Research

Bioautography of dewandaru leaf ethanol extract thin-layer chromatography against *Pseudomonas aeruginosa*

I Wayan Lolik Lesmana, I Wayan Putu Sutirta Yasa, Ni Putu Oktaviani Rinika Pranitasari
Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine, Udayana University / Udayana University General Hospital, Badung, Indonesia

ABSTRACT

Background: Ear infections are a significant health concern due to their impact on hearing function. One ear infection that frequently occurs is Chronic Suppurative Otitis Media (CSOM), which is commonly caused by Pseudomonas aeruginosa. The development of therapies using natural ingredients appears to be a promising option for treating various infectious diseases. The potential of the Dewandaru leaves (Eugenia uniflora L.) has long been recognized, and several studies have demonstrated its antibacterial properties. Dewandaru leaves extract contains several active compounds with antibacterial activity, as evidenced by various in vitro studies. **Purpose:** To test the antibacterial activity of flavonoids, saponins, tannins, and terpenoids from Dewandaru leaves extract against American Type Culture Collection (ATCC) strain of *Pseudomonas aeruginosa,* ATCC 9027, as the most common cause of CSOM. **Method:** Antibacterial activity was assessed using the Thin-Layer Chromatography–Bioautography (TLC–bioautography) contact method using a clear area on the TLC plate. **Result:** Dewandaru leaf extract showed antibacterial activity against *Pseudomonas aeruginosa* with a significant zone of inhibition. The phytochemical substances in the extract that exhibit antibacterial activity were flavonoids. **Conclusion:** Dewandaru leaves had the potential to be a natural antibacterial agent against *Pseudomonas aeruginosa*.

Keywords: dewandaru, TLC-Bioautography, *Pseudomonas aeruginosa*

ABSTRAK

Latar belakang: *Infeksi telinga merupakan permasalahan kesehatan yang signifikan karena dampaknya terhadap fungsi pendengaran. Penyakit infeksi telinga yang umum terjadi adalah Otitis Media Supuratif Kronis (OMSK), yang seringkali disebabkan oleh Pseudomonas aeruginosa. Perkembangan terapi menggunakan bahan alami menunjukkan manfaat yang menjanjikan untuk menatalaksana berbagai penyakit infeksi. Potensi daun Dewandaru (Eugenia uniflora L.) telah diketahui sejak lama, dan beberapa studi telah membuktikan sifat antibakteri yang dimiliki oleh daun tersebut. Ekstrak daun Dewandaru mengandung beberapa zat aktif dengan aktivitas antibakteri yang dibuktikan melalui berbagai studi in vitro.* ***Tujuan:*** *Untuk menguji aktivitas antibakteri dari flavonoid, saponin, tanin, dan terpenoid, dari ekstrak daun Dewandaru terhadap* American Type Culture Collection (ATCC) ATCC *9027 (Pseudomonas aeruginosa), sebagai penyebab yang paling sering dari OMSK.* ***Metode:*** *Aktivitas antibakteri dinilai melalui metode kontak Thin Layer Chromatography-Bioautography (TLC-Bioautography) menggunakan area bersih pada* plate *TLC.* ***Hasil:*** *Ekstrak daun Dewandaru menunjukkan aktivitas anti-bakteri terhadap Pseudomonas aeruginosa dengan zona inhibisi yang signifikan. Zat fitokimia dalam ekstrak daun Dewandaru yang menunjukkan aktivitas antibakteri adalah flavonoid.* ***Kesimpulan:*** *Daun Dewandaru memiliki potensi sebagai unsur antibakteri alami terhadap Pseudomonas aeruginosa.*

Kata kunci: *dewandaru, biootografi* TLC, *Pseudomonas aeruginosa*

Correspondence address: I Wayan Lolik Lesmana. Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine Udayana University / Udayana University General Hospital. Email: Loliklesmana22@gmail.com

INTRODUCTION

Ear infections are a significant health issue and are a primary concern due to their impact on hearing function.[1] A common ear infection in Indonesia is Chronic Suppurative Otitis Media (CSOM), characterized by ear inflammation with perforation of the eardrum, with or without ear discharge, lasting more than two months.[1,2] The World Health Organization (WHO) reports that Indonesia has a high morbidity rate for CSOM, approximately 2-4%.[3] Research conducted in several provinces in Indonesia in 1996 found that the percentage of CSOM infections was 3.8%.[2,4]

Chronic Suppurative Otitis Media can result from bacterial or fungal infections. The most common bacterial causes of CSOM are *Pseudomonas aeruginosa* (22-44%), *Staphylococcus aureus* (17-37%), and *Klebsiella pneumoniae* (4-7%).[5] The management of CSOM involves eradicating the causative bacteria, typically through antibiotic ear drops. However, these are recommended only for short-term use, less than two weeks, due to the risk of ototoxicity.[5,6]

The development of herbal therapies using natural ingredients appears to be a promising option for treating various infectious diseases in Indonesia. The potential of the Dewandaru leaves has long been recognized, and several studies have demonstrated its antibacterial properties.[7] Research by Lesmana (2023) highlighted the potential of Dewandaru leaf extract (*Eugenia uniflora L.*) against *Pseudomonas aeruginosa*.[7,8] However, no study has yet identified the active compounds responsible for its antibacterial activity.

This study was a follow-up investigation into the benefits of Dewandaru leaves extract for treating ear, nose, and throat infections. The results are expected to provide a foundation for developing relatively safe and effective treatments for CSOM.

METHOD

This was an experimental laboratory study on the antibacterial activity and bioautographic thin-layer chromatography (TLC) analysis of active compounds such as flavonoids, alkaloids, saponins, tannins, and terpenoids from the Dewandaru leaves (*Eugenia uniflora L.)* against *Pseudomonas aeruginosa*. The study was conducted from March 2024 until September 2024 at the Pharmacy Laboratory of the Faculty of Mathematics and Natural Sciences and the Microbiology Laboratory, of the Faculty of Medicine, Udayana University.

The research sample was the American Type Culture Collection (ATCC) strain of *Pseudomonas aeruginosa* (ATCC 9027), which causes CSOM. The ATCC *Pseudomonas aeruginosa* bacterial culture was obtained from the Microbiology Laboratory of the Faculty of Medicine, Udayana University.

Herbal material and extract preparation

Dewandaru leaves (*Eugenia uniflora L.*) were collected from local farmers in Bali, specifically from Menanga village, Rendang, Karangasem, Bali.

A total of 2.21 kg of Dewandaru leaves was processed through several steps, including wet sorting, washing, draining, shredding, drying, dry sorting, and powdering. This process resulted in 600 grams of Dewandaru leaf powder. The powder was then macerated with 96% ethanol as the solvent, with a ratio of 1:5. The maceration lasted for 3 days, with stirring for 15 minutes on the first and second days. On the third day, the mixture was

filtered, and the filtrate was further processed by evaporation using a rotary evaporator for 120 minutes. This process resulted in 80 grams of concentrated Dewandaru leaves extract.

Preparation of agar media

Thirty-eight grams of *Mueller Hinton* powder was mixed with 1 liter of sterile distilled water, and then sterilized in an autoclave at 121°C for 15 minutes. The agar solution was poured into 100 mm petri dishes, each receiving 20 mL, until it solidified. Afterward, the petri dishes were placed in an incubator at 37°C for 18-24 hours to perform quality testing of the medium. The medium was deemed suitable for use if no contamination, shown by the presence of colonies, was detected after the incubation period.

Preparation of bacterial suspension

To prepare the test bacteria, a bacterial suspension of *Pseudomonas aeruginosa* (ATCC 9027) was created to match a 0.5 McFarland standard. This suspension was then evenly swabbed across all four sides of the agar medium using a cotton swab.

Phytochemical testing of Dewandaru leaves extract

Silica gel plate 60-F_{254} were used which were activated using a TLC heater for 10 minutes at 110 °C, to eliminate moisture. Next, the plates were spotted with5 µL, 10 µL, and 20 µL of Dewandaru leaves extract (three plates for each volume), positioned 1 cm from the bottom of the plate using an Automatic TLC Sampler.

The first, second, and third plates were placed in a chamber filled with elution solvent and allowed to saturate for 30 minutes. The eluents used included toluene, ethyl acetate, and diethylamine or ammonia (70:20:10) for alkaloids; ethyl acetate, formic acid, acetic acid, and water (100:11:11:26) for flavonoids; and chloroform, ethanol, and water (70:30:4) for saponins. The test was stopped after elution reached 1 cm from the top edge of the plate. The plates were then air-dried and observed under UV light, at 254 nm and 366 nm.

To visualize the active compounds from the Dewandaru leaves extract, the surface of the plates was sprayed with Aluminum Chloride (AlCl3) reagent for flavonoids, ammonia vapor for tannins, and anisaldehyde sulfuric acid reagent for saponins and triterpenoids; observed under UV light at 254 nm and 366 nm. The second and third plates, which did not receive the spray, were later tested for antibacterial potency using the Thin-Layer Chromatography (TLC) bioautography contact method against *Pseudomonas aeruginosa* (ATCC 9027) in vitro.

Contact bioautography

The antibacterial activity test of the active compounds in Dewandaru leaves extract (flavonoids, tannins, saponins, and terpenoids) was performed using contact bioautography method. The plates coated with Dewandaru leaves extract (*Eugenia uniflora L.*) were sterilized with UV light. The underside of the plates was cleaned with 70% alcohol to eliminate bacterial contamination.

The agar medium was sterilized in an autoclave at 121°C for 15 minutes, and then allowed to cool properly. After cooling, a suspension of *Pseudomonas aeruginosa* was applied to the agar medium using a cotton swab. The chromatographic plates, which contain flavonoid, alkaloids, saponin, tannin, and terpenoid compounds from the Dewandaru leaf extract, were placed on the medium with the inner side of the plate facing down, to ensure good contact with the agar surface. The plates were then refrigerated for 15–30 minutes before being removed from the agar medium. After that, the Petri dishes were incubated for 24 hours at 37°C, and the clear zones on the plates were examined to assess antibacterial activity.

Antibacterial activity of active components of Dewandaru leaves extract

Data were presented descriptively regarding the clear zones indicating the inhibitory effect of active compounds flavonoids, alkaloids, tannins, saponins, and terpenoids from ethanol extract of Dewandaru leaves (*Eugenia uniflora L.*) on the *Mueller Hinton* agar against the *Pseudomonas aeruginosa* (ATCC 9027).

RESULT

Results of phytochemical screening of Dewandaru leaf extract *(Eugenia uniflora L.)*

The extraction process from Dewandaru leaves (*Eugenia uniflora L.*) resulted in 80 grams of concentrated extract. Phytochemical analysis of this extract showed the presence of several compounds, such as alkaloids, saponins, flavonoids, tannins, and terpenoids. These findings were determined through color changes observed after the addition of specific reagents, as detailed in Table 1.

Table 1. Results of phytochemical screening of Dewandaru leaf extract (*Eugenia uniflora L.*)

Parameter	Reagent	Result	Description
Flavonoids	Ammonia vapor	+	Yellow-green color under white light
	Citrobore reagent	+	Yellow color under UV light (366 nm)
	AlCl3	+	Yellow color under white light
Alkaloids	Wagner's reagent	+	Light brown color under white light
	Dragendroff	-	No brown-orange color under white light
Saponins	Lieberman-Burchard reagent	+	Blue color under UV light (366 nm)
	1% Vanillin-sulfuric acid	+	Blue color under UV light (366 nm)
Tannins	Ammonia vapor	+	Purple color under UV light (366 nm)
	2% $FeCl_3$	+	Blue to brown color under white light
Terpenoids	Anisaldehyde-sulfuric acid	+	Purple-red color under UV light (366 nm)
	1% Vanillin-sulfuric acid	+	Purple color under UV light (366 nm)

Thin-Layer Chromatography contact method test for *Pseudomonas aeruginosa*

Dewandaru leaves extract (*Eugenia uniflora L.*) was fractionated using a suitable eluent for the tested compounds. The plate with the test compounds were then evaluated for antibacterial activity against *Pseudomonas aeruginosa* (ATCC 9027) in vitro through contact bioautography.

Thin-Layer Chromatography (TLC) of active compound in Dewandaru leaves extraxt

The TLC plates with flavonoids, alkaloids, saponins, tanins, and terpenoid compounds extracted from Dewandaru leaves (*Eugenia uniflora L.*) were analyzed using a bioautographic TLC assay through the contact method against the test bacterium *Pseudomonas aeruginosa* (ATCC 9027). The results were illustrated in Figure 1. In the figure, a clear zone was visible only on the flavonoid plate with 10 µl (Figure 1.D) and 20 µl (Figures 1.E and 1.F), as highlighted by the red box.

No clear zones were visible on the plates containing alkaloids, saponins, tannins, and terpenoids compounds from the extract of Dewandaru leaves (*Eugenia uniflora L.*) for any volumes.

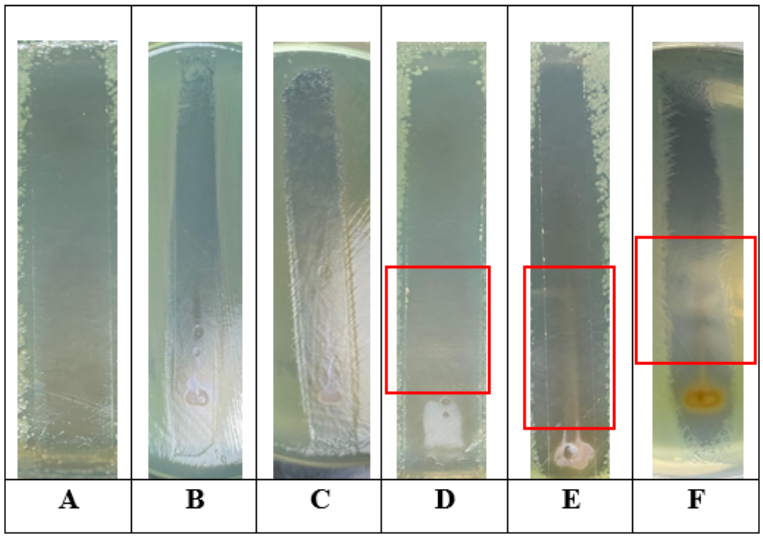

Figure 1. TLC Bioautography assay using the contact method of flavonoid compounds from ethanol extract of Dewandaru leaves against Pseudomonas aeruginosa. (A) and (B) TLC bioautography plates with 5 µl flavonoid, (C) and (D) TLC bioautography plates with 10 µl flavonoid, (E) and (F) TLC bioautography plates with 20 µl flavonoid

DISCUSSION

The development of therapies using natural ingredients appears to be a promising option for treating various infections. The extraction of bioactive compounds from the plant matrix depends on factors such as the chemical structure of the compounds and the solvents used.[9] The potential of Dewandaru leaves (*Eugenia uniflora L.*) has long been recognized, and several studies had demonstrated their antibacterial potency. The extract of Dewandaru leaves contains several active compounds with antibacterial activity, as evidenced by various in vitro studies.[7]

The test results showed that the extract of Dewandaru leaves (*Eugenia uniflora L.*) contained flavonoids, alkaloids, saponins, tannins, and terpenoids. This was based on the research findings by the yellow color, observed under white light on the flavonoid chromatogram after adding AlCl3 reagent. Alkaloid compounds were identified using Wagner's reagent, which resulted a brown color. Saponin identification was carried out with Liebemann-Buchard reagent, leading to a blue color. Tannin identification was performed using ammonia vapor, resulting in a purple color under UV 366 nm, with a positive result indicated by a blue to brown color under white light with 2% $FeCl_3$ reagent. Terpenoids were positive with anisaldehyde-sulfuric acid reagent, producing a reddish-purple color under UV 366 nm, and a purple color under UV 366 nm with 1% vanillin-sulfuric acid reagent. Phytochemical screening of Dewandaru leaves extract had revealed the presence of flavonoids, alkaloids, saponins, tannins, and terpenoids, as previously reported by Lesmana (2023) for this species.[8]

Thin-Layer Chromatography (TLC)-bioautography test was performed on the Dewandaru leaf extract against the *Pseudomonas aeruginosa* to found the compounds that contribute antibacterial activity. The contact bioautography method was used for this research, as it yielded reliable results and facilitated the effective transfer of antibacterial components from the chromatogram to the agar medium.[10] -

The results of the Thin-Layer Chromatography (TLC) bioautography test in a study showed an inhibition zone against the growth of *Pseudomonas Aeruginosa*, indicated by a clear area on the flavonoid chromatogram. Several factors influence the antibacterial activity observed through the TLC-bioautography method. Poor bacterial culturing and nutrient conditions could inhibit bacterial growth on the Petri dish medium.[11]

Considering the chemical composition of Dewandaru leaves, flavonoids were the metabolites of particular interest.[7] Flavonoids were found in many plant extracts, and were frequently the focus of pharmacological studies. Despite their well-documented antioxidant activity, they also exhibit numerous other properties, including anti-inflammatory, anti-aging, anti-cancer, anti-allergic, hypocholesterolemic, and antibacterial.[12] Flavonoids are the major components of Dewandaru leaves, have garnered significant interest because they can disrupt bacterial membranes and deplete essential substrates. The mechanisms by which flavonoids act as antibacterial can be categorized into three main actions: inhibiting nucleic acid synthesis, disrupting cell membrane function, and inhibiting energy metabolism.[13] The Gram-negative strain *Pseudomonas aeruginosa* exhibits differences in cell wall peptidoglycan content, resulting in a unique composition contributing to its intrinsic resistance to most antibiotics.[14] These findings suggest that flavonoids may compromise the cell membrane of P. aeruginosa, a process influenced by the low permeability of the outer membrane of this bacterial strain.[13]

In conclusion, Dewandaru leaves (*Eugenia uniflora L.)* contain active flavonoid compounds that may serve as effective natural antibacterial agents against *Pseudomonas aeruginosa.*

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