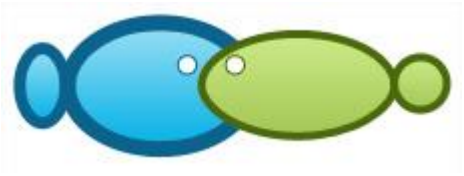

Evaluation of antibacterial activity of different solvent extract from *Coffea canephora* leaves against *Edwardsiella tarda* and *Streptococcus agalactiae*

Hapsari Kenconojati, Mohammad F. Ulkhaq, Muhammad H. Azhar, Darmawan S. Budi

Faculty of Fisheries and Marine, Campus Banyuwangi, Airlangga University, Banyuwangi, Indonesia. Corresponding author: H. Kenconojati, hapsari@fpk.unair.ac.id

Abstract. The enhancement of bacterial resistance due to the use of antibiotics has become a major concern in aquaculture industry. Eco-friendly products are crucially needed to replace antibiotics in sustainable disease management. This research aims to determine the antibacterial activity of coffee leaves, extracted by different solvents, against *Edwardsiella tarda* and *Streptococcus agalactiae*. Both bacteria in this study have been known as pathogenic in fish culture. Through this research, an enhancement of coffee leaves waste is expected, in order to be used as natural antibiotics for infected fish treatment. Coffee leaves extract was obtained by maceration whilst the antibacterial activity was performed by disk diffusion and tube dilution methods. Ethyl acetate extract of coffee leaves has the highest antibacterial activity on both bacteria. Meanwhile, n-hexane extract has no antibacterial activity. The minimum inhibitory concentration (MIC) of ethyl acetate fraction is 6.25 mg mL^{-1} and the minimum bactericidal concentration (MBC) is 12.5 mg mL^{-1}. In conclusion, ethyl acetate fraction of coffee leaves could be used as future sources of antibacterial agent for infected fish treatment.
Key Words: Coffee leaves, extract, antibiotics, bacteria, fish pathogen.

Introduction. Infection is a class of diseases with a frequent negative impact on the aquaculture activities, causing significant losses. Some pathogenic bacteria in fish are *Edwardsiella tarda* and *Streptococcus agalactiae*. *E. tarda* is a member of the Enterobacteriaceae family. These are motile, facultative anaerobic and gram-negative bacteria (Yuji et al 2015), at the origin of edwardsiellosis in salmon, tilapia and striped bass. This bacterial infection causes necrosis, hyperplasia, edema and hemosiderin deficit in the gills, liver and kidneys of fish (Abraham et al 2015). Moreover, *E. tarda* is classified as a zoonotic bacteria causing gastroenteritis and meningitis in humans (Lima et al 2008).

Besides the gram-negative, the infection in fish can also be caused by gram-positive bacteria, for example, *S. agalactiae*, a group B streptococcus which can cause Streptococcosis in fish. These bacteria lead to pneumonia and meningitis in human and mastitis in cows (Abuseliana et al 2011). The infected fish showed hemorrhagic skin symptoms, swelling of internal organs, exophthalmy, and whirling. This disease caused many losses to the cultivation of tilapia (Wang et al 2013).

A standard treatment of bacterial diseases uses antibiotics such as oxytetracycline and chloramphenicol, sometimes leading to the accumulation in the fish's body and increases the risk of bacterial resistance (Rasul & Majumdar 2017). Therefore, recent research focuses on discovering natural products having antibacterial activity. One alternative that can be done is using medicinal plants such as coffee.

Coffee is a pharmacologically active tropical plant known as the most consumed beverage in the world (Valduga et al 2019). Some studies suggested that coffee has some compounds that have specific activity, such as antibacterial (Kenconojati et al 2019; Mohammed & Al-Bayati 2009), antioxidant (Khotimah 2014), anti-diabetic

(Retnaningtyas et al 2015), and anti-inflammatory (Galam et al 2013). The Arabica coffee bean extract is compatible with biomedical therapy applications, according to the study conducted by Runti et al (2015) on its antibacterial activity against *Staphylococcus epidermidis* and *Enterococcus faecalis,* with a low cytotoxicity. The antibacterial activity is presumably derived from the high caffeine content in coffee beans even though the content of other chemical compounds such as chlorogenic acid, trigoneline, flavonoids, and phenolic compounds also contribute to the antibacterial activity (Fardiaz 1995; Hasanah et al 2016; Nayeem et al 2011). Many previous studies on antibacterial activity from coffee focus on the use of the coffee beans. Nevertheless, the study of the antibacterial activity of the leaves of coffee is still limited, especially in aquaculture. Coffee leaf is a coffee plantation waste produced by pruning, due to the agronomic management practices (Martinez et al 2019). The large amount of coffee leaves waste can become environmental problems if discarded inadequately. Therefore, this study investigated the antibacterial activity of the coffee leaves extract against pathogenic bacteria in freshwater fish, with results is expected to increase the knowledge about natural, alternative antibiotics in fish infections treatment, based on coffee leaves waste applications.

Material and Method

Preparation of the coffee leaves extract. Coffee leaves extract were obtained by maceration using different polarity of solvents as reported by Bhat et al (2018). The fresh leaves of coffee were cleaned and cut into small pieces than dried at room temperature. The dried coffee leaves were ground into powder using blender. The 500 g sample powder was macerated with 1.5 L of solvent at room temperature for 24 hours. The solvent used for extraction were hexane and ethyl acetate and the essays were performed serially, ordered by polarity increase the. The mixture was filtered using Whatmann filter paper. The liquid extract was evaporated in rotary vacuum evaporator to give crude extracts. The extracts were stored at 4^{o}C.

Antibacterial activity test with disk diffusion method. The antibacterial activity test of coffee leaves extract was performed using the disc diffusion method as previously described, with slight modification (Indu et al 2006). *E. tarda* and *S. agalactiae* cultures were obtained from the culture collection of Bogor Agricultural University. The diffusion method was conducted using Nutrient Agar sterile medium. The microbes were suspended in physiologic solution and were measured until optical density (OD) value was 0.5 McFarland standard (equivalent with 10^8 CFU mL^{-1}, $\lambda_{625\ nm}$=0.08-0.1). Microbe suspension (100 µL) was grown on a petri dish in a sterile Nutrient Agar medium by pour plate method until the medium solidified. Filter paper discs of 6 mm diameter were prepared and sterilized. The coffee leaves extract was prepared in 4 different concentrations (200, 400, 600 and 800 mg mL^{-1}) and as much as 20 µL of coffee leaves extract was impregnated in sterile filter paper disc. Each paper disc was placed on the medium containing tested microbe and incubated at 37^{o}C for 24 hours. The inhibition diameter was then measured. Ethyl acetate and n-hexane were used as negative control in each treatment and chloramphenicol was used as a positive control. The best antibacterial activity extract on this test was continued in tube dilution test to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. The MIC and MBC test was performed using broth tube dilution method as reported by Genovese et al (2012). The microbes were diluted to a concentration of McFarland no. 0.5. The coffee leaves extract was gradually diluted in Nutrient Broth until it reached various concentrations (6.25, 12.5, 25, 50, 100, 200 mg mL^{-1}) and the microbe suspension was injected. The highest concentration of the coffee leaves extract on the broth tube dilution test was determined from the smallest concentration which can inhibit the growth of microbes on the disc diffusion test. Medium

containing tested microbe were incubated at 37°C for 24 hours. For the measurement of the MIC and MBC, all of the culture solutions were grown on nutrient agar medium and incubated at 37°C for 24 hours. The MIC was determined as the lowest extract concentration that still showed microbial growth, while the MBC was determined as the lowest extract concentration which killed 99.9% microbes.

Results

Solvent extraction. The use of different polarity of solvent on coffee leaves extraction produced variations of the extract yield. The highest extract yield (2.01%) was obtained from n-hexane, while the lowest extract yield (1.56%) was obtained from ethyl acetate.

Disk diffusion method. The formation of clear zone around the paper disc has shown the presence of antibacterial activity of coffee leaves extract. The result revealed that ethyl acetate extract showed the best activity for inhibiting the growth of both bacteria. The inhibition zone has begun to appear for a concentration of 200 mg mL^{-1} of ethyl acetate extract. However, hexane extract of coffee leaves at the same concentration did not demonstrate inhibition activity towards both of tested bacteria. The size of inhibition zone grew with the increase of the extract concentration. The largest inhibitory zone of ethyl acetate extract was obtained at 800 mg mL^{-1}, 17 mm in *E. tarda* and 10.25 mm in *S. agalactiae*. Conversely, 800 mg mL^{-1} of hexane extract produced only a small inhibitory zone in *E. tarda* (5.25 mm) and no inhibition zone in *S. agalactiae*. Antibacterial activities of coffee leaves extract are shown in Table 1.

Table 1

Antibacterial activity of coffee leaves extract by disk diffusion method

Type	*Concentration (mg mL^{-1})*	*Diameter of inhibition zone (mm)*	
		Edwardsiella tarda	*Streptococcus agalactiae*
n-hexane extract	0	0	0
	200	0	0
	400	0	0
	600	1.50	0
	800	5.25	0
Ethyl acetate extract	0	0	0
	200	4.38	2.50
	400	8.38	5.88
	600	9.25	7.25
	800	17.00	10.25
Chloramphenicol 30 µg	-	13.75	31.50

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The result of the tube dilution test showed clear media at all concentration treatments, except the negative control. After it grew on a nutrient agar medium, the bacterial growth still occurred at a concentration of 6.25 mg mL^{-1}, while at a concentration of more than 12.5 mg mL^{-1} there was no bacterial growth anymore. These results were obtained from both of the tested bacteria, *E. tarda* and *S. agalactiae*. Therefore, the MIC of ethyl acetate extract of the coffee leaves is 6.25 mg mL^{-1} and the MBC is 12.5 mg mL^{-1}.

Discussion. Based on the disc diffusion test, all the coffee leaves extract and the chloramphenicol, as positive control, were absorbed and diffused on Nutrient Agar medium containing the tested bacteria. The measurement of the inhibition zone showed that its diameter for both bacteria increased with the extracts concentration increase. The increased concentration of the extract generated more active compounds which diffused into the medium. Ethyl acetate extract of coffee leaves has been proved more effective in the inhibition of *E. tarda* and *S. agalactiae* than the hexane extract. Ethyl acetate extract

formed an inhibition zone on a medium containing *E. tarda* and *S. agalactiae,* at the lowest concentration (200 mg mL^{-1}). Meanwhile, n-hexane extract began to show an inhibition zone on *E. tarda* at 600 mg mL^{-1} and no inhibition zone on *S. agalactiae,* at any concentration.

Compared with *S. agalactiae*, *E. tarda* was more sensitive to the coffee leaves extracts. This result suggested that coffee leaves extract is more effective against gram negative than against gram positive bacteria. The type of bacteria can be related to its susceptibility to the plant extract, due to the difference in the cell walls components, for the two bacteria (Alabri et al 2014; Runti et al 2015). An extract that contains certain active compounds may inhibit bacterial growth through various mechanisms such as the inhibition of bacterial cell wall, of protein, or of DNA and RNA synthesis, and also of the changes in cell membrane permeability and antimetabolite (Tortora et al 2002). *E. tarda* is classified as gram-negative where the peptidoglycan layer of the cell wall is thinner than gram positive's so that the active compounds will be able to diffuse easily into the cell (Santos et al 2014). The ease of an active compound diffusion into the bacterial cell wall is not only caused by the component of the cell wall, but also depends on the lipophilicity of the active compound. Lipophilicity is defined as the tendency of a compound to be distributed in the matrix of lipid and water (Grant & Higuchi 1990). Highly polarized compounds will be more difficult to penetrate the semi-polar cell membrane whereas high lipophilic compounds will tend to be easily oxidized before entering the cell membrane (Kerns & Di 2008).

The different results are shown against the values for the chloramphenicol, where the larger inhibition zone was produced in *S. agalactiae,* which is a gram positive. Chloramphenicol is an antibiotic that has been used since 1948 and known as broad spectrum antibiotic. Chloramphenicol was used as feed additives in fish and livestock feed. But in the 1990's, the use of chloramphenicol as feed additives began to be banned because of the harmful effects to human (Shukla et al 2011). Based on the categories specified by National Committee for Clinical Laboratory Standards (NCCLS 2002), *E. tarda* has been resistant to the chloramphenicol. Bacterial resistance to an antibiotic can occur through various mechanisms, one of which is an increased ability to close the pores of the bacterial cell wall, thus decreasing the amount of antibiotics that will pass through the membrane (Peleg & Hooper 2010).

Using plant material as medicinal plant is known for a long time in most of the countries. Phytochemical content in plant, such as alkaloids, flavonoids, phenolic compound and terpenoid, has been recognized as the responsible agent for its biological activity such as antimicrobial, antioxidant, and antifungal (Alabri et al 2014; Hossain & Nagooru 2011). The amount and composition of the extracted phytochemical content from the plant was induced by solvent in extraction process because of its solubility. Some phytochemical compounds like steroids and terpenoids will be more soluble in non-polar solvents, whilst phenolic compound will be more soluble in polar solvents (Dirar et al 2019). In this study, the use of different solvent in coffee leaves extraction produced various extract yield.

Based on the MIC and MBC of coffee leaves extract against both bacteria, it was demonstrated that coffee leaves extract has been able to inhibit bacterial growth at low concentrations. The lower MIC and MBC indicate that the antibacterial activity of the extract is higher. Hasanah et al (2017) reported that the coffee leaves extract contained several secondary metabolite compounds such as alkaloids, flavonoids, phenolic compounds. These compounds are considered to have contributed to the inhibition of the bacterial growth (Munfaati et al 2015).

This study revealed that ethyl acetate extract of coffee leaves has a good potential for inhibiting the growth of pathogenic bacteria in fresh-water fish, namely *E. tarda* and *S. agalactiae*.

Conclusions. The current antibacterial study on different solvent extracts of *C. canephora* leaves informed that the ethyl acetate extract from coffee leaves has a higher antibacterial activity than the hexane extract, against *E. tarda* and *S. agalactiae*. The difference of inhibitory activity is related to the extracted phytochemical content into the

solvent. Therapeutic efficacy of coffee leaves extract was demonstrated in fresh-water fish infections, especially for edwardsiellosis and streptococcosis treatment. Further investigations are needed to isolate and characterize the active antimicrobial phytochemical compound on ethyl acetate extract. A comprehensive analysis of the safety and toxicity of the coffee leaves extract in fish is also required.

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Authors:
Hapsari Kenconojati, Airlangga University, Faculty of Fisheries and Marine, Campus Banyuwangi, Banyuwangi, Indonesia, e-mail: hapsari@fpk.unair.ac.id
Mohammad Faizal Ulkhaq, Airlangga University, Faculty of Fisheries and Marine, Campus Banyuwangi, Banyuwangi, Indonesia, e-mail: m-faizalulkhaq@fpk.unair.ac.id
Muhammad Hanif Azhar: Airlangga University, Faculty of Fisheries and Marine, Campus Banyuwangi, Banyuwangi, Indonesia, e-mail: hanifazhar@fpk.unair.ac.id
Darmawan Setia Budi, Airlangga University, Faculty of Fisheries and Marine, Campus Banyuwangi, Banyuwangi, Indonesia, e-mail: darmawansetiabudi@fpk.unair.ac.id

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