In vitro study of an ethanolic extract of coffea leaves to inhibit freshwater pathogenic bacteria

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Abstract. Bacteria are a disease agent that can cause a loss in cultivation. The use of antibiotics as bacterial disease control is still an option, although it has adverse side effects on fish and on the environment. Therefore an alternative antibacterial agent is needed that is safe and environmentally friendly. Coffea leaves contain flavonoids, alkaloids, tannins, and phenolic compounds that are considered to have antibacterial activity and thus, properties. The aim of this study was to determine the antibacterial activity of the ethanolic extracts of coffee leaves against Edwardsiella tarda, Aeromonas hydrophila, and Streptococcus agalactiae. The ethanolic extract of coffee leaves was obtained using the maceration method and the antibacterial activity test was performed using the disc diffusion method. The result showed that the ability of this extract to inhibit the growth of all bacteria increased with the increasing concentration. The best performance of the ethanolic extract of coffee leaves was where it was shown to inhibit Aeromonas hydrophila.

1. Introduction
Diseases are related to the success of fish farming. Pathogenic bacteria will easily attack the fish when the environment quality decreases. Aeromonas hydrophila, Edwardsiella tarda, and Streptococcus agalactiae are types of bacteria that often infect freshwater fish. Aeromonas hydrophila is Gram negative and is normally found in the aquatic environment. This is known to cause Motile Aeromonad Septicemia (MAS) disease which is characterized by wounds on the body surface, ulcers and hemorrhages in the gills [1]. This virulence is caused by production of extracellular compounds such as aerolycin, hemolycin, protease, adhesion, enterotoxin, phospholipase and lipase [2]. Sarkar and Rashid [3] reported that Aeromonas hydrophila caused deaths at a percentage between 60-100% in catfish, carps, and perch populations. Moreover, these bacteria can also infect humans and can cause gastroenteritis and meningitis [4, 5].

Edwardsiella tarda is a family of Enterobacteriaceae which are motile, facultative anaerobes classified as Gram negative [6]. These bacteria cause Edwardsiellosis in salmon, tilapia, and striped bass. This bacterial infection induces necrosis, hyperplasia, edema and hemosiderin in the gills, liver, and kidneys [7]. This family is also classified as zoonotic which can lead to gastroenteritis and meningitis in human [8].

Streptococcus agalactiae is classified as Streptococcus B that causes Streptococcus in fish. Furthermore, these bacteria induce pneumonia and meningitis in infants and mastitis in cattle [9]. The infected fish showed skin hemorrhages, swelling of the internal organs, exophthalmia and whirling. Wang et al [10] said that this bacteria has caused a lot of losses in tilapia cultivation.
The treatment of bacterial diseases is carried out using antibiotics. However, antibiotics have adverse side effects in fish and in the environment. Antibiotics can accumulate in the fish’s body and increase the risk of bacterial resistance. Because of that, it is necessary to explore natural compounds that have antibacterial activity and that are environmentally friendly using medicinal plants such as coffee. Some studies have reported that coffee contains active compounds that have certain properties; for example, antibacterial, antioxidant, and anti-inflammatory [11,12]. Yaqin and Nurmilawati [13] reported that robusta coffee bean extract inhibited the growth of Staphylococcus aureus with a minimum concentration of 12.5%. Runti et al. [14] also stated that the arabica coffee bean extract demonstrated antibacterial activity against Staphylococcus epidermidis and Enterococcus faecalis with low cytotoxicity. This is evidence that it is appropriate to apply in biomedicine or treatment.

Antibacterial activity from this extract is allegedly derived from the caffeine content in the coffee beans, although the other metabolite compounds such as chlorogenic acid, flavonoid, and phenolic compounds contributed to the antibacterial activity [15, 16]. There have been many studies on the antibacterial activity of coffee bean extracts but the study of the antibacterial activity of coffee leaves is still limited. This research will therefore study the antibacterial activity of coffee leaf extracts against pathogenic bacteria in freshwater fish.

2. Methodology
2.1. Research method
The study was conducted in the Instrumental Laboratory of Universitas Airlangga Campus Banyuwangi. The phytochemical screening test was carried out at the Testing Service Unit of the Pharmacy Faculty, Universitas Airlangga, Surabaya. The data was analyzed using the descriptive method.

2.2. Materials
The sample of coffee leaves was collected from a coffee plantation in Licin, Banyuwangi. Aeromonas hydrophila, Edwardsiella tarda, and Streptococcus agalactiae cultures were obtained from the culture collection of Bogor Agricultural Institute. The chemicals used in this research consisted of ethanol, Triptic Soy Agar (TSA) medium, Nutrient Broth medium, and a chloramphenicol disk. These chemicals were pro-analysis grade. Ethanol was used as a solvent for extraction and was technical grade.

2.3. Research procedure
2.3.1. Preparation of coffee leaves extract
The coffee leaf extract was obtained from the maceration method. Fresh coffee leaves were cleaned and cut into small pieces, and then dried at room temperature for about 5 days. The dried coffee leaves were ground into powder. About 500 grams of coffee leaf powder were soaked in ethanol solvent for 1 x 24 hours. The mixture was filtered using Whatman filter paper no. 1. The liquid extract was concentrated using a rotary vacuum evaporator to produce an ethanol crude extract.

2.3.2. Phytochemical screening tests
The phytochemical screening test of the ethanolic coffee leaf extract was carried out using the screening method put forward by Harborne [17]. The phytochemical tests include alkaloids, flavonoids, polyphenol, saponins and steroids/terpenoids.

2.3.3. Disk diffusion method
The antibacterial activity of the coffee leaf extract was performed using the disc diffusion method. Filter paper discs 6 mm in diameter were prepared and sterilized. The paper disc was soaked in a concentration of coffee leaf extract (20%, 40%, 60%, 80%, and 100%) for 5 minutes. Ethanol was used as a negative control and chloramphenicol was used as a positive control. The discs were then placed over nutrient agar plates seeded with the respective test microbes and incubated in 37°C for 24
The inhibition diameter was then measured in mm. Inhibition zones with a diameter that was less than 12 mm were considered as having no antibacterial activity. Diameters between 12 and 16 mm were considered to be moderately active and diameters of more than 16 mm were considered to be highly active [18].

3. Results and discussion
From this study, the ethanolic extract of coffea leaves obtained a 3.82% yield. The concentrated extract was blackish green. Based on the phytochemical screening test, the ethanolic extract of the coffea leaves contained some phytochemical compounds such as flavonoids, steroid/terpenoids, polyphenols and saponin. Campa et al. [19] said that coffea leaves has a varied phytochemical content such as alkaloid, flavonoid, terpenoids, amino acid, sucrose, tannin, xanthonoid, phenolic acid, and catechin. The profile of the phytochemical content depended on the leaf development stage. Young leaves have more phytochemical content than mature leaves [20, 21]. Phytochemicals are essential for plants to defend themselves from disease. Many previous studies have explained that the varied phytochemical content also correlates with varied biological activity, especially antibacterial activity [22].

The antibacterial activity of ethanolic coffee leaf extract can be known from the presence of a clear zone around the paper discs. The antibacterial activities of ethanolic coffee leaf extracts have been shown in Figure 1.

![Figure 1. Antibacterial activity of ethanolic coffee leaves extract by disk diffusion test.](image)

Based on the results, the clear zone had begun to appear when given the extract concentration of 20%. The diameter of the clear zone increased with the increasing concentration extract on the media containing Aeromonas hydrophila and Streptococcus agalactiae. This result proved that in a higher concentration, more active compounds can diffuse into the media and inhibit the growth of bacteria. Different results happened in the media containing Edwardsiella tarda. Antibacterial activity increased at a concentration of 20% up to 80%, but it decreased at a concentration of 100%. The reduction of this activity occurred because of the enhancement of the bacterial ability to tolerate antibiotics. Vranakis et al [23] stated that bacteria have ability to change their membrane permeability toward antibiotics so then the antibiotics can no longer diffuse into the membrane.

The activity of the coffee leaf extract inhibits the growth of bacteria in different ways. The widest clear zone was shown in the media containing Aeromonas hydrophila. The clear zone in the media
containing Edwardsiella tarda and Streptococcus agalactiae were almost the same size. This result revealed that Aeromonas hydrophila is the most susceptible bacteria. Aeromonas hydrophila and Edwardsiella tarda are classified as Gram negative bacteria. The outer membrane of Gram-negative bacteria contributed to the diffusion activity of the polar compounds into the cell. The outer membrane is selectively permeable because it has porins or a certain protein membrane that facilitates the hydrophilic or polar compounds to diffuse into the cell [24]. Nevertheless, Aeromonas hydrophila and Edwardsiella tarda have a different structure in their outer protein membrane that can lead to differential antibacterial compound diffusion into the cell [25, 26].

The antibacterial activity of the ethanolic coffee extract was suspected due to its varied secondary metabolite content. Some of the previous studies mentioned that the caffeine and chlorogenic acid in coffee leaf extracts have antibacterial activity. Caffeine is an alkaloid that is synthesized in the young leaves, and it is accumulated in mature leaves [27]. Prutviraj et al. [28] stated that caffeine performs the role of an inhibitor of adenine and thymidine incorporation so then the protein and DNA synthesis of bacteria can be disturbed. Moreover, the presence of caffeine enhances genotoxicity after the DNA damaged. Besides caffeine, chlorogenic acid (CGA) is also found in coffee leaves. CGA is one of the phenolic compounds that have antibacterial activity. According to Naveed et al [29], CGA inhibits antibiotic efflux systems in multi-drug resistant bacteria.

Based on the results, the antibacterial activity of ethanolic coffee leaf extract is weaker than chloramphenicol as a positive control. According to Indu et al [18], ethanolic coffee leaf extract at 100% was classified as having no antibacterial up to moderate activity. Meanwhile, chloramphenicol has a moderate up to highly active level of activity against the bacteria. Chloramphenicol was initially isolated from Streptomyces venezuelae and synthetically produced from 1948 up to now. Nevertheless, this antibiotic has begun to be banned because it has a harmful effect in humans [30].

4. Conclusion
This study reported that the ethanolic extract of coffee leaves has antibacterial activity that can inhibit the growth of Aeromonas hydrophila, Edwardsiella tarda, and Streptococcus agalactiae. The best performance of the ethanolic extract of coffee leaves was shown in how it inhibited Aeromonas hydrophila. Ethanolic extracts of coffee leaves are a promising natural product for use as an antibacterial agent, especially for fish. Therefore, further study is needed to determine its potential for therapeutic application in fish.

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6. References
[1] Vijayakumar S, Vaseeharan B, Malaiakozhundan B, Gobi N, Ravichandran S, Karthi S, Ashokkumar B and Sivakumar N. (2017). Microb. Pathog, 110, 140-151
[2] Parker JL and Shaw JG. (2011). J. Infect, 62, 109-118
[3] Sarkar MJ and Rashid MM. (2012). JBAU, 10, 157-161
[4] McCoy AJ, Koizumi Y, Toma C, Higa N, Dixin V, Taniguchi S, Tschopp J, Suzuki T. (2010). Eur. J. Immunol, 40, 2797-2803
[5] Albarral V, Sanglas A, Palau M, Minnana-Galbis D and Fuste MC. (2016). Can. J. Microbiol, 62, 296-306
[6] Hirai Y, Asahata-Tago S, Ainoda Y, Fujita T and Kikuchi K. (2015). Can. J. Infect. Dis. Med. Microbiol, 26, 313-318
[7] Abraham TJ, Mallick PK, Adikesavalu H and Banerjee S. (2015). Arch. Pol. Fish, 23, 141-148
[8] Lima LC, Fernandes AA, Costa AAP, Velasco FO, Leite RC and Hackett JL. (2008). Arq. Bras. Med. Vet. Zootec, 60, 275-277
[9] Abuseliana AF, Daud HHM, Aziz SA, Bejo SK and Alsaid M. (2011). JAVA, 10, 914-919
[10] Wang K, Chen D, Huang L, Lian H, Wang J, Xiao D, Geng Y, Yang Z and Lai W. (2013). Afr. J. Microbiol. Res., 7:317-323
[11] Mohammed MJ and Al-Bayati FA. (2014). Int. J. Green Pharm., 3, 52-57
[12] Galam NZ, Gambo IM, Rabiu A, Chinejo N and Dami S. (2013). J. Nat. Sci. Res., 3, 191-193
[13] Yaqin MA and Nurmilawati M. (2015). Effect of Robusta (Coffea robusta) Coffee Extract as a Growth Inhibitor of Staphylococcus aureus. Seminar Nasional XII Pendidikan Biologi FKIP UINS: 867-872
[14] Runti G, Pacor S, Colomban S, Gennaro R, Navarini L and Scocchi M. (2015). LWT-Food Sci. Technol., 62, 108-114
[15] Fardia S. (1995). ASEAN Food J., 10, 103-106
[16] Nayeem N, Denny G and Mehta SK. (2011). Pharm. Lett., 3, 292-297
[17] Harborne JB. (1987). Phytochemical Method Guide to Modern Ways to Analyze Plants. Bandung: ITB Press
[18] Indu MN, Hatha AAM, AbiroshC, Harsha U and Vivekanandan G. (2006). Braz. J. Microbiol., 37, 153-158
[19] Campa C, Mondolot L, Rakotondravao A, Bidel LP, Gargadennec A, Couturon E, LaFisca P, Rakotomalala JJ, Jay-Aleem C and Davis AP. (2012). Ann. Bot., 110, 595-613
[20] Mondolot L, La Fisca P, Buatois B, Talansier E, de Kochko A and Campa C. (2006). Ann. Bot., 98, 33-40
[21] Chen XM, Ma Z and Kitts DD. (2018). Food Chem., 249, 143-153
[22] Liu Q, Meng X, Li Y, Zhao CN, Tang GY and Li HB. (2017). Int. J.Mol. Sci., 18, 1283
[23] Vranakis I, Goniotakis I, Psaroulaki A, Sandalakis V, Tseletis Y, Gevaert K and Tsiotis G. (2014). J. Proteomics, 97, 88-99
[24] Santos RX, Oliveira DA, Sodre GA, Gosmann G, Brendel M and Pungartnik C. (2014). Genet. Mol. Res., 13, 7725-7735
[25] Aoki T and Holland BI. (1985). FEMS Microbiol. Lett., 27, 299-305
[26] Purkait S, Abraham TJ, Karmakar S, Dey B and Roy A. (2018). J. Aquac. Res. Dev., 95, 24
[27] Patay EB, Bencsik T and Papp N. (2016). Asian Pac. J. Trop. Med., 9, 1127-1135
[28] Pruthviraj P, Suchita B, Shital K and Shilpa K. (2011). Int. J. Res. Ayurveda Pharm., 2, 1354-1357
[29] Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, Ahmad F, Babazadeh D, FangFang X, Modarresi-Ghazani F et al. (2018). Biomed. Pharmacother, 97, 67-74
[30] Shukla P, Bansode FW and Singh RK. (2011). JMMS, 2, 1313-1316