Walisongo Journal of Chemistry
Walisongo Journal of Chemistry Vol. 7 Issue 2 (2024), 221-230
ISSN: 2621-5985 (online); 2549-385X (print)
DOI: https://doi.org/10.21580/wjc.v7i2.22354

ANTIBACTERIAL ACTIVITY OF RESIDUAL SEEDS AND PODS OF ANNATTO (*Bixa orellana L.*) AFTER PIGMENT EXTRACTION

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Abstract

Recent studies have demonstrated that annatto seeds contain a variety of metabolites with antimicrobial properties. Correspondingly, this research investigated the antimicrobial activity of extracts from annatto (Bixa orellana) seeds, pods, and their combination. As part of a strategy to convert waste into valuable resources, this study evaluated the antimicrobial potential of residual extracts derived from annatto seeds and pods. Ethanol extracts of pigment-extracted annatto seeds, pods, and their mixture were tested against Escherichia coli, Streptococcus faecalis, Staphylococcus aureus, and Bacillus subtilis using the agar well diffusion method. Streptomycin and ampicillin (10 mg/mL each) served as positive controls, while an ethanol-water mixture (1:1) served as the negative control. All three extracts exhibited varying activity levels against the tested bacteria. They were most effective against B. subtilis, with inhibition zones measuring 11.5 ± 0.5 mm, 14.5 ± 0.5 mm, and 23.5 ± 0.5 mm for the seed, pod, and seed-pod extract mixture, respectively. In contrast, they exhibited the least activity against E. coli, with inhibition zones of 6.0 ± 2.0 mm, 5.5 ± 1.5 mm, and 11.0 ± 0.0 mm for the respective extracts. Post-hoc Tukey's test revealed that the combination of seed and pod extracts demonstrated significantly higher antimicrobial activity than the individual extracts ($p < 0.001$) against all tested organisms. Notably, the combined extract demonstrated comparable activity to ampicillin against B. subtilis ($p > 0.05$) and significantly higher activity than streptomycin ($p < 0.001$) against all tested strains except S. aureus. The pronounced antimicrobial efficacy of the seed and pod extracts, especially when combined, contributes to the growing body of evidence supporting the potential of Bixa orellana as a natural antimicrobial agent effective against both Gram-positive and Gram-negative bacteria.

Keywords: *Bixa orellana, Gram-positive, Gram-negative, antimicrobial, bacteria, extracts, residual, ethanol, controls.*

Introduction

The rise in multidrug resistance has hindered the development of new synthetic antimicrobial drugs and prompted the exploration of alternative sources for new antimicrobials (Salam et al., 2023). Natural compounds represent a rich source of therapeutic agents. Recent advancements in drug discovery from natural sources have yielded compounds under development for the treatment of cancer, drug-resistant bacteria, viruses, and immunosuppressive disorders (Uddin et al., 2021). Phytochemicals derived from medicinal plants, which exhibit antimicrobial

properties, have the potential to meet this demand due to their distinct chemical structures compared to conventional microbial sources. The presence of various bioactive metabolites in certain plants suggests promising potential as sources of new antimicrobial agents with both general and specific activities (Abdallah, 2023). The screening of active compounds from plants has led to the discovery of new medicinal drugs that play effective protective and therapeutic roles against various diseases (Riaz et al., 2023). Numerous reports in the literature document the presence of antimicrobial compounds in various plants.

In this regard, extracts from the seeds of the tropical bush *Bixa orellana* L. contain carotenoid-type pigments that provide unique flavor and color to foods. These extracts are extensively used as colorants, primarily in dairy products such as cheese and butter (Aluko, 2024). Earlier studies have shown that commercial annatto extracts exhibit biological activities against microorganisms relevant to food fermentation, preservation, and safety. Every part of the *Bixa orellana* plant—including the root, leaves, pod, and foliage—has been historically used for medicinal purposes. These include stimulating digestion, lowering blood pressure, protecting the liver, reducing inflammation, treating coughs, cleansing the blood, soothing membranes, alleviating fevers, promoting wound healing, and addressing snake bites (Rather & Mohammad, 2016).

Annatto seeds have traditionally been used for therapeutic purposes due to their effectiveness against bacteria, parasites, and germs (Khan et al., 2024), with numerous studies supporting these claims. However, there is a lack of research on the antimicrobial activity of the pods bearing the seeds or the post-pigment extraction waste. The present study, therefore, evaluated the respective and combined antibacterial actions of the seeds and pod extracts of annatto from the perspective of waste recycling.

Figure 1. Bixa orellana seeds in their pods

Materials and Method

Sample Collection and Preparation

Residual seeds and pods from pigment-extracted *Bixa orellana* were collected in February 2024 during the dry season. The samples were obtained from Alagbaka, located 12 km south of the Federal University of Technology, Akure, Nigeria. As a tropical country in West Africa, Nigeria has a warm climate with distinct wet and dry seasons, characterized by abundant rainfall in the south and a hot, dry climate in the north. The collected samples were washed with distilled water, dried, and finely blended. Each 20 g sample was dissolved in 100 mL of ethanol at room temperature for 24 hours, followed by filtration. The residue was further washed with ethanol while being stirred to ensure complete extraction. A combined extract (1:1 ratio) of seeds and pods was prepared by mixing 20 mL of each extract. Figure 2 illustrates the visual appearance of the ethanol extracts derived from *Bixa orellana* seeds and pods.

All extracts were stored at 4°C for 5 days prior to antibacterial analysis. Low-temperature storage is known to preserve bioactive metabolites by reducing degradation caused by heat or enzymatic activity (Montiel et al., 2024). However, extended storage may gradually affect metabolite stability, potentially influencing antibacterial efficacy (Postružnik et al., 2024).

Figure 2. Ethanol extracts of Bixa orellana seeds and pods (BS: Bixa Seed Extract; BP: Bixa Pod Extract; BSBP: Bixa Seed and Pod Extract Mixture; Blank: Ethanol-Water Mixture)

Antibacterial Analysis

The antibacterial analysis was conducted using the agar-well diffusion method, following the procedure outlined by the Clinical and Laboratory Standards Institute (CLSI, 2022). This method allowed antimicrobial compounds in the extracts to diffuse into a medium seeded with bacterial strains. The resulting inhibition zones, where bacterial growth was prevented, appeared uniformly circular. The diameters of these inhibition zones were measured in millimeters to assess the antibacterial effectiveness of the extracts. The bacterial inoculum was standardized to a concentration of 10^6 CFU/mL using the 0.5 McFarland standard, corresponding to approximately 10^6 CFU/mL for most bacterial strains.

Preparation of Agar Medium

The agar medium was prepared by dissolving 33.9 g of commercially available Mueller-Hinton Agar Medium (HiMedia) in 1000 mL of distilled water. The dissolved medium was autoclaved at 15 psi pressure at 121°C for 15 minutes. After autoclaving, the medium was mixed thoroughly and poured into 100 mm Petri dishes (25–30 mL per dish) while still molten (CLSI, 2022).

Preparation of Nutrient Broth

One liter of nutrient broth was prepared by dissolving 13 g of commercially available nutrient medium (HiMedia) in 1000 mL of distilled water and boiling to dissolve the medium completely. The medium was dispensed as needed and sterilized by autoclaving at 15 psi pressure (121°C) for 15 minutes. All experiments were conducted in triplicate.

Analysis Procedure

Petri dishes containing 20 mL of Mueller-Hinton medium were inoculated with 24-hour cultures of bacterial strains: *Escherichia coli*, *Streptococcus faecalis*, *Staphylococcus aureus*, and *Bacillus subtilis*. The bacterial inocula was prepared by adjusting the concentration of bacterial suspensions to 10^6 CFU/mL using the 0.5 McFarland standard. These bacterial strains were obtained from the Microbiology Department of the Federal University of Technology, Akure**.** Wells were carefully cut into the agar using a sterile 6 mm cork borer, and 20 µL of ethanolic extracts from *Bixa* seed and pod were added to each well. The plates were incubated at 37°C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around each well (CLSI, 2022). Ampicillin and streptomycin, each at a concentration of 10 mg/mL, were used as positive controls. A mixture of ethanol and distilled water (1:1) served as the negative control, with 20 µL added to each well (Nunes et al., 2017). Statistical analysis was performed using one-way ANOVA to compare the mean inhibition zones recorded in triplicate for each extract. Post-hoc Tukey's test was applied to determine significant differences between groups, with a significance level set at $p < 0.05$.

Results and Discussion

Statistical Analysis

The obtained values were analyzed using the mean and standard deviation of triplicate determinations. A one-way ANOVA for the activity recorded in Table 1 revealed significant differences among treatments, with extremely high F-statistics and very low p-values ($p < 0.001$) for all bacteria. Particularly strong effects were observed for *B. subtilis*, which had the highest F-statistic (816.9018), while *E. coli* exhibited the lowest F-statistic (79.8708), though it remained highly significant. The effect sizes ($\eta^2 > 0.97$ for all strains)

indicated that over 97% of the variance in antimicrobial activity could be attributed to treatment differences. Post-hoc Tukey's test showed that the combination of seed and pod extracts demonstrated significantly higher antimicrobial activity than individual extracts ($p < 0.001$) across all tested organisms. The combined extract exhibited comparable activity to ampicillin against *B. subtilis* ($p > 0.05$) and significantly higher activity than streptomycin ($p < 0.001$) against all tested strains except *S. aureus*.

Antibacterial Activity of Extracts

Figure 3 illustrates the inhibition zone of *S. faecalis* observed with the *Bixa orellana* seed extract, while Figure 4 shows the inhibition zone of *S. aureus* with the pod extract. The clear zones around the wells indicated bacterial inhibition by the extracts. Figure 5 presents a comparative chart of the antibacterial activities of the various extracts and controls against the tested bacterial strains. The individual pod and seed extracts demonstrated moderate antimicrobial activity, consistently outperforming the negative control (Ethanol + Water), which showed no inhibition zones (0.0 ± 0.0 mm) across all bacterial strains. This finding confirmed the validity of the assay and ascertained that the inhibition zones resulted from the active extracts. The seed-pod extract combination exhibited superior activity compared to individual extracts across all bacterial strains, with particularly notable activity against *B. subtilis* (23.5 ± 0.5 mm), approaching the effectiveness of ampicillin.

All extracts were most effective against *B. subtilis*, with inhibition zones measuring 11.5 ± 0.5 mm, 14.5 ± 0.5 mm, and 23.5 ± 0.5 mm for the seed, pod, and seed-pod extract mixture, respectively. However, they exhibited the least activity against *E. coli*, with inhibition zones of 6.0 ± 2.0 mm, 5.5 ± 1.5 mm, and 11.0 ± 0.0 mm for the same extracts, respectively. It has been reported that Gram-negative bacteria, such as *E. coli*, possess an outer lipopolysaccharide layer in addition to their peptidoglycan layer, making their cell walls more complex and nearly impermeable to many environmental substances and antibiotics (Maldonado et al., 2016). In contrast, Gram-positive bacteria lack this outer lipopolysaccharide layer and rely solely on peptidoglycan in their cell structure. This difference in cell wall composition contributes to Gram-positive bacteria being generally more sensitive to various agents due to less resistance to permeability (Gauba & Rahman, 2023; Paracini et al., 2022). The efflux pump mechanism might also help to explain the resistance of *E. coli* to the antimicrobial extracts from *Bixa orellana* seeds and pods. As a Gram-negative bacterium, *E. coli* possesses efflux pumps that actively expel antimicrobial compounds from the cell, reducing the internal concentration of the bioactive substances in the extracts (Karpov et al., 2024). This condition results in a diminished antimicrobial effect, even when the extracts are potent against other bacteria (Huang et al., 2022).

The relatively higher standard deviations observed in some measurements, particularly against *E. coli* (SD ranging from 1.5 to 2.0 mm), could be attributed to several factors. These included the natural variation in the concentration of active compounds in different batches of plant extracts (Bhalodia & Shukla, 2011) and potential interactions between multiple bioactive compounds in the crude extracts. Additionally, as a Gram-negative bacterium, *E. coli* possesses a more complex cell wall structure, which can lead to variable responses to antimicrobial agents and differing levels of permeability to the active compounds (Shilhavy et al., 2010). This structural complexity often results in varied zones of inhibition. Such variability is commonly observed in antimicrobial studies using plant extracts and even standard antibiotics against Gram-negative bacteria (Chalo et al., 2017).

The significant antimicrobial activity exhibited by the seed and pod extracts, particularly their combined synergistic effect, adds to the growing body of evidence supporting *Bixa orellana*'s potential as a natural antimicrobial agent. These results suggest that, while the extracts might not match the efficacy of ampicillin, they still

demonstrated promising antimicrobial properties, particularly when combined. This finding indicates potential therapeutic applications, especially when alternative antimicrobial agents are needed. Previous studies have revealed that extracts from the leaves and seeds of *B. orellana* exhibited significant antimicrobial activity against a range of pathogens, including Gram-positive and Gram-negative bacteria (Dos Santos et al., 2022; Franklin et al., 2023). Vaou et al. (2021) highlight that the effectiveness of medicinal plant extracts in inhibiting bacterial growth is often linked to the synergistic effects of their active compounds. Furthermore, Kar et al. (2022) demonstrated that ethanolic extracts of *B. orellana* leaves could exhibit effective antibacterial activity against multi-drug-resistant pathogens. Similarly, Hajoori and Dasgupta (2021) reported the activity of *B. orellana* seeds against *B. subtilis*.

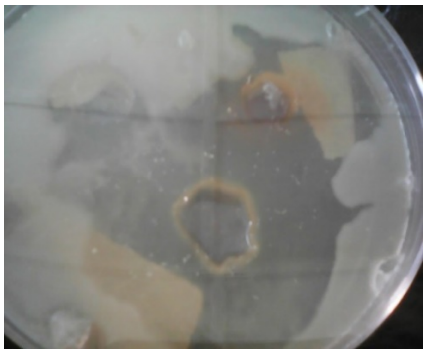

Figure 3. Zone of inhibition of S. faecalis by Bixa orellana seed extract

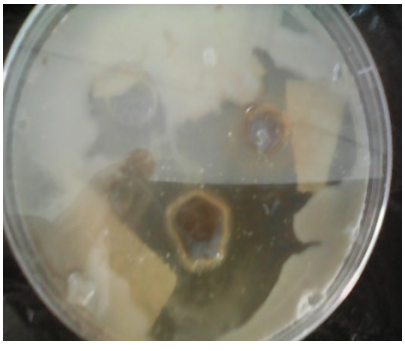

Figure 4. Zone of inhibition of S. aureus by Bixa orellana pod extract

Discussion

Several plants rich in alkaloids, flavonoids, tannins, and glycosides have been shown to possess antimicrobial activity against a wide range of microorganisms (Inusa et al., 2018; Othman et al., 2019). The antibacterial activity observed in the seed and pod extracts of *Bixa orellana* was likely attributed to the presence of flavonoids and tannins, which are well-known bioactive compounds. These compounds exert antimicrobial effects through distinct yet complementary mechanisms, potentially explaining the observed antibacterial activity. Flavonoids**,** known for their broad spectrum of biological activities, may contribute to the antibacterial effects by disrupting bacterial cell membranes (Zhou et al., 2023), thereby increasing permeability (Abayomi et al., 2014). This disruption facilitates the penetration of other antimicrobial agents, such as tannins.

Flavonoids can inhibit bacterial enzymes involved in cell wall synthesis and DNA replication, thus interfering with bacterial growth and reproduction (Zhou et al., 2023). For instance, flavonoids like quercetin, which have been reported in *Bixa orellana*, may inhibit enzymes such as DNA gyrase or topoisomerase, ultimately leading to bacterial cell death (Ullah et al., 2020). Conversely, tannins are polyphenolic compounds that can form complexes with bacterial proteins, enzymes, and metal ions (Adamczyk, 2017). This interaction inhibits bacterial enzyme functions, particularly those involved in nutrient uptake and cell wall biosynthesis, ultimately impairing bacterial growth (Molnar et al., 2024). The antimicrobial activity of tannins is particularly effective against Gram-positive bacteria due to their ability to bind to and disrupt cell wall components (Othman et al., 2019; Ullah et al., 2020).

Moreover, synergism between flavonoids and tannins in the *Bixa orellana* seed and pod extracts might exist. The combined action of these two classes of compounds could result in a more potent antibacterial effect than when either compound acts alone. Flavonoids may enhance the uptake of tannins into bacterial cells, enabling tannins to interfere more effectively with bacterial metabolic

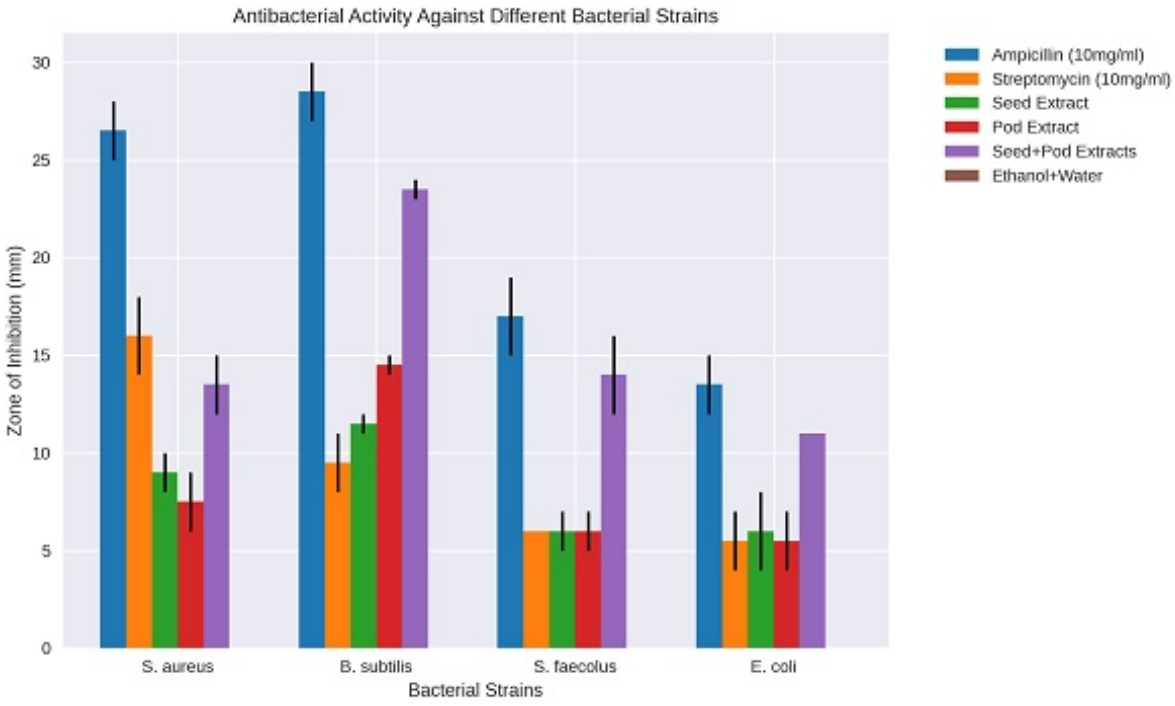

Figure 5. Antibacterial activity against bacterial strains

Table 1. Antibacterial activity of Bixa orellana seed and pod

	Zone of Inhibition (in mm)			
	S. aureus	**B. subtilis**	***B. subtilis***	***E. coli***
Ampicillin (10 mg/mL)	26.5 ± 1.5	28.5 ± 1.5	17.0 ± 2.0	13.5 ± 1.5
Streptomycin (10 mg/mL)	16.0 ± 2.0	9.5 ± 1.5	6.0 ± 0.0	5.5 ± 1.5
Seed Extract	9.0 ± 1.0	11.5 ± 0.5	6.0 ± 1.0	6.0 ± 2.0
Pod Extract	7.5 ± 1.5	14.5 ± 0.5	6.0 ± 1.0	5.5 ± 1.5
Seed + Pod Extracts	13.5 ± 1.5	23.5 ± 0.5	14.0 ± 2.0	11.0 ± 0.0
Ethanol + Water	0.0	0.0	0.0	0.0

**Values represent the mean ± standard deviation (SD) of three replicates. Significance levels were tested at $p < 0.05$*

processes. This synergistic effect has been observed in other plant extracts rich in both flavonoids and tannins (Klongsiriwet et al., 2015; Kováč et al., 2022), suggesting that such interactions may play a key role in the efficacy of *Bixa orellana* extracts.

While the potential synergism between flavonoids and tannins in *Bixa orellana* seed and pod extracts was plausible, further studies are necessary to isolate and test individual compounds, as well as to explore their combined effects more comprehensively. Such investigations could include testing individual flavonoid and tannin concentrations and performing synergy assays (e.g., checkerboard assays) to quantify the interactions between these two classes of compounds.

Although this study provides valuable insights into the antimicrobial potential of *Bixa orellana* extracts, certain limitations must be acknowledged. The absence of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) testing limited the researchers' ability to quantify the precise antimicrobial potency of the extracts. These tests are essential to determine the optimal concentrations required to inhibit bacterial growth or achieve bactericidal effects. Hence, incorporating MIC and MBC testing in future studies would provide a clearer understanding of the extracts' therapeutic potential and further validate their effectiveness for clinical and industrial applications.

Conclusion

Residues from pigments extracted from Bixa orellana seeds and pods exhibited notable antimicrobial properties. They could effectively combat a range of Gram-positive and Gram-negative bacteria, including B. subtilis, S. aureus, S. faecalis, and E. coli. The significant antimicrobial activity demonstrated by the seed and pod extracts, particularly their combined synergistic effect, adds to the growing body of evidence supporting B. orellana's potential as a natural antimicrobial agent. Furthermore, the utilization of these by-products, traditionally considered waste, highlights the value of recycling and valorizing food and agricultural residues, offering a sustainable avenue for developing natural antimicrobial agents. These extracts hold promise for various applications, including the development of pharmaceutical products, food preservatives, and natural therapeutic agents, paving the way for eco-friendly alternatives to synthetic chemicals. To further advance this research, future studies should focus on isolating and characterizing the active compounds in Bixa orellana extracts, exploring their potential in pharmaceutical, food preservation, and therapeutic applications, and developing scalable, standardized extraction methods for commercial use.

Acknowledgment

The authors express sincere gratitude to the Department of Chemistry, the Department of Microbiology, the School of Science, and the School of Postgraduate Studies at the Federal University of Technology, Akure, for their support in the successful completion of this research.

Disclosure Statement

Conflict of interest: The authors declare no conflict of interest in the preparation of this publication. Ethical approval: All ethical guidelines have been adhered to.

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 | ISSN 2621-5985 (online) | ISSN 2549-385X (print)
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