REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: 06/01/2024 Revised: 19/01/2024 Accepted: 01/02/2024

In Vitro Effectiveness Evaluation of Eco-Enzyme to Inhibit the Growth of *Aspergillus sp.* Fungi that Isolated from Dog's Skin

Nevi[1], Hapsari Mahatmi[2] , Ketut Tono Pasek Gelgel[2], Putu Henrywaesa Sudipa[2*], Sri Kayati Widyastuti[3], I Nyoman Suartha[3]

[1]Student of Bachelor of Veterinary Medicine Study Program, Udayana University, Bali, Indonesia
[2]Laboratory of Veterinary Bacteriology and Mycology, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia
[3]Laboratory of Veterinary Internal Medicine, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia

Corresponding Author: Putu Henrywaesa Sudipa
*henrywaesa@unud.ac.id

ABSTRACT
Dermatitis is an inflammation of the skin that often occurs in dogs. The fungus of *Aspergillus sp.* is reported to be one of the causes of dermatitis. Eco-enzyme is a herbal substance believed to be used as an antifungal. The eco-enzyme in this study were made from neem leaves (*Azadirachta indica A. Juss*), papaya peels (*Carica papaya L.*), soursop peels (*Annona muricata L.*), and citronella (*Cymbopogon nardus L.*). This study aims to determine the inhibitory power of eco-enzyme in inhibiting the growth of *Aspergillus sp.* isolated from a dog's epidermis. This study used a quantitative method with modification of agar plate diffusion (Kirby Bauer) with well diffusion techniques. This experimental study used a completely randomized design with six treatments; eco-enzyme concentrations of 25%, 50%, 75%, 100%, positive control, and negative control. The effectiveness of eco-enzyme was determined by observing and calculating the inhibition zones formed on SDA media. Data analysis used ANOVA and continued with the Games- Howell test. The results showed that eco-enzyme made from neem leaf extract, papaya peels, soursop peels, and citronella effectively inhibited the growth of *Aspergillus sp.* at 25%, 50%, 75%, and 100%. It is necessary to test eco-enzyme effectiveness in dogs infected *with Aspergillus sp.* using *in vivo* testing to see the ability to inhibit its growth.

Keywords: *Aspergillus sp.*, eco-enzyme, concentration, inhibition zone

Introduction

Dermatitis is a skin disorder caused by various agents, one of which can be caused by a fungus. Fungal species that can cause dermatitis and can infect the respiratory system, namely *Aspergillus sp.* The fungus *Aspergillus sp.* is a normal flora often found and is included in opportunistic pathogenic fungi that harm patients with immune system disorders (1).. 53.3% of the dog population in Bali was positively infected with the fungus *Aspergillus sp.* (2). Modern dermatitis treatment in dogs generally uses topical and orally chemical-based antifungals, such as ketoconazole, miconazole, fluconazole, and itraconazole (3). Inappropriate use of chemical drugs, as well as irrational doses, can cause resistance. Resistance to antifungals is one of the causes of failure in the clinical treatment of fungal infections; other factors can also be caused due to weak immunity, fast drug metabolism, and low drug absorption rates (4). Based on this, herbal medicines can be recommended as an innovation to replace chemical-based antifungals. Eco enzymes are herbal medicines that can be antifungals (5). Eco-enzyme is a product of the fermentation of organic waste, water, and brown sugar. Eco-enzymes are in great demand because the manufacturing process is easy, economical, and environmentally friendly. Eco-enzymes can be used as antimicrobials because they have very high antimicrobial activity (6). The content of acetic acid (CH_3COOH) and specific enzymes contained in eco-enzymes can kill disease-causing microorganisms (7). Based on the background above, this research was conducted to know the effectiveness of eco-enzymes made from papaya peel (*Carica papaya L.*), soursop (*Annona muricata L.*), neem leaves (*Azadirachta indica A. Juss*), and fragrant citronella (*Cymbopogon nardus L*) on the growth of *Aspergillus sp.* isolated from dog skin ectodermal tissue in vitro.
Based on the above background, this research was conducted to know the in vitro effectiveness of eco enzymes made from papaya peel (*Carica papaya L.*), soursop (*Annona muricata L.*), neem leaves (*Azadirachta indica A. Juss*), and fragrant citronella (*Cymbopogon nardus L*) on the growth of *Aspergillus sp.* isolated from dog skin ectodermal tissue.

Methods

Research Object

The objects of this research were eco enzyme solutions (concentrations of 25%, 50%, 75%, 100%) and the fungus *Aspergillus sp.* isolated from dog skin in vitro.

Research design

This study uses an experimental method. The design was completely randomized with six treatments: eco-enzymes with

concentrations of 25%, 50%, 75%, and 100%, and itraconazole solution as a positive control and distilled water as a negative control. The concentration of eco-enzymes in this study was based on research by (5) that neem leaf extract could inhibit fungal growth at a concentration of 25%. The positive control used itraconazole because it is known to treat cutaneous aspergillosis caused by Aspergillus fumigatus (8), and the negative control used distilled water. Then each treatment received four repetitions. Determination of repetitions using the formula: t (r-1) ≥ 15, where t is the number of treatments and r is the number of repetitions sought (9).

Research Variables
The independent variables in this study were eco-enzymes with concentrations of 25%, 50%, 75%, and 100%, itraconazole solution as a positive control, and negative controls using distilled water. The dependent variable in this study was the formation of an inhibition zone around the wells given eco-enzymes against *Aspergillus sp.* The control variables in this study were the fungi growing media, incubation time, and *Aspergillus sp.*

Data Collection
Data was collected using the Mackenzie method by using a toothbrush rubbed over the skin area previously examined microscopically to identify the fungus *Aspergillus sp.* Then the toothbrush was rubbed several times on the Sabouraud Dextrose Agar medium. On the fourth day, the fungi will grow thickly in the media.

Preparing Sabouraud Dextrose Agar (SDA) Media
A weight of 9.1 grams of SDA is mixed with 200 ml of distilled water and put into the Erlenmeyer. Homogenize the media using a digital magnetic stirrer. Cover the homogeneous media using aluminum foil and then sterilize it using an autoclave at 121°C for 15 minutes. Pour the media into each 20 ml/petri dish. Wait for the media to solidify.

Preparing Ecoenzyme Concentration and pH evaluation
Make dilutions at each eco-enzyme concentration of 10 ml. The 25% eco-enzyme concentration is made by dissolving 2.5 ml eco-enzyme and 7.5 ml distilled water, 50% eco-enzyme concentration is made by dissolving 5 ml eco-enzyme and 5 ml distilled water, 75% eco-enzyme concentration is made by dissolving 7.5 ml eco-enzyme and 2, 5 ml of distilled water, 100% eco-enzyme concentration, no need for dissolution. Prepare a closed container, then put each eco-enzyme into the container. Use a pH meter to measure the pH at each eco-enzyme concentration by turning on the pH meter, inserting it into a container filled with eco-enzymes with various concentrations, then waiting for the pH meter until the number indicating pH stops.

Sensitivity Test Method
The method used in this study is modified agar plate diffusion (Kirby-Bauer), a direct sensitivity test method using the good diffusion technique (10). Labels were given to each petri dish according to the six treatments. Spread the fungi grown previously using a loop on SDA media. Then make a hole with a diameter of 5 mm on the SDA media using a cork borer. The holes were filled with eco-enzyme with different concentrations (25%, 50%, 75%, and 100%), itraconazole solution was given with a volume of 0.5 µL in the positive control hole, and distilled water in the negative control hole. Then incubate at room temperature for 24 hours, observe until an inhibition zone is formed, then measure the formed inhibition zone.

Sensitivity Test Observation
Observations were made by looking for the *Aspergillus sp.* around the wells that have been given eco-enzymes on Sabouraud Dextrose Agar media. Positive results were indicated by the formation of barriers around the wellbore, which were not overgrown by fungi. The inhibition zone formed can be measured using a vernier caliper in millimeter units (11).

Data Analysis
The data obtained from the calculations were analyzed using Analysis of Variance (ANOVA), followed by the Games-Howell test using the Statistical Product and Service Solutions (SPSS) version 26 application.

Result
The results of the effectiveness test showed that eco-enzymes could inhibit the growth of the *Aspergillus sp*. at concentrations of 25%, 50%, 75%, and 100 to produce an inhibition zone (figure 1) each of 6.73mm; 9.12mm; 9.67mm; 10.41mm and significantly different from the negative control ($P<0.05$), the measurement results can be seen in table 1.
Checking the pH on the negative control showed a pH of 7, and on the inhibition test, no barriers were formed on the SDA media. This is because the negative control uses distilled water which is distilled water that is free from compounds inhibiting fungal growth. The antifungal used as a positive control in this study was itraconazole.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: 06/01/2024 Revised: 19/01/2024 Accepted: 01/02/2024

Pharmacologically, itraconazole effectively inhibits fungal growth by reducing the synthesis of ergosterol, which can inhibit the formation of fungal cell membranes.

Table 1. In vitro diameter data of the ecoenzyme inhibition zone against *Aspergillus sp*. isolated from dog skin.

Treatment	Average Inhibit Zone (mm)
Control (-)	0^{a}
Eco-enzyme 25%	$6,73 \pm 0,58^{b}$
Eco-enzyme 50%	$9,12 \pm 0,32^{b}$
Eco-enzyme 75%	$9,67 \pm 0,41^{b}$
Eco-enzyme 100%	$10,41 \pm 0,22^{b}$
Control (+)	$11,43 \pm 0,26^{b}$

Different alphabets in a column indicate significant difference (P<0.05).

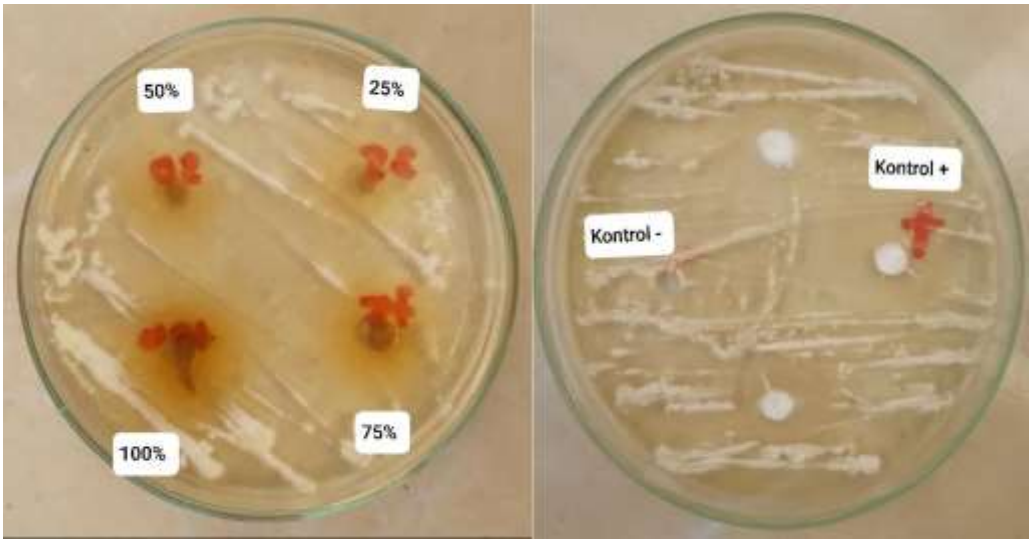

Figure 1. Ecoenzyme inhibition zone (25%, 50%, 75%, 100%, positive control and negative control on Sabouraud Dextrose Agar media).

Discussion

Eco-enzymes are effective in inhibiting *Aspergillus sp.* because it has the CH_3COOH compound, which can kill microorganisms such as fungi by damaging cell membranes and disrupting cell metabolic activities (7). The resulting pH determines the success of eco-enzyme products, that is, with a pH below 4 (12), while the fungus *Aspergillus sp.* optimal growth at pH 5 to 7 and lowest at pH 10 (13). This aligns with the results of checking the pH, namely eco-enzymes with a concentration of 25%: 50%; 75%; and 100%, respectively, showing a pH of 2.6; 2.5; 2.5; and 2,4. Apart from the pH, the inhibition zone formed in each treatment was due to the presence of active substances contained in eco-enzymes, such as alkaloids, flavonoids, steroids, saponins, tannins, citronellal, and geraniol, which can inhibit the growth of the Aspergillus sp.

The mechanism of action of alkaloid compounds contained in eco-enzymes is that they can inhibit fungal cell respiration (14), inhibit the synthesis of nucleic acids, proteins, and phospholipid membranes so that they interfere with the formation and function of these substances which lead to total cell damage. This follows (15) statement that the mechanism of antifungal action includes neutralizing enzymes that play a role in fungal invasion, damaging fungal cell membranes so that hyphal tips are not formed, and affecting nucleic acid and protein synthesis. Flavonoid compounds can cause cell permeability disturbances because of their ability to denature proteins by forming complex bonds (16). Disruption of fungal cell permeability causes damage to the plasma membrane so that the fungal cell membrane becomes lysis. The lysis of the cell membrane of the fungus *Aspergillus sp*. causes death in mushrooms (17). Steroid compounds are active compounds that can function as antifungals. These compounds are hydrophobic or lipophilic, which can result in the inhibition of spore germination and mycelium multiplication in fungi (18). Saponin compounds can cause cell membrane lysis. This affects cell stability due to losing important components in fungal cells, such as proteins, nucleic acids, and nucleotides (19). Tannin compounds can function as antifungals because they inhibit the synthesis of chitin, which is used to form cell walls in fungi. This compound can also damage cell membranes, inhibiting fungal growth (20). Citronellal and geraniol compounds can inhibit the metabolic processes of fungal cells, disrupting cell growth. If this compound is excessive, it will lead to cell death (21).

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No. 2 (2024)
http://www.veterinaria.org
Article Received: 06/01/2024 Revised: 19/01/2024 Accepted: 01/02/2024

Conclusion

Ecoenzyme effectively inhibits the growth of *Aspergillus sp.* at a concentration of 25% with a pH of 2.6 and an inhibition zone of 6.73mm, at a concentration of 50% with a pH of 2.5 and an inhibition zone of 9.12mm, at a concentration of 75% with a pH of 2.5 and an inhibition zone of 9.67mm, and a concentration of 100% with a pH of 2.4 and an inhibition zone of 10.41mm. Eco-enzyme concentrations of 25%, 50%, 75%, and 100%, and the positive control were significantly different from the negative control ($P<0.05$).

Acknowledgement

Thank you to the Dean of The Faculty of Veterinary Medicine and Institute for Research and Community Service Udayana University who have facilitated and funded this research with contract :B/78.141/UN14.4A/PT.01.03/2022.

References

1 Patterson, K., Strek, M.E. (2010). Allergic bronchopulmonary aspergillosis. *Proceedings of American Thoracic Society* 7(3): 237-44. doi: 10.1513/pats.200908-086AL. PMID: 20463254.

2. Sudipa, P. H., Puja, I. K., Dharmayuda, A. A. G. O. ., Gunawan, I. W. N. F., Sudimartini, L. M. ., Jayanti, P. D., Sukernayasa, I. W., & Mufa, R. M. D. (2023). Identification and Prevalence of Aspergillus sp Isolated from Bali Dog's Skin. *Jurnal Riset Veteriner Indonesia (Journal of The Indonesian Veterinary Research)*, *7*(1). https://doi.org/10.20956/jrvi.v7i1.21594
3. Dengen, C.L.L. (2017). Diagnosis dan Terapi Kasus Malasseziais pada Anjing Golden Retriever di Mutiara Pet Klinik Bandung Jawa Barat. [Skripsi]. Fakultas Kedokteran Hewan. Universitas Hasanudin.
4. Loeffler, J., Stevens, D.A. (2003). Antifungal Drug Resistance, *Clin Infect Dis*, 36 (1): S31- 41. doi: 10.1086/344658. PMID: 12516028
5. Putri, A.C.A., Suartha, I.N., Merdana, I.M., Sudimartini, L.M. (2018). Ekstrak Daun Mimba Efektif terhadap *Microsporum gypseum* yang Diisolasi dari Dermatitis pada Anjing. *Indonesia Medicus Veterinus* 7(6): 608-615. DOI: 10.19087/imv.2018.7.6.608.
6. Arifin, L.W., Syambarkah, A., Purbasari, H.S., Ria, R., Puspita, V.A. (2009). Introduction of Eco-enzyme to Support Organic Farming in Indonesia. *Asian Journal Of Food and Agro-Industry*, 357-358.
7. Mavani, H.A.K., Tew, I.M., Wong, L., Yew, H.Z., Mahyuddin, A., Ghazali, R.A., Pow, E.H.N. (2020). Antimicrobial Efficacy of Fruit Peels Eco-Enzyme against Enterpcoccus faecalis: An In Vitro Study. *International Journal of Environmental Research and Public Health* 17, 5107. doi: 10.3390/ijerph17145107. PMID: 32679828; PMCID: PMC7400228.
8. Setyaningrum, T., Pratiwi, K. D. (2019). Aspergillus fumigatus as an agent of cutaneous aspergillosis in immunocompetent patient: A rare case. *Dermatology Reports*, 11(s1). https://doi.org/10.4081/dr.2019.8089
9. Sampurna, I.P., Nindia, T.S. (2009). *Metodologi Ilmiah dan Rancangan Percobaan*. Fakultas Kedokteran Hewan: Universitas Udayana
10. Akeel, R.A., Mateen, A., Janardhan, K., Gupta, V.C. (2017). Analysis of Anti-bacterial and AntiOxidative Activity of Azadiractha indica Bark Using Various Solvent Extracts. *Saudi Journal of Biological Science* 24(1): 11-14. doi: 10.1016/j.sjbs.2015.08.006
11. Surjowardojo, P., Susilorini, T.E., Benarivo, V. (2016). Daya Hambat Dekok Kulit Apel Malang (Malus sylvestris Mill) Terhadap Pertumbuhan Escherichia coli dan Streptococcus agalactiae Penyebab Mastitis Pada Sapi Perah. *Ternak Tropikal 17(1)*: 11-21. Doi: https://doi.org/10.21776/ub.jtapro.2016.017.01.2
12. Win, Y.C. (2011). *Ecoenzyme Activating The Earth's Self-Healing Power*. Alih Bahasa: Gan Chiu Har. Malaysia: Summit Print SDN. BHD; 6, 8, 9-14
13. Hakim, L., Kurniatuhadi, R., Rahmawati. (2020). Karakteristik Fisiologis Jamur Halofilik berdasarkan Faktor Lingkungan dari Sumur Air Asin di Desa Suak, Sintang, Kalimantan Barat. Bioma: *Jurnal Biologi Makassar* 5(2) : 227-232. Doi: https://doi.org/10.20956/bioma.v5i2.11299
14. Aniszewki, T. (2007). Alkaloid-secrets of life: Aklaloid Chemistry, Biological Significance, Applications and Ecological Role. *Elsevier*, ISBN: 9780080475332.
15. Djunaedy, A. (2008). Aplikasi Fungisida Sistemik dan Pemanfaatan Mikoriza dalam Rangka Pengendalian Patogen Tular Tanah pada Tanaman Kedelai (Glycine max L.). *Journal Embryo*, 5(2) : 1-9.
16. Munawwaroh, R. (2016). Uji Aktivitas Antifungi Jamu Madura Empot Super terhadap Jamur Candida albicans. Fakultas Sains dan Teknologi, Universitas Islam Negeri Malang.
17. Febriani, TH. (2014). Uji Daya Antifungi Jus Buah Pare (Momordica Charantia L) terhadap Daya Hambat Pertumbuhan Candida Albicans secara In Vitro. [Skripsi]. Fakultas Kedokteran Gigi. Universitas Muhammadiyah Surakarta.
18. Natta LO, Krittika, Pantip. 2008. Essensial Oil from Zingiberaceae for Anti Food Borne Bacteria. *International Food Research Journal*, 15(3): 337-346.
19. Bayuaji, T., Astuti, I., Dhiani, B. (2012). Aktivitas Antifungi Krim Daun Ketepeng Cina (Senna alata L. Roxb.) Terhadap Trichophyton mentagrophytes. *PHARMACY: Jurnal Farmasi Indonesia (Pharmaceutical Journal of*

Indonesia), 9(03). doi:http://dx.doi.org/10.30595/pji.v9i3.745
20. Watson, R.R., Preedy, P.R. (2007). Bioactive foods in promoting health: probiotics and prebiotics. USA: Academic Press.
21. Nurmansyah. 2010. Efektifitas Minyak Sereh Wangi dan Fraksi Sitronellal terhadap Pertumbuhan Jamur Phytophthora Palmivora Penyebab Penyakit Busuk Buah Kakao. Balai Penelitian Obat dan Aromatik, 21(1): 43-52.