BIOVALENTIA: BIOLOGICAL RESEARCH JOURNAL
***e*-ISSN: 2477-1392**
Vol. 8 No. 1, May 2022

Antibacterial Activities of Ciherang Rice (*Oryza sativa* L. Var. Ciherang) Ethanol Extract against *Enterotoxigenic Escherichia coli* (ETEC)

Dinda Sari[1], Bening Fitri Rini[1], Alifia Anisya[1], Feby Oktavia[1], Ali Zainal Abidin[1], Marieska Verawaty[1]*

Sriwijaya University, Faculty of Science, Department of Biology, Jalan Raya Palembang-Prabumulih km 32, Indralaya, South Sumatra 30662, Indonesia.

*Corresponding author

E-mail address: marieskaverawaty@unsri.ac.id (Marieska Verawaty).
Peer review under responsibility of Biology Department Sriwijaya University

Abstract

Enterotoxigenic Escherichia coli (ETEC) is one of the most common bacteria that cause a large number of diarrhea in infants, children and adults living in developing countries. Potential activity of Ciherang rice's (Oryza sativa L. var. Ci-herang) leaves' extract as antibacterial agent against the ETEC was investigated in this research. The secondary metabolite was extracted, its antibacterial activity, and its minimum inhibitory concentration (MIC) against the ETEC were determined. This study indicated the leaf extract of Ciherang rice in the ethanol fraction contained tannins, saponins, flavonoids, alkaloids, and terpenoids. It showed strong antibacterial activity against ETEC (at concentration of 100%); the extract activity test had diameter of inhibition zone around 11.12 mm with MIC value of 11.12 mm. This study suggested the ethanol leaf extract at various concentrations showed significant effect ($p<0.05$) toward the diameter of the inhibition zone. The Ethanol leaves extract of Ciherang rice at 100% of concentration has a MIC value of 11.37 mm.

Keywords : Antibiotic, Ciherang Rice, Diarrhea, ETEC, Minimum Inhibitory Concentration

Received: November 27, 2021, Accepted: May 27, 2022

1. Introduction

Indonesia is one of the world's largest rice producing countries after China and India. Its rice production increased by 1.00% in year 2020, which was 31.63 million tons, compared to the year of 2019 which was 31.31 million tons [1], make rice become potential source of some of the valuable metabolites product for many industrial purposes.

Diarrhea is still one of the main causes of children under-five mortality in Indonesia. It is the second-largest cause of death in children aged below five, which is around 525,000 people with 1.7 billion cases every year [2]. This infectious digestive tract infection was caused by food or drink contaminated with bacteria, viruses and parasitic organisms. Diarrhea can cause serious problem and life-threatening if it is not treated immediately [3], [4].

Types of *Escherichia coli* can cause diarrhea, one of them is the Enterotoxigenic *Escherichia coli* (ETEC); it is known as the common cause in travelers in developing countries [5], [6].

Antibiotics are widely used to treat diseases including diarrhea caused by *Escherichia coli*; however, currently, there is an increasing trend of antibiotics resistance bacterial cases worldwide, including for combating diarrheal diseases. Therefore, exploration for new antibiotics candidates from natural compounds extracted from plants and microbial sources are still highly in demand [7]. This study, focused on the exploration of the Ciherang rice (*Oryza sativa* L. Var. Ciherang) leaf extract for its metabolite activity against ETEC. This rice is one of Indonesian staple food. Some studies suggested a unique characteristic of the rice such as secondary metabolites which potential for health supplements and antimicrobial products such as its tannins and flavonoids [8]. In relation to that, there is still limited information about potency of the Ciherang rice (*Oryza sativa* L. Var Ciherang) leaf extract antibacterial activity, especially against Enterotoxigenic *Escherichia coli* (ETEC).

2. Materials and Methods

2.1 Preparation and Extraction of Ciherang Rice Leaves (*Oryza sativa* L. Var. Ciherang) Crude Extract

Ciherang leaves (Oryza sativa L. Var. Ciherang) used in this study was obtained from the Agrotech Training Center (ATC) field Faculty of Agriculture, Sriwijaya University. Crude extraction of plant metabolite was done according to Sarker et al [9]; the crude extract preparation was done by measuring the leaves (1 kg) and then air dried for 4 days. The dried leaves were mashed by using a blender to obtain simplicia (dry powder). The simplicia was then macerated using ethanol solvent and filtered. The solvent was evaporated using a rotary evaporator to obtain ethanol crude extract.

2.2 Qualitative Screening of Secondary Meta-bolic Compounds of the Leaves Extract

Qualitative screening of the metabolites was done according to method done by Safeena and Kalinga 2020 [10] for testing flavonoid, tannin, terpenoid, steroid, alkaloid and saponin as follows: Flavonoid test: 2 mL of extract was put into a test tube, then 5 ml of 30% methanol was added. Then it was heated at 50^0C for 5 minutes. The formed filtrate was added with 3 drops of concentrated sulfuric acid. The presence of flavonoids is indicated by the formation of a red or yellow color precipitate [5, 25]. Tannin test: 2 mL of extract was put into a test tube, and then added 5 drops of 1% (w/v) FeCl3 solution.

The presence of tannins was indicated by a change in the color of the extract to dark blue or black [1]. Terpenoid and Steroid Test: These tests were done according to Lieberman-Burchad, the method described in Parbuntari et al [11]; 2 mL of extract was put into a test tube, then 1 mL of glacial acetic acid and 1 ml of concentrated sulfuric acid were added. The presence of terpenoids was indicated by a change in the color of the extract to red or purple, while the presence of steroids was indicated by a green or blue color. Alkaloid Test: This test was done according to Method of Culvenor-Fitzgeraid described in Parbuntari et al [11]; 2 mL of extract was put into a test tube, then added with 10 mL of chloroform and 3 drops of ammonia.

The chloroform fraction obtained was separated and acidified with 2 drops of H2SO42 M. The acid fraction was divided into 3 tubes and each tube was added with 3 drops of Dragendorf, Meyer, and Wagner reagents. The presence of alkaloids is indicated by the formation of white precipitate, red precipitate, and brown precipitate. Saponin test: 2 mL of extract was put into a test tube, then added with 5 mL of distilled water, then heated for 5 minutes. Then the extract was shaken for 5 minutes. The presence of saponins was indicated by the formation of a stable foam after being left for 10 minutes Parbuntari et al [11].

2.3 Bacterial Suspension Culture Preparation

Enterotoxigenic Escherichia coli (ETEC) isolate was obtained from the Microbiology Laboratory, Faculty of Medicine, University of Indonesia. The ETEC was suspended into a test tube containing 6 ml of 0.9% NaCl solution and vortexed for 2 minutes. The obtained suspension turbidity was then visually compared with a standard McFarland 0.5 solution which was equivalent to 1.5 x 108 CFU/mL [12], [13].

2.4 Antibacterial Activity Test and Determination of Minimum Inhibition Concentration (MIC)

Antibacterial activity test and its MIC were conducted in accordance with Huey The et al., 2017 [13]. ETEC suspension was re-grown in Mueller Hinton Agar (MHA) media. A total of 0.1 mL suspension was inoculated into the Nutrient Agar (NA) medium in each petridish and spread evenly using a Drigalsky spatula let dried. After that, the disc papers were dripped into each of the extract concentration (25%, 50%, 75%, 100%), and also 0.5% of levofloxacin as a positive control. Dimethyl sulfoxide (DMSO) was used as a negative control (10 µL). Each of the petridishes was incubated for 24 hours at 37ºC. After being incubated for 24 hours, the inhibition zone formed was measured.

The highest and strongest antibacterial activity for the ethanol extract against the tested bacteria was subjected to serial dilution using sterile DMSO. Each of serial dilution concentration was dripped as much as 10 µL into a paper disc and directly attached to the MHA medium contained the tested grown bacteria. Then they were incubated at 37°C for 24 hours. The inhibition zone formed was observed and subjected to diameter measurement.

2.5 Experiment Design and Data Presentation

The research design carried out in this study was a Completely Randomized Design (CRD). Data analysis was carried out using SPSS 16 Software. Data for calculating the total number of Enterotoxigenic *Escherichia coli* (ETEC) and antibacterial activity tests were analyzed using ANOVA at $\alpha = 0.05$. If the treatment has a significant effect, it will be continued with Duncan's further test at $\alpha = 0.05$. In this study, the data collection from the results was presented descriptively and displayed in the form of tables and figures. The data obtained from the results of the antibacterial activity test and the MIC value determination test was processed to obtain the average value and standard deviation value.

3. Results and Discussion

Secondary Metabolites of Ciherang Rice Leaf

Based on the qualitative screening test that has been carried out, the Ciherang rice leaf extract has several secondary metabolites, which can be seen in Table 1. It is known that Ciherang rice leaf extract contains tannin, saponin, flavonoid, alkaloid, and terpenoid.

Table 1. Qualitative Screening Results of the Ciherang Rice Leaf Extract Secondary Metabolites

Testing Method	Secondary Metabolites	Test Results
Qualitative	Tannin	+
Qualitative	Saponin	+
Qualitative	Flavonoid	+
Qualitative	Terpenoid	+
Qualitative	Steroid	-
Qualitative	Alkaloid	+

Positive result in the tannin test were indicated by a change in the color of the Ciherang rice leaf extract to dark blue or black, while for the saponin test were indicated by the formation of stable foam on the rice leaf extract after being left for 10 minutes. For the flavonoid test, it was indicated by the formation of red or yellow precipitates on the Ciherang rice leaf extract after reacting with concentrated hydrochloric acid. Lastly, the terpenoid positive test was signed by the change in the color of the extract to red or purple.

The negative result of the steroid test were indicated by the absence of a change in the Ciherang rice leaf extract to green or blue. This happens because of the use of ethanol as a solvent which is polar, while steroid is a non-polar compound. Positive alkaloid test result were indicated by the formation of a white precipitate with the addition of Mayer's reagent, a red precipitate with the addition of Dragendorff's reagent, and a brown precipitate with the addition of Wagner's reagent.

Antibacterial Activity Test of Ciherang Rice Leaf Extract

The results of the antibacterial activity of Ciherang rice leaf extract can be seen in Table 2. and Figure 1. The diameter of the inhibition zone ranges from 11.12 mm to 2.62 mm. Ciherang rice leaf extract at a concentration of 100% was categorized as having strong activity, with a zone of inhibition diameter of 11.12 mm. The criteria for antibacterial activity were based on the diameter of the inhibition zone, namely >20 mm (very strong), 11-20 mm (strong), 6-20 mm (moderate), 5 mm (weak) [14], [15]. The ANOVA results showed that Ciherang rice leaf extract at various concentrations had a significant effect ($p<0.05$) on the diameter of the inhibition zone.

Study showed tannin have the ability to inhibit the adhesion of ETEC to intestinal epithelial cells and inhibit the formation of biofilms. Biofilms facilitate the exchange of plasmids containing antibiotic resistance genes, so those genes inactivate antibiotics and reduce the ability to penetrate antibacterial compounds into bacterial cells [17]. Tannins inhibited the formation of biofilms by ETEC at 25 °C and 37 °C [18].

Table 2. Antibacterial Activity Test of Ciherang Rice Leaf Extract against Enterotoxigenic *Escherichia coli*

No	Ciherang Rice Leaf Extract	Inhibition Zone Diameter (mm)	Criteria for Antibacterial Activity
1	100%	**$11.12^{a} \pm 0.94$**	**Strong**
2	75%	$8.75^{b} \pm 0.64$	Moderate
3	50%	$6.12^{c} \pm 0.85$	Moderate
4	25%	$2.62^{d} \pm 1.93$	Weak
5	Levofloxacin (Positive Control)	$35.37^{e} \pm 1.31$	Very Strong
6	DMSO (Negative Control)	$0^{f} \pm 0$	Weak

The results of Duncan's test showed that Ciherang rice leaf extract at various concentrations had a significant effect on the diameter of the inhibition zone. The difference in Duncan's test results is thought to be related to the concentration of Ciherang rice leaf extract. The higher the concentration of ciherang rice leaf extract, the higher the antibacterial activity, indicated by the formation of a wide diameter of inhibition zone around the paper disc. This is related to the large amount of bioactive compounds contained in the Ciherang rice leaf extract. According to Mulyatni *et al*. (2012) [16], the ability of antibacterial to inhibit bacterial growth is directly proportional to the concentration of the sample extract.

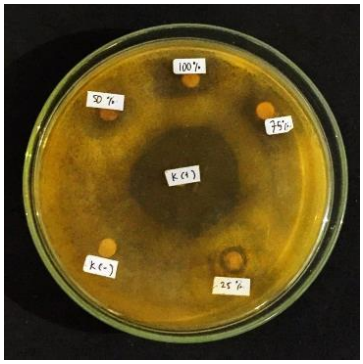

Figure 1. Antibacterial Activity Test of Ciherang Rice Leaf Extract against Enterotoxigenic *Escherichia coli*

The saponin's antibacterial activity is believed to be related to its ability in damaging the bacterial cell walls and in disrupting the stability and permeability of ETEC cell membrane. The saponin also known for its ability for reducing the surface tension of the ETEC cell walls and result in the disruption of stability of the cell membrane [19]. Flavonoid was reported to cause physiology disturbances in ETEC cells. It is affecting the cell through these three inhibition mechanisms such as in nucleic acid synthesis, membrane function, and metabolic processes [20]. One of study also reported that it can inhibit DNA gyrase activity during replication and transcription processes [21]; The alkaloid also reported can cause the DNA synthesis disruption through topoisomerase enzyme intervention [5]. Lastly, triterpenoid was known to can damage porins in the ETEC cell wall, and cause the growth inhibition and further the death of the cell [22].

Determination of Minimum Inhibitory Concentration (MIC) Ciherang Rice Leaf Extract

Table 3. Minimum Inhibitory Concentration of Ciherang Rice Leaf Extract against Enterotoxigenic *Escherichia coli*

Ciherang Rice Leaf Extract	Inhibition Zone Diameter (mm)
100%	11,37 ± 1,25
50%	6,25 ± 0,64
25%	2,87 ± 1,65
12,5%	0,75 ± 0,64
6,25	0,00 ± 0,00
Levofloxacin (Positive Control)	36,12 ± 0,85
DMSO (Negative Control)	0,00 ± 0,00

This study indicated the higher the leaf extract, the the higher the inhibition activity will be (Table 3). The extract concentration of 100% has the highest MIC value of 11.37mm. This result inline with other studies that also indicated the higher the concentration of ciherang rice leaf extract, the higher the antibacterial activity [16,[23].

4. Conclusion

This study showed qualitative phytochemical screening of secondary metabolites from Ciherang rice of ethanol leaves extract contained tannins, saponins, flavonoids, alkaloids, and terpenoids. The the the higher the inhibition activity will be (100% extract with 11.12 mm of inhibition zone diameter). The ethanol extract showed a significant effect ($p<0.05$) toward the inhibition zone diameter.

5. Acknowledgements

This research is supported by the Ministry of Education and Culture (Kemendikbud) Republik Indonesia (RI) under the Student Creativity Program 2021 (Program Kreativitas Mahasiswa), and our sincere appreciation to PKM reviewers who have evaluated our research so that this research is qualified to be published.

References

[1]. BPS Kab Bandung, "Harvest Area and Rice Production in Indonesia 2020," 2020. [Online]. Available:https://www.bps.go.id/pressrelease/2020/10/15/1757/luas-panen-danproduksi-padi-pada-tahun-2020-mengalami-kenaikan-dibandingkan-tahun2019-masing-masing-sebesar-1-02-dan-1-02-persen-.html.

[2]. N. Ribek, "Gambaran Perawatan Anak Diare Di Puskesmas Provinsi Bali," *Ejournal.Poltekkes-Denpasar.Ac.Id*, vol. 13, no. 1, pp. 21–28, 2020, [Online]. Available: http://ejournal.poltekkes-denpasar.ac.id/index.php/JGK/article/view/1139.

[3]. WHO, "Diarrhoeal Disease," 2017. https://www.who.int/news-room/factsheets/detail/diarrhoeal-disease.

[4]. J. Barker, "Diarrhea Symptoms: When Is It Something More Serious?," 2021. https://www.webmd.com/digestive-disorders/diarrhea-symptoms.

[5]. E. B. Hartadi, M. H. Effendi, H. Plumeriastuti, E. D. Sofiana, F. M. Wibisono, and A. R. Hidayatullah, "A review of enterotoxigenic *Escherichia coli* infection in piglets: Public health importance," *Systematic Reviews in Pharmacy*, vol. 11, no. 9, pp. 687–698, 2020, doi: 10.31838/srp.2020.9.100.

[6]. M. Stoppato *et al.*, "Oral administration of an anti-CfaE secretory IgA antibody protects against Enterotoxigenic *Escherichia coli* diarrheal disease in a nonhuman primate model," *Vaccine*, vol. 38, no. 10, pp. 2333–2339, 2020, doi: 10.1016/j.vaccine.2020.01.064.

[7]. N. Debora, W. C. Prabowo, A. Ibrahim, and L. Rijai, "Uji efek antidiare kombinasi ekstrak biji pepaya (*Carica papaya* L.) dengan daun kesumba keling (*Bixa orellana* L.) pada mencit (*Mus musculus*)," pp. 20–21, 2016, doi: 10.25026/mpc.v4i1.188.

[8]. R. Bocco, C. B. Gandonou, F. Gbaguidi, and A. C. Ahouansou, "Phytochemical screening and quantitative variation of some secondary metabolites in five cultivated rice varieties.," *Journal of Applied Biosciences*, vol. 113, pp. 11146–11157, 2017.

[9]. S. D. Sarker, Z. Latif, and A. I. Gray, *Natural Products Isolation*, 2nd ed. Totowa, New Jersey: Humana Press, 2006.
[10]. M. Safeena and J. Kalinga, "Qualitative and quantitative screening of secondary metabolites in selected medicinal plants of Sri Lanka," ~ *94* ~ *Journal of Medicinal Plants Studies*, vol. 8, no. 5, 2020.
[11]. H. Parbuntari, Y. Prestica, R. Gunawan, M. N. Nurman, and F. Adella, "Preliminary Phytochemical Screening (Qualitative Analysis) of Cacao Leaves (Theobroma cacao L.)," *EKSAKTA: Berkala Ilmiah Bidang MIPA*, vol. 19, no. 2, pp. 40–45, 2018, doi: 10.24036/eksakta/vol19-iss2/142.
[12]. S. Aryal, "McFarland Standards- Principle, Preparation, Uses, Limitations," 2021. https://microbenotes.com/mcfarland-standards/.
[13]. C. H. Teh, W. A. Nazni, A. H. Nurulhusna, A. Norazah, and H. L. Lee, "Determination of antibacterial activity and minimum inhibitory concentration of larval extract of fly via resazurin-based turbidometric assay," *BMC Microbiology*, vol. 17, no. 1, pp. 1–8, 2017, doi: 10.1186/s12866-017-0936-3.
[14]. W. W. Davis and T. R. Stout, "Disc plate method of microbiological antibiotic assay. II. Novel procedure offering improved accuracy.," *Applied microbiology*, vol. 22, no. 4, pp. 666–670, 1971, doi: 10.1128/aem.22.4.666-670.1971.
[15]. CLSI, *Performance Standards for Antibiotic Disk Susceptibility Tests: Approved Standard*, 11th ed. Wayne PA: CLSI, 2012.
[16]. A. S. Mulyatni, A. Budiani, and D. Taniwiryono, "Aktivitas antibakteri ekstrak kulit buah kakao (*Theobroma cacao* L.) terhadap *Escherichia coli*, Bacillus subtilis, dan Staphylococcus aureus," *E-Journal Menara Perkebunan*, vol. 80, no. 2, pp. 77–84, 2016, doi: 10.22302/ppbbi.jur.mp.v80i2.39.
[17]. D. Bakkiyaraj, J. R. Nandhini, B. Malathy, and S. K. Pandian, "The anti-biofilm potential of pomegranate (*Punica granatum* L.) extract against human bacterial and fungal pathogens," *Biofouling*, vol. 29, no. 8, pp. 929–937, 2013, doi: 10.1080/08927014.2013.820825.
[18]. D. Shao *et al.*, "Inhibition of Gallic Acid on the Growth and Biofilm Formation of *Escherichia coli* and *Streptococcus mutans*," *Journal of Food Science*, vol. 80, no. 6, pp. M1299–M1305, 2015, doi: 10.1111/1750-3841.12902.
[19]. K. Sudarmi, I. B. G. Darmayasa, and I. K. Muksin, "Uji fitokimia dan daya hambat ekstrak daun juwet (*Syzygium cumini*) terhadap pertumbuhan *Escherichia coli* dan *Staphylococcus aureus* ATCC," *SIMBIOSIS Journal of Biological Sciences*, vol. 5, no. 2, p. 47, 2017, doi: 10.24843/jsimbiosis.2017.v05.i02.p03.
[20]. R. P. Rijayanti, "Uji Aktivitas Antibakteri Ekstrak Etanol Daun Mangga Bacang," *Skripsi, Universitas Tanjungpura*, pp. 13–14, 2014.
[21]. N. Mufti, E. Bahar, and D. Arisanti, "Artikel Penelitian Uji Daya Hambat Ekstrak Daun Sawo terhadap Bakteri *Escherichia coli* secara In Vitro," *Artikel Penelitian*, vol. 6, no. 2, pp. 289–294, 2017.
[22]. H. Halimah, D. Margi Suci, and I. Wijayanti, "Study of the Potential Use of Noni Leaves (*Morinda citrifolia* L.) as an Antibacterial Agent for *Escherichia coli* and Salmonella typhimurium," *Jurnal Ilmu Pertanian Indonesia*, vol. 24, no. 1, pp. 58–64, 2019, doi: 10.18343/jipi.24.1.58.
[23]. R. A. Lolongan, O. Waworuntu., and C. N. Mintjelungan, "Minimum inhibitory concentration (MIC) test of water henna leaf extract (*Impatiens balsamina* L.) on the growth of *Streptococcus mutans*," *Jurnal e-GiGi*, vol. 4, no. 2, 2016.