT

Hydrophobicity of *Streptococcus pyogenes* cell surface

The hydrophobicity test aims to determine the ability to inhibit the hydrophobicity activity of *Streptococcus pyogenes* after interacting with the ethanol extract of *Ziziphus mauritiana* leaves. The hydrophobicity value is reported in percentage (index).

A) 75%; (B) 50%; (C) 25%); (D) 12,5%); (E) 6,25%; (F) 3,125%; and (G) positive control (Amoxicillin). Clear color (hydrophobic area); blue color (hydrophobic ring); and yellow color (hydrophilic area). In general, all concentrations are hydrophobic to the surface of *Streptococcus pyogenes* cells.

The results in Figure 2 and Table 1. show that the ethanol extract of Ziziphus mauritiana leaves has a strong influence on the hydrophobicity activity of the cell surface of *Streptococcus pyogenes*. All concentrations of test and positive control material (Amoxicillin) had a strong hydrophobicity index at the 24-hour incubation time, except at a concentration of 50%. Meanwhile, at the incubation time of 48 hours there was a decrease, especially at the concentrations of 12.5% and 3.125%.

The Phospholipase Index of Streptococcus Pyogenes

The phospholipase index assessment was used to measure the ability of *Streptococcus pyogenes* to break down phospholipids as a source of nutrition. Phospholipase values are reported in percentage (index).

The results of the study in Figure 3. shows that the ethanol extract of *Ziziphus mauritiana* leaves can increase the inhibition of activity phospholipase of *Streptococcus pyogenes*. The 50% concentration has a lower inhibitory ability than other concentrations. It shows that the ethanol extract of *Ziziphus mauritiana* leaves can be antibacterial by preventing the expression of activity phospholipase at the cellular level of *Streptococcus pyogenes*.

The hydrophobicity index between 24 hours and 48 hours did not significantly differ based on the independent sample T test ($p>0.05$; 0.965). There was no significant difference in the hydrophobicity index based on the Kruskal-Wallis analysis on the concentration of Ziziphus mauritiana leaves ethanol extract ($p>0.05$; 0.689). Both incubation times may determine the degree of hydrophobicity

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Table 2. **Phospholipase index distribution of *Ziziphus mauritiana* leaves ethanol extract against *Streptococcus pyogenes***

Concentration	24 Hours				48 Hours				P-value
	N	P-Index	SDV	Scale	N	P-Index	SD	Scale	
75	7	0.727	0.010	Strong	7	0.828	0.010	Strong	Incubation Time P>0.05 (0.683)
50	7	0.427	0.110	Moderate	7	0.330	0.012	Low	
25	7	0.736	0.020	Strong	7	0.644	0.011	Strong	
12.5	7	0.789	0.230	Strong	7	0.822	0.270	Strong	
6.25	7	0.707	0.022	Strong	7	0.797	0.122	Strong	Concentration P>0.05 (0.162)
3.125	7	0.659	0.031	Strong	7	0.786	0.091	Strong	
K+	7	0.584	0.050	Strong	7	0.668	0.012	Strong	

Note: Phospholipase index: >0.5= strong;0.35-4.9= moderate; and<0.35=low: OD (*Optical Density*)

activity of the *Streptococcus pyogenes* cell surface, although there was no significant difference (p>0.05). However, the ethanol extract of *Ziziphus mauritiana* leaves has strong properties to reduce hydrophobicity activity of *Streptococcus pyogenes* cell surface based on the concentration of the test material and the incubation time.

Table 2 shows that all concentrations of the ethanol extract of *Ziziphus mauritiana* leaves have the same ability to prevent the expression of the *Streptococcus pyogenes* phospholipase enzyme. The table data shows that the incubation time (p>0.05; 0.683) and the concentration (p>0.05; 0.162) did not have a significant difference in the degree of inhibition of phospholipase enzyme expression. These results indicate that the ethanol extract of *Ziziphus mauritiana* leaves can prevent one of the virulence factors of *Streptococcus pyogenes* involved in the pathogenesis of tonsillitis. Figure 3 shows that the ethanol extract of *Ziziphus mauritiana* leaves has a strong ability to inhibit the Streptococcus pyogenes phospholipase enzyme expression.

DISCUSSION

Phytochemical compounds from various plants, including *Ziziphus mauritiana*, can be directly bactericidal, such as preventing the adherence of bacteria to the surface of the pharyngeal mucosa, skin, and tooth surfaces, inhibition of glycolytic enzymes and lowering pH, reducing biofilm and plaque formation, and decreasing cell surface hydrophobicity. Collectively, findings from various studies suggest that phytochemicals can be used as drugs to eliminate infections with minimal side effects.[12,14] Khaleel et al. reported that differences in antibacterial properties of *Ziziphus sp* depend on the content of the active compound both in quality and quantity.[15] Elaloui et al. added that the extract of Ziziphus sp leaf has a strong inhibition zone. In addition, the test material has strong cytotoxicity against bacteria (*Escherichia coli, Staphylococcus aureus,* and *Klebsiella pneumonia*) and fungal groups (*Fusarium culmorum, Fusarium solani,* and *Botrytis cinerea*).[16]

Figure 1 shows that the ethanol extract of *Ziziphus mauritiana* leaves has a strong influence on the hydrophobicity activity of *Streptococcus pyogenes* cell surface. This shows the ethanol extract of *Ziziphus mauritiana* leaves can suppress the ability of *Streptococcus pyogenes* by excreting protein M and LTA, thus interferes with *Streptococcus pyogenes* to carry out hydrophobicity properties on the surface of the host cell. These conditions reduce the ability of *Streptococcus pyogenes* to form biofilms as the first initiation of quorum sensing formation in the pathogenesis of mucosal cell infections. In addition, the phytochemical properties of the flavonoids contained in the ethanol extract of *Ziziphus mauritiana* leaves can disrupt the permeability of the membrane structure, inhibit protein synthesis, and the production of coenzyme folate, nucleic acid, and peptidoglycan as virulence factors.[17]

Hydrophobicity of *Streptococcus pyogenes* is facilitated by LTA, in addition to the protein M family consisting of Emm (protein M), Mrp (M-related protein), Enn (M-like protein), and *Streptococcus* (Spa) protective antigen reportedly also involved in hydrophobicity activity of *Streptococcus pyogenes* cell surface.[5] Protein M is reported to be the main virulence factor of *Streptococcus pyogenes,* which acts by limiting phagocytosis, interferes with complement function, and is responsible for adhesion.[18] The function of *Streptococcus pyogenes* LTA is not only hydrophobin but also mediates the adhesion of organisms to various host cells. The hydrophobicity of a number of *Streptococcus pyogenes* serotypes depends on the expression of surface proteins that form complexes with LTA, allowing the ester-linked fatty acids of LTA to be exposed to the bacterial surface.[5] This explanation implies that the role of *Ziziphus mauritiana* (as reported in table 1) able to reduce the hydrophobicity of *Streptococcus pyogenes*, meaning that the ethanol extract of *Ziziphus mauritiana* leaves can inhibit LTA as the hydrophobin protein of these bacteria, however, this needs further study. This assumption correlates with the hydrophobicity index of the concentration of each test material for the ethanol extract of *Ziziphus mauritiana* leaves.[15]

The active compound coating of Ziziphus mauritiana on the surface of the host cell can also close the hydrophilic ion channels from the cell, thus disrupt the potential reaction atmosphere on the surface of the host cell.[15] The concentration of the test material also determines the hydrophobicity inhibition ability of bacterial cell surface. The extract solution concentration can affect the absorption and penetration properties of the cell surface as an antibacterial.[19] The results are shown in Figure 1, that at the 50% concentration of *Ziziphus mauritiana*

leaves, ethanol extract had a lower ability at the 24-hour incubation time. Even at the 48-hour incubation time, there was a decrease, although it was not significant. This shows the test material has an active phase for 24 hours. In addition, based on the findings of this study, it was reported that the lowest concentration of the test material still showed effective anti-hydrophobicity, especially 24 hours. Araújo et al. reported that the minimum inhibitory concentration and post-antibiotic effect of antimicrobial agents should be considered when determining the appropriate dosage in studies of cell surface hydrophobicity and bacterial adhesin of group B *Streptococcus* strains in vitro.[20] Active molecules from these plant extracts have ligands on cells to create oxidative stress through changes in the passive diffusion mechanism of pathogenic cell membranes, causing cells to undergo lysis and a decrease in oxygen uptake.[21,22]

Figure 2 shows that the hydrophobicity index is shown in areas stained with crystal violet. The hydrophobic ring area reacts between the active compound of the test material and xylene to form a semipolar region that maintains the balance of the polar region (hydrophilic). The intensity of this hydrophobic nature becomes a reference to measure its effect on the protection of host cells from bacterial infection because the higher the hydrophobicity on the surface of bacterial cells, the more difficult it is for bacteria to communicate between other bacteria, including the formation of quorum sensing and biofilms on the mucosal surface. The principle of hydrophobicity index can be used to measure the absorption power from a solution of medicinal substances, including natural substances used as the test material in this study.[23]

Based on the research findings, there was no significant difference in the hydrophobicity index activity of *Ziziphus mauritiana* leaves ethanol extract between 24 hours and hours. This shows that time does not have a significant effect on the activity of the ethanol extract of *Ziziphus mauritiana* leaves in responding to the hydrophobicity of the cell surface of *Streptococcus pyogenes;* thus, it is understandable that some active compounds contained in the ethanol extract of *Ziziphus mauritiana* leaves have stability in responding to *Streptococcus pyogenes*.[8] In addition, the two incubation periods determined the degree of hydrophobicity inhibition of *Streptococcus pyogenes* cell surface, although there was no significant difference ($p>0.05$).

Testing the effect of *Ziziphus mauritiana* leaves ethanol extract on *Streptococcus pyogenes* phospholipase enzyme has a relationship with these virulence factors in the pathogenesis of laryngeal and tonsil infection. Oda et al. reported *Streptococcus pyogenes* gene was associated with inducing the expression of intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) via arachidonic acid signaling cascade. ICAM 1 and VCAM 1 cells have an association with the mucosal defense system against pathogens present in the respiratory tract.[24] Cellularly, the active molecules found in plants, including *Ziziphus mauritiana,* can inhibit the *Streptococcus pyogenes* phospholipase gene, thus inhibit cyclooxygenase-2 from preventing adhesion.[25]

The preceding theoretical principle is relevant to the results of this study, where in vitro experiments showed the ethanol extract of *Ziziphus mauritiana* leaf could prevent the activity of the *Streptococcus pyogenes* phospholipase enzyme, thus enabling bacteria to break phospholipids as an energy source. This potential indicates that the ethanol extract of *Ziziphus mauritiana* leaf can be used as an active material to prevent the adhesion of *Streptococcus pyogenes* on vascular cells. In terms of biological principles, the ethanol extract of *Ziziphus mauritiana* leaf has good bio-response properties to reduce the phospholipase enzyme activity, thus preventing inflammation of vascular endothelial cells.[26] As reported in this study, the ethanol extract of *Ziziphus mauritiana* leaves can inhibit the formation of hydrophobicity and the Streptococcus pyogenes phospholipase enzyme activity. This result is in line with the previous theory, which describes the ethanol extract of *Ziziphus mauritiana* leaves to be bactericidal.

Phospholipase index assessment is used as a reference to determine *Streptococcus pyogenes* to break phospholipids for nutrient sources and defend against the influence of the host immune system. Bandana et al. explained that phospholipases have a role in catalyze-hydrolysis phospholipids, a key component of eukaryotic cell membranes. Metabolites produced after hydrolysis function as secondary messengers, which are then involved in signal transduction, membrane changes, and cell proliferation. This enzyme is considered an important virulence factor as it assists bacterial pathogens in various ways, such as the invasion of host cells, modulating its membrane phospholipid content. In addition, this enzyme is essential in the pathogenesis of certain bacteria because of its role in detaching itself from host defense mechanisms.[27]

The test data of *Ziziphus mauritiana* leaves ethanol extract against hydrophobicity and phospholipase have the same properties, where at a concentration of 50%, there is a decrease in its bio-response. Both virulence factors have the same response to the test material, so thus it is explainable that the concentration does not influence the strength of the bio-response of the test material because it is not significant at various test concentrations. This shows that all concentrations respond to the decrease in two virulence traits of *Streptococcus pyogenes* with varying tendencies. The ability to inhibit the *Streptococcus pyogenes* phospholipase enzyme can be assumed that the test material can prevent inflammatory activity triggered by these bacteria because, in addition to the role of providing nutritional sources, the phospholipase enzyme expressed by *Streptococcus pyogenes* can increase lipolytic activity and pro-inflammatory activity on the surface of vascular cells in the pathogenesis of respiratory tract infection.[26,28]

Based on the results of this study, the ethanol extract of *Ziziphus mauritiana* leaves can reduce the virulence activity of *Streptococcus pyogenes* by inhibiting phospholipase because the inhibitory ability of this enzyme is relatively strong. According to Cheesman et al., this strength is influenced by the quality of the active compounds contained in these

plants.[29] In addition, the antagonistic properties of each active molecule are also a determinant of the strength in reducing bacterial virulence properties.[30] Ashraf et al. reported that *Ziziphus mauritiana* has a number of active compounds with low molecular weight such as phytol which can penetrate the surface of the pathogenic cell wall.[24,31,32] In general, the active compounds from plant extracts including *Ziziphus mauritiana* with low molecular weight have antibacterial properties.[6] Low molecular antibiotics produced by these plants are classified according to two types, phytoanticipins which are involved in the inhibitory action of microbes including by inhibiting phospholipase as reported in this study, and phytoalkesins which are generally antioxidants synthesized by plants in response to microbial infections.[33]

The limitation in this study is no further research has been done on how much (in milliliters) the ethanol extract contains *Ziziphus mauritiana* in products such as mouth rinse which is needed as tonsillitis therapy.

CONCLUSION

The ethanol extract of *Ziziphus mauritiana leaves* can inhibit the hydrophobicity and phospholipase activity of *Streptococcus pyogenes* cell surface with different quantity and quality at each concentration and incubation time virulence.

CONFLICT OF INTEREST

There was no conflict of interest to disclose.

FUNDING

There were no financial interests involved.

ETHICAL STATEMENT

KEPPKN Registration Number: 1171012P.

AUTHOR CONTRIBUTION

All of the authors are equally contributed to the study.

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