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TO INVESTIGATE THE ANTIBACTERIAL AND ANTIBIOFILM EFFICACY OF *URARIA PICTA* AND ITS ADULTERANT SPECIES AND THEIR EFFECTIVENESS AGAINST MULTIDRUG-RESISTANT BACTERIA

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Keywords:

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ABSTRACT: An ayurvedic nutritional tonic, *i.e.*, Dashmularishta, is made up of ten different plants that support gut health and restore appetite by relieving indigestion, bloating, *etc*. *U. picta* is one of the important medicinal plant used in this formulation. Due to ever increasing demand *U. picta* is now in the status of endangered. As a result, many of its similar species are being used as its adulterant in the formulation. A comparative study has been performed on *U. picta* and its adulterant species (*D. gangaticum, D. puchellum, D. velutinum, D. longipes,* and *L. reticulate*) for their effectiveness against multidrug-resistant bacteria. The plant extracts from these plants were screened against ten different clinical isolates of multidrug-resistant bacteria. *U. picta, D. gangaticum,* and *D. puchellum* exhibited antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. However, *U. picta* presented minimum inhibitory concentrations (MICs) of 25 mg/ml and 12.5 mg/ml against *Staphylococcus aureus* and *Escherichia coli*, respectively. In contrast, *D. gangaticum* presented MICs of 12.5 mg/ml and 50 mg/ml against *Staphylococcus aureus* and *Escherichia coli*, whereas *D. puchellum* presented an MIC of 50 mg/ml against *Staphylococcus aureus*. Compared with its adulterant species, *U. picta* showed strong antibacterial and antibiofilm activities against the test bacteria. A major decrease in biofilm formation was detected via both qualitative and quantitative tests against *S. aureus* and *E. coli*. Furthermore, light microscopic analysis confirmed a substantial reduction in the density and thickness of the biofilm matrix against *S. aureus* and *E. coli*.

INTRODUCTION: Infections caused by bacteria are indeed a significant health concern. The overuse of antibiotics in humans and animals leads to genetic mutation or the acquisition of resistance genes [1].

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Thus, bacterial strains develop resistance mechanisms against locally available antibiotics, making them difficult to treat.

Owing to the rise of antibiotic-resistant bacteria globally, the search for alternative treatments has led researchers back to an ancient source: the natural world. Multidrug-resistant (MDR) bacteria pose a major challenge to modern antibiotics; thus, there is an urgent need for some effective antibacterial agents [2]. Historically, plants have played a major role in the treatment of various diseases [16].

To overcome this challenge, naturally derived compounds or plant sources can be used as substitutes for antibiotics. This study explored the antibacterial and antibiofilm potency of *U. picta* and its adulterant species, such as *Dsemodium gangaticum, D. puchellum, D. velutinum, D. longipes* and *L. reticulata,* against several MDR bacteria. The plant *U. picta,* commonly known as 'Prishnaparni', is found throughout India and in tropical Africa. Traditionally, the root of this plant is used for the treatment of fatigue, mouth ulcers, and various gynecological diseases [4].

It is one of the key ingredients of 'Dashmoolarista', which is a well-known medicine in Ayurveda. Pharmacologically, the plant is used for the treatment of UTIs (urinary tract infections), edema, tumors, etc., because of the presence of several phytochemical constituents, such as phenols, flavonoids, and terpenoids [5]. It also exhibits anti-inflammatory activities, hepatoprotective effects, anti-acaricidal activity, antimicrobial efficacy, antinociceptive effects, antioxidant activity, fracture healing activity, anticancer activity, protective effects, antidiabetic activity, and anxiolytic activity in the treatment of Alzheimer's disease [6].

The formulation of Dashmoolarista by Ayurvedic science is a boon to modern society, and *U. picta* is one of the major ingredients. The formulation is used to restore the normal health of postpartum females, is effective in treating pain, and has been proven to cure rheumatoid arthritis and indigestion [7].

Owing to the increase in the demand for Dashmoolarista, companies have started to find adulterant species for *U. picta.* Most plant species, namely, *Dsemodium gangaticum, D. puchellum D. velutinum, D. longipes* and *L. reticulata,* are adulterant species in Dashmoolarista. The above mentioned plants have several pharmacological activities, but despite their beneficial activities, they are considered adulterant species. This situation generally arises from their tendency to interfere, particularly in herbal medicine. As a result, the advantageous properties that *U. picta* can deliver may be unnoticed due to concerns about the quality and purity of the products they affect. Therefore, it is crucial to evaluate and manage these plant species carefully to ensure that their medicinal benefits can be utilized effectively while reducing their potential negative impacts. Hence, this article provides a detailed view of the antibacterial and antibiofilm efficacy of *U. picta* and its adulterant species against MDR bacteria.

MATERIALS AND METHODS:

Antimicrobial Activities of *U. picta* and its Adulterant Species:

Bacterial Culture Maintenance: To determine the antimicrobial potential of methanolic extracts of the medicinal plants collected from the Gandhamardan hills of western Odisha, which includes *U. picta, D. gangaticum, D. puchellum, D. velutinum, D. longipes,* and *L. reticulate* against bacterial pathogens, clinical bacterial strains were collected from Veer Surendra Sai Institute of Medical Sciences and Research (VIMSAR), Burla, Sambalpur, Odisha, India. The bacterial strains used for preliminary screening were *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Shigella* sp., *Proteus mirabilis Salmonella typhi, S. paratyphi A, S. paratyphi B.* The bacterial strains were cultured and maintained in Luria Bertani (LB) broth media.

Preliminary Antimicrobial Screening: For preliminary antimicrobial screening, an agar well diffusion assay was performed. The experimental setup was prepared according to previous methods[3]. The wells were prepared and supplemented with different concentrations of the plant extracts, and 5% DMSO was used as a control. The molten agar plates were incubated overnight at 37 °C, and the holo zones around the wells were measured in mm.

Determination of the Minimum Inhibitory Concentrations (MICs): The MIC was determined using 2-fold serial microdilution method against the test pathogens. The plant extracts (20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml and 0.156 mg/ml) were inoculated with the bacterial culture (0.5 McFarland) [18]. The experimental setup was incubated at 37 °C for 16 hrs to observe bacterial growth. The antibiotic imipenem (IP) was used as a positive control, and the plant extracts without bacterial culture were used as a negative control.

The absorbance was measured at 600 nm in a microplate reader. An agar well diffusion assay was used to determine the antibacterial activity of the plant extracts and IP at the MIC. The results were taken in triplicate.

Anti-biofilm Efficacy of *U. picta* and its Adulterant Species:

Qualitative Biofilm Inhibition Assay: The effects of the extracts and IP on the formation of biofilm matrices in the selected bacterial strains were evaluated *via* a tube adhesion assay *via* the crystal violet staining method [19, 20].

Moreover, the tube adhesion assay revealed the ability of bacteria to adhere to different surfaces through biofilm matrix formation when treated with or without plant extracts and IP.

Quantitative Biofilm Inhibition Assay: For the quantitative estimation of biofilm formation after treatment with sub-MIC levels of extracts and IP, the biofilm formation ability was determined via the crystal violet staining method. Briefly, the selected bacterial strains were grown in sterile LB broth supplemented with or without plant extracts at 37 °C overnight.

After the completion of the incubation period, the culture media was discarded, and the attached biofilm matrices were carefully washed 2--3 times in saline phosphate buffer (PBS) solution (pH 7.0) and successively stained with 0.1% crystal violet for approximately 20 min.

The excess strains were removed, 95% ethanol was added to the stained biofilm matrices over the polystyrene surface, and the optical density (OD) was measured at 540 nm [21].

$$\% \text{ biofilm inhibition} = [OD_{(Control)} - OD_{(Treatment)} / OD_{(Control)}] \times 100$$

Microscopic Analysis of Bacterial Biofilms: For light microscopic analysis, the selected bacterial strain was grown in sterile LB broth supplemented with or without sub-MIC concentrations of the extracts and IP in a 6-well microtiter plate containing sterile coverslips. The 6-microtiter plates were incubated overnight at 37 °C for the growth of biofilm matrices. After incubation, the culture medium was discarded, and the attached biofilm matrices were washed with sterile PBS. Afterward, the biofilm matrices that were attached to the coverslip were stained with 0.1% crystal violet for 20 min. The stained coverslips were then mounted onto the slides and visualized under a light microscope (Quasmo, PZRM-26) [17].

RESULTS:

Priliminary Antibacterial Screening of *U. picta* and its Adulterant Species against Clinical Strains: The antimicrobial activities of different extracts of *U. picta*, *D. gangaticum*, *D. puchellum*, *D. velutinum*, *D. longipes*, and *L. reticulate* were screened against ten different clinically isolated bacteria, i.e., *S. aureus*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *Shigella* sp., *P. mirabilis*, *S. typhi*, *S. paratyphi A*, and *S. paratyphi* B. However, *U. picta*, *D. gangaticum*, and *D. puchellum* exhibited antibacterial activity against *S. aureus* and *U. picta*, and *D. gangaticum* showed potential antibacterial activity against *E. coli* **Table 1.**

TABLE 1: PRIMARY SCREENING OF *U. PICTA* AND ITS ADULTERANT SPECIES *D. GANGATICUM, D. PUCHELLUM, D. VELUTINUM, D. LONGIPES,* AND *L. RETICULATE* AGAINST 10 DIFFERENT CLINICAL ISOLATES OF MULTIDRUG-RESISTANT BACTERIA

	U. picta	*D. gangaticum*	*D. puchellum*	*D. velutinum*	*D. longipes*	*L. reticulata*
S. aureus	✓	✓	✓	NR	NR	NR
E. faecalis	NR	NR	NR	NR	NR	NR
P. aeruginosa	NR	NR	NR	NR	NR	NR
K. pneumonaie	NR	NR	NR	NR	NR	NR
E. coli	✓	✓	NR	NR	NR	NR
P. mirabilis	NR	NR	NR	NR	NR	NR
Shigella Sp.	NR	NR	NR	NR	NR	NR
S. typhi	NR	NR	NR	NR	NR	NR
S. paratyphi A	NR	NR	NR	NR	NR	NR
S. paratyphi B	NR	NR	NR	NR	NR	NR

✓: The plant sample inhibited the bacterial isolates. NR: No Result.

Determination of MIC and zone of Inhibition (ZOI): The MIC of *U. picta* and *D. gangaticum* against *S. aureus* was 12.5 mg/ml, whereas for *D. puchellum*, it was 25 mg/ml. The extracts of *U. picta* and *D. gangeticum* presented the minimum inhibitory concentration of 12.5 mg/ml against *E. coli* **Table 2.** The minimum bactericidal concentration (MBC) was found to be 25 mg/ml, 12.5 mg/ml and 50 mg/ml against *S. aureus* for *U. picta*, *D. gangaticum* and *D. puchellum*, respectively. Furthermore, the extracts of *U. picta* and *D. gangaticum* had MBCs of 12.5 mg/ml and 50 mg/ml against *E. coli*, respectively **Table 3.** At the MIC, *U. picta*, *D. gangaticum*, and *D. puchellum* inhibited *S. aureus* by 15.4 ± 0.4 mm, 11.2 ± 0.9 mm, and 10.9 ± 0.7 mm, whereas *U. picta* and *D. gangaticum* inhibited *E. coli* by 13.2 ± 0.2 mm and 10.7 ± 0.3 mm, respectively. In contrast, the antibiotic imipenem (IP) had a zone of inhibition of 17.9 ± 0.7 mm and 16.2 ± 1.24 mm against *S. aureus* and *E. coli*, respectively **Table 4.**

TABLE 2: MINIMUM INHIBITORY CONCENTRATIONS (MG/ML) OF PLANT EXTRACTS OF *U. PICTA* AND ITS ADULTERANT SPECIES *D. GANGATICUM, D. PUCHELLUM, D. VELUTINUM, D. LONGIPES,* AND *L. RETICULATE* AGAINST 10 DIFFERENT CLINICAL ISOLATES OF MULTIDRUG-RESISTANT BACTERIA

	U. picta	*D. gangaticum*	*D. puchellum*	*D. velutinum*	*D. longipes*	*L. reticulata*
S. aureus	12.5	12.5	25	NR	NR	NR
E. faecalis	NR	NR	NR	NR	NR	NR
P. aeruginosa	NR	NR	NR	NR	NR	NR
K. pneumonaie	NR	NR	NR	NR	NR	NR
E. coli	12.5	12.5	NR	NR	NR	NR
P. mirabilis	NR	NR	NR	NR	NR	NR
Shigella Sp.	NR	NR	NR	NR	NR	NR
S. typhi	NR	NR	NR	NR	NR	NR
S. paratyphi A	NR	NR	NR	NR	NR	NR
S. paratyphi B	NR	NR	NR	NR	NR	NR

TABLE 3: MINIMUM BACTERICIDAL CONCENTRATIONS (MG/ML) OF PLANT EXTRACTS OF *U. PICTA* AND ITS ADULTERANT SPECIES, NAMELY, *D. GANGATICUM, D. PUCHELLUM, D. VELUTINUM, D. LONGIPES,* AND *L. RETICULATE,* AGAINST 10 DIFFERENT CLINICAL ISOLATES OF MULTIDRUG-RESISTANT BACTERIA

	U. picta	*D. gangaticum*	*D. puchellum*	*D. velutinum*	*D. longipes*	*L. reticulata*
S. aureus	25	12.5	50	NR	NR	NR
E. faecalis	NR	NR	NR	NR	NR	NR
P. aeruginosa	NR	NR	NR	NR	NR	NR
K. pneumonaie	NR	NR	NR	NR	NR	NR
E. coli	12.5	50	NR	NR	NR	NR
P. mirabilis	NR	NR	NR	NR	NR	NR
Shigella Sp.	NR	NR	NR	NR	NR	NR
S. typhi	NR	NR	NR	NR	NR	NR
S. paratyphi A	NR	NR	NR	NR	NR	NR
S. paratyphi B	NR	NR	NR	NR	NR	NR

TABLE 4: ZONE OF INHIBITION (IN MM) OF THE PLANT EXTRACT OF *U. PICTA* AND ITS ADULTERANT SPECIES *D. GANGATICUM, D. PUCHELLUM, D. VELUTINUM, D. LONGIPES, AND L. RETICULATE* AGAINST 10 DIFFERENT CLINICAL ISOLATES OF MULTIDRUG-RESISTANT BACTERIA

	Imipenem	*U. picta*	*D. gangaticum*	*D. puchellum*	*D. velutinum*	*D. longipes*	*L. reticulata*
S. aureus	15.02 ±0.2	14.65± 0.36	15.68± 0.31	14.36± 0.29	NR	NR	NR
E. faecalis	NR	NR	NR	NR	NR	NR	NR
P. aeruginosa	NR	NR	NR	NR	NR	NR	NR
K. pneumonaie	NR	NR	NR	NR	NR	NR	NR
E. coli	10.45±0.26	10.03 ±0.32	9.95 ± 0.24	NR	NR	NR	NR
P. mirabilis	NR	NR	NR	NR	NR	NR	NR
Shigella Sp.	NR	NR	NR	NR	NR	NR	NR
S. typhi	NR	NR	NR	NR	NR	NR	NR
S. paratyphi A	NR	NR	NR	NR	NR	NR	NR
S. paratyphi B	NR	NR	NR	NR	NR	NR	NR

Antibiofilm Activities of *U. picta* and its Adulterant Species: At sub-MIC (1/2 MIC) concentrations of the plant extracts of *U. picta* and its adulterant species, a significant decrease in the production of exopolysaccharides was observed, as evidenced by the presence of heavy rings on the surface of the tubes.

The tube adherence assay revealed the presence of minor rings at the liquid–air interface and slight or no attachments at the surface or bottom surface when bacterial strains were supplemented with sub-MICs of *U. picta, D. gangaticum, D. puchellum* and the standard drug imipenem (IP).

The results revealed the inhibition of biofilm matrix formation in comparison with the presence of major ring and matrix formation in the untreated control **Fig. 1A**. Furthermore, the extracts of *U. picta, D. gangaticum* and *D. puchellum* resulted in a reduction in biofilm formation, which was quantified *via* the crystal violet staining method **Fig. 1B**. The biofilm inhibition potential of the extracts was further confirmed via light microscopic observations.

Compared with a thicker biofilm matrix with evenly distributed aggregates of bacterial cells in the untreated control and IP groups, *U. picta, D. gangaticum* and *D. puchellum* extract-treated bacterial cells were comparatively less aggregated with thin nonuniform matrices **Fig. 1C.**

In contrast, the remaining plant extracts, i.e., *D. velutinum, D. longipes,* and *L. reticulate,* did not inhibit biofilm formation against the bacterial pathogens.

(A) Test Tube Adhesion Assay:

FIG. 1A: IN THE TEST TUBE METHOD, THE HEAVY ATTACHMENTS ON THE SURFACE AND BOTTOM OF THE TUBES IN THE CONTROL TUBES INDICATE THE PRODUCTION OF EPS. IN CONTRAST, THE TUBES TREATED WITH THE ANTIBIOTIC IMIPENEM (IP) AND PLANT EXTRACTS (AT SUBMICS) HAD MINOR ATTACHMENTS ON THE SURFACE AND BOTTOM OF THE TUBES. (A) *U. PICTA* (B) *D. GANGETICUM* (C) *D. PUCHELLUM* (D) *D. VELUTINUM* (E) *D. LONGIPES* (F) *L. RETICULATE*

(B) Crystal Violet Staining Method:

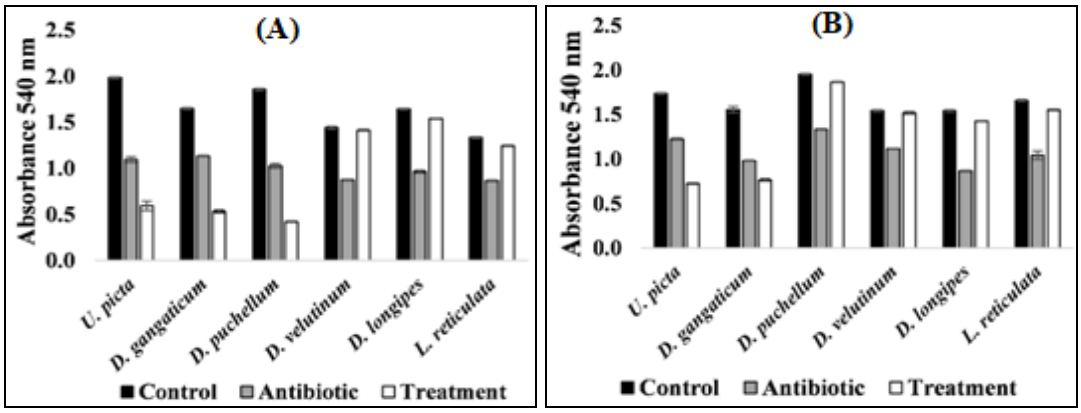

FIG. 1B: THE GRAPH SHOWS THE EFFECTIVENESS OF THE PLANT EXTRACTS IN INHIBITING BIOFILM FORMATION. HIGHER ABSORBANCE VALUES INDICATE GREATER BIOFILM FORMATION, WHEREAS LOWER ABSORBANCE VALUES SUGGEST THE INHIBITION OF BIOFILM FORMATION. THE GRAPH DEPICTS THE EFFECTS OF SUB-MIC CONCENTRATIONS OF *U. PICTA*, *D. GANGETICUM*, AND *D. PUCHELLUM* ON (A) *S. AUREUS* AND (B) *E. COLI* VIA THE QUANTITATIVE CRYSTAL VIOLET STAINING METHOD. IN CONTRAST, *D. VELUTINUM*, *D. LONGIPES*, AND *L. RETICULATA* DID NOT EFFECTIVELY INHIBIT BIOFILM FORMATION

(C) Light Microscopic Assay:

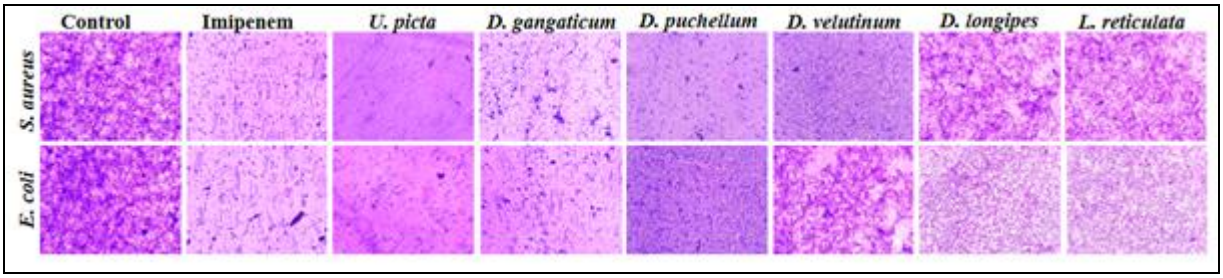

FIG. 1C: THE BIOFILM INHIBITION POTENTIAL OF THE PLANT EXTRACT WAS FURTHER CONFIRMED VIA LIGHT MICROSCOPIC OBSERVATIONS. COMPARED WITH THE THICKER BIOFILM MATRIX FORMED BY *S. AUREUS* AND *E. COLI* WITH EVENLY DISTRIBUTED AGGREGATES OF BACTERIAL CELLS OBSERVED IN THE UNTREATED CONTROL, FEWER AGGREGATED BACTERIAL CELLS WITH THIN NONUNIFORM MATRIX FORMATIONS WERE OBSERVED IN THE ANTIBIOTIC AND *U. PICTA*, *D. GANGETICUM*, AND *D. PUCHELLUM* TREATMENT GROUPS. IN CONTRAST, *D. VELUTINUM*, *D. LONGIPES* AND *L. RETICULATA* DID NOT EFFECTIVELY INHIBIT BIOFILM FORMATION

DISCUSSION: Antibacterial resistance, often referred to as antibiotic resistance, is an important worldwide health challenge that arises when bacteria develop mechanisms to endure the effects of medicines designed to destroy them or hinder their growth. This sensation can lead to treatment failure, extended illness, and increased death[10]. Antibiotic resistance arises from numerous interconnected issues, worsening the problem. One of the chief causes is the overuse and misuse of antibiotics in healthcare. Unsuitable prescriptions for viral contamination and poor treatment routes permit bacteria to survive and adjust [11]. The use of natural products as replacements for antibiotics is gaining increasing attention because of the increasing risk of antibiotic resistance. Many plant products have inherent antibacterial properties that can efficiently fight infections without side effects related to conventional antibiotics [12]. Compounds identified from natural sources have the potential to hinder bacterial development and disrupt biofilm formation, making them valuable contenders for new therapeutic approaches. The antibacterial efficacy of *Uraria picta* has been supported by various studies that provide measurable data on its effectiveness against different bacterial strains. Methanolic extracts of *Uraria picta* showed minimum inhibitory concentrations (MICs) ranging from 31.25 to 125 µg/mL against *Staphylococcus aureus* and *Escherichia coli* [13].

Furthermore, another study confirmed that the ethanolic extract of *Uraria picta* considerably repressed biofilm formation, reducing biofilm formation by up to 60% in the tested bacterial strains, which is vital for preventing chronic contagions [14]. Phytochemical analysis revealed the presence of bioactive compounds such as flavonoids, alkaloids, and saponins, which are known for their antibacterial properties. Moreover, a study revealed that when combined with standard antibiotics, *Uraria picta* extracts had synergistic effects, increasing overall antibacterial activity and potentially reducing the required dosages of conventional antibiotics [15].

These findings suggest that *Uraria picta* not only acts as an antibacterial agent but also may help to reinstate the effectiveness of existing antibiotics. The addition of adulterant species to Dasmoolarishta can markedly worsen its therapeutic properties. Dasmoolarishta is known for its synergistic properties resulting from an exact mixture of ten medicinal plants, which contributes to its effectiveness in endorsing overall health, predominantly in the treatment [9]. When adulterant species are familiar, they can dilute the attentiveness of active compounds vital for the therapeutic benefits of the formulation. This dilution can decrease the strength of the formulation, leading to reduced therapeutic effects. Moreover, some adulterants may have pharmacological activities that could counter or inhibit the proposed effects of Dasmoolarishta [8]. Moreover, adulteration can disturb the quality, stability, and bioavailability of the active ingredients of a formulation. This can lead to differences in efficacy and could confuse the standardization of the product, making it problematic to ensure reliable therapeutic results. Thus, maintaining the integrity of Dasmoolarishta by avoiding adulteration is crucial for preserving its medicinal properties and ensuring patient safety in Ayurvedic practice.

CONCLUSION: In conclusion, examination of the antibacterial and antibiofilm effectiveness of *Uraria picta* highlights the important potential of these plants as alternatives in the fight against multidrug-resistant bacteria. These findings suggest that *Uraria picta* has prominent antibacterial properties compared with those of its adulterant species, with the ability to interrupt biofilm formation, which is important for addressing persistent bacterial infections. This investigation highlights the importance of discovering natural products as feasible therapeutic agents, particularly in an era where conventional antibiotics are progressively losing their efficacy. Future studies should focus on isolating specific bioactive compounds, understanding their mechanisms of action, and leading clinical trials to assess their therapeutic potential. This work contributes to the increasing body of information designed to combat antibiotic resistance and endorses the sustainable use of herbal drugs in present healthcare.

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Data Availability Statement: The authors declare that the data generated during the current study are provided within the manuscript.

CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest.

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