Pigment-Producing Endophytic Fungus Arcopilus cupreus strain T8: Characterization, Bioactivity, and Application as a Natural Textile Dye

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Abstract

Endophytic fungi are promising sources of natural pigments for sustainable textile dyeing and functional biomaterials. In this study, a pigment-producing fungal strain (T8) isolated from healthy leaves of *Rhodomyrtus tomentosa* was identified as *Arcopilus cupreus* based on morphology and ITS rDNA sequencing (100% similarity). The strain produced a vivid extracellular red pigment during submerged fermentation, yielding 1.47 g per gram of dried biomass. The pigment was highly water-soluble and exhibited a maximum absorption at 500 nm. It showed excellent thermal and photostability, while pH influenced color variation from red to violet and brown. LC–MS/MS analysis identified oosporein as the predominant secondary metabolite, consistently detected in both ionization modes at 6.2 min with the highest signal intensity, indicating its role as the primary pigment compound. The pigment demonstrated moderate antioxidant activity (IC_{50} = 59.45 μg/mL) and low cytotoxicity toward human dermal fibroblasts (> 85% viability at 2 mg/mL). Cotton dyeing showed strong color uptake, enhanced by alum mordant, and pH-dependent color variation, with selective antibacterial activity against *Staphylococcus aureus*. These findings highlight *A. cupreus* T8 as a promising source of stable, multifunctional pigments for eco-friendly textile applications, although further safety evaluation is required.

Introduction

Natural pigments derived from plants, animals, and microorganisms have long been utilized as colorants in food, cosmetic, pharmaceutical, and textile applications; however, pigments from plant and animal sources present significant limitations, including low extraction yields, dependence on seasonal and geographical factors, and the need for large quantities of raw materials to obtain small amounts of pigment, resulting in high production costs and restricted scalability. Increasing environmental and health concerns surrounding synthetic dyes further intensify the demand for natural alternatives, as many synthetic colorants have been associated with carcinogenicity, hyper-allergenicity, and other toxicological risks[1,2]. Moreover, industrial dyeing processes are notably inefficient, with approximately 10–35% of dyes being lost in textile effluents and up to 50% of reactive dyes remaining in dye bath wastewater[3], contributing substantially to environmental pollution and generating hazardous degradation products. These limitations overall underscore the urgent need for safe, sustainable, and environmentally benign pigment sources.

Microbial pigments have emerged as promising alternatives due to several advantages, including stable year-round production, rapid growth, high pigment yield, and ease of cultivation on inexpensive substrates. Microorganisms are capable of synthesizing a wide variety of pigment classes, such as carotenoids, flavins, melanins, quinones, monascin, violacein, phycocyanin, and indigo[4,5]. Many of these pigments also exhibit bioactivities beneficial for food preservation, cosmetic formulation, and biomedical applications. Nevertheless, large-scale pigment production may be influenced by strain characteristics, fermentation conditions, nutrient composition, and cellular growth dynamics. Improvements in productivity can be achieved through strain selection, mutagenesis, genetic engineering, and optimization of fermentation processes[3,6].

Among microbial pigment producers, filamentous fungi represent one of the most important groups due to their chemical diversity and high pigment-producing capacity. Numerous genera, including *Alternaria*, *Aspergillus*, *Ashbya*, *Blakeslea*, *Curvularia*, *Epicoccum*, *Fusarium*, *Monascus*, *Nigrospora*, *Penicillium*, and *Trichoderma*, are known to generate industrially relevant pigments[7]. Several fungal pigments, such as monascin, ankaflavin, monascorubin, rubropunctatin, Arpink Red™, β–carotene, lycopene, and riboflavin, are currently commercialized as natural food colorants. Their application in textile dyeing has also been demonstrated, especially on cotton, silk, and wool, where fungal pigments exhibit strong fiber affinity, good solubility, stability, and colorfastness[8,9]. Moreover, the intrinsic antimicrobial and antioxidant properties of fungal pigments further support their use in the development of functional and hygienic textiles.

Medicinal plants represent a valuable reservoir of endophytic fungi due to their rich microecological interactions. These fungi inhabit plant tissues without causing disease and are well known for their ability to synthesize structurally diverse secondary metabolites, including pigments[10]. *Rhodomyrtus tomentosa* (Aiton) Hassk., commonly known as downy rose myrtle, is a medicinal plant widely used in Southeast Asian traditional medicine. It contains a broad spectrum of phytochemicals, including flavonoids, triterpenoids, phenolics, meroterpenoids, and the antibacterial compound rhodomyrtone[11]. The presence of these bioactive metabolites suggests that the plant's endophytic fungi may also produce valuable compounds, including natural pigments suitable for textile dyeing and functional applications[12]. Given the growing demand for safe, non-toxic, and environmentally friendly colorants, pigment-producing endophytic fungi from *R. tomentosa* represent a promising source of natural dyes with potential functional benefits. Optimization strategies, including strain improvement, medium formulation, and controlled fermentation, remain essential for enhancing pigment yield and improving the feasibility of large-scale production.

The present study aimed to characterize pigment-producing endophytic fungi from *R. tomentosa* and evaluate the potential of their extracellular pigments as natural textile dyes. The specific objectives were to: (i) extract and characterize the fungal pigment; (ii) apply the pigment to textile dyeing and assess dyeing performance; (iii) evaluate the antimicrobial activity of dyed fabrics; and (iv) characterize the chemical composition, antioxidant activity, antimicrobial activity, and cytotoxicity of the pigment extract. This work contributes to the development of sustainable, bioactive fungal pigments as alternatives to synthetic dyes currently used in the textile industry.

Results

Identification and pigment production of fungal strain T8

An endophytic fungal isolate, designated T8, was recovered from surface-sterilized leaf tissues of *Rhodomyrtus tomentosa*. This isolate demonstrated the ability to produce a prominent extracellular red pigment. On PDA, colonies of T8 reached 75–80 mm in diameter after 7 days and exhibited a characteristic morphology of flat colonies with a brown, densely myceliated central region and white

peripheral margins (Fig. 1A and 1B). The culture medium surrounding the colony became intensely red, indicating secretion of a diffusible pigment. Microscopic examination revealed ascocarps that were rounded, ovoid, or obovate, possessing ostiolate ascomata covered with distinctive hairs typical of the *Chaetomiaceae* family (Fig. 1C–1E). The asci of *A. cupreus* are clavate, thin-walled, and typically short-stipitate, arising singly within the ostiolate ascomata. Each ascus contains eight lemon-shaped, smooth-walled ascospores that gradually become brown at maturity (Fig. 1F and 1G). Molecular identification based on ITS rDNA sequencing revealed that strain T8 showed 100% sequence similarity to *Arcopilus cupreus*, supporting its assignment to this species (Fig. 1H). The ITS rDNA sequence of *A. cupreus* has been deposited in the GenBank database under accession number PZ292733. Pigment production was further assessed in submerged fermentation using 500-mL Erlenmeyer flasks containing 300 mL of PDB. After 1 week of incubation, the culture filtrate exhibited intense red coloration, and lyophilization of the filtrate yielded a crude pigment corresponding to 1.47 g of pigment per 1 g of dried mycelial biomass. This high pigment-to-biomass ratio highlights the strong pigment-producing capability of *A. cupreus* T8 and its potential as a viable source of natural colorants.

Characterization of crude fungal pigment

Solubility of crude T8 pigment

The solubility of the crude T8 pigment in different solvents is summarized in Table S1. and illustrated in Fig. 2A. The pigment exhibited strong solubility in distilled water, where it dissolved completely and displayed a dark red coloration with a maximum absorbance at 500 nm. Moderate solubility was observed in absolute methanol (70% dissolved) and slight solubility in 95% ethanol (50% dissolved), with both solvents producing a red to dark-red solution. In contrast, no solubility occurred in ethyl acetate or hexane, indicating that the pigment is highly polar and water-soluble.

Stability of crude T8 pigment

The stability of the pigment under various pH conditions revealed notable sensitivity to pH changes (Table S2; Fig. 2B). The crude T8 pigment extract exhibited a neutral pH of 4.5 prior to pH adjustment. The pigment appeared red at acidic pH (pH 4), shifted to violet or purple between pH 5–8, and underwent a marked color loss at alkaline pH values (pH 9–11), where it turned brown. This behavior suggests structural alterations or partial degradation under alkaline conditions. The pigment was most stable in acidic to neutral environments, with maximal absorbance near 500 nm, indicating that its chromophore is preserved at lower pH values. Thermal stability testing demonstrated that the pigment retained high stability across all tested temperatures (40–120 °C), with no major shifts in color and >90% stability maintained even at 120 °C (Table S3; Fig. 2C). Similarly, exposure to sunlight, UV light, and autoclaving resulted in minimal degradation, with stability values ranging from 93% to 98% (Table S3; Fig. 2D). These findings confirm that the red pigment of *A. cupreus* T8 is thermally robust and photostable, making it suitable for applications requiring heat or light exposure, such as textile dyeing and food processing.

Antioxidant activity of crude T8 pigment

The DPPH radical scavenging activities of *A. cupreus* T8 pigment and L–ascorbic acid (positive control) are presented in Table 1. L–ascorbic acid showed strong antioxidant activity with an IC_{50} value of 5.82 µg/mL, consistent with its well-established potency. The crude pigment displayed moderate but significant antioxidant activity, with an IC_{50} of 59.45 µg/mL. Although less potent than L–ascorbic acid, the pigment demonstrated clear radical-scavenging capacity, indicating that bioactive compounds within the extract contribute meaningfully to antioxidant activity. The increasing inhibition percentages observed across concentrations reflect effective electron-donating or hydrogen-donating capabilities of the pigment molecules. The highest concentration tested (10 µg/mL) yielded 78% inhibition for T8 and 88% inhibition for L–ascorbic acid, supporting the pigment's capacity to quench DPPH radicals. These findings suggest that the red pigment from *A. cupreus* T8 possesses valuable antioxidative properties, which may enhance its functional utility in natural dye formulations.

Table 1. DPPH radical scavenging activity of *A. cupreus* T8 pigment and L–ascorbic acid

Concentration (µg/mL)	**% Inhibition (Mean ± SD)**	
	L–ascorbic acid	**T8 crude pigment**
0.3125	−1.26 ± 1.89[a]	6.19 ± 3.47[a]
0.625	2.34 ± 2.74[a]	7.61 ± 5.22[a]
1.25	8.82 ± 2.93[b]	15.75 ± 2.46[b]
2.50	20.72 ± 2.09[c]	26.96 ± 2.34[c]
5.00	42.61 ± 4.40[d]	47.27 ± 1.28[d]
10.00	87.83 ± 1.24[e]	77.50 ± 2.93[e]
IC_{50} (µg/mL)	**5.82**	**59.45**

Cytotoxicity of the crude T8 pigment

The cytotoxicity of the crude fungal pigment from *A. cupreus* T8 was assessed using normal human dermal fibroblasts (NHDFs) to evaluate its safety for potential skin–contact applications. As shown in Table 2, NHDFs maintained high viability following 24-hour exposure to all tested pigment concentrations and under the tested conditions. The highest viability (109.44%) occurred at 0.125 mg/mL, indicating a possible stimulatory or hormetic effect on cell metabolism, while the lowest viability (85.80%) was observed at the maximum concentration of 2 mg/mL. Even at this highest concentration, cell viability remained well above the 70% threshold commonly used to classify materials as non-cytotoxic. The results support the pigment's biocompatibility and safety, suggesting its suitability for applications involving direct or indirect contact with human skin.

Table 2. Percentage cell viability of NHDFs after 24 h exposure to *A. cupreus* T8 crude pigment

T8 concentration (mg/mL)	% Cell viability
NT (non-treated control)	100 ± 0.00[c]
0.0156	99.32 ± 5.15[c]
0.0313	99.15 ± 5.46[c]
0.0625	103.11 ± 6.47[c]
0.1250	109.44 ± 7.73[d]
0.2500	104.91 ± 6.06[c]
0.5000	93.33 ± 5.58[b]
1.0000	92.34 ± 5.71[b]
2.0000	85.80 ± 13.44[b]
PC (Positive control)	2.86 ± 0.76[a]

Cell viability ≥70% indicates non-cytotoxicity according to ISO 10993–5 guidelines ISO 10993, 2009[13].

Characterization of T8–dyed textile

Dyeing performance and color variation of T8 pigment

In this study, alum mordants at concentrations of 5%, 10%, and 15% markedly improved pigment uptake on cotton fabrics compared with unmordanted controls (Fig. 3A). Among the treatments, 5% alum produced the most intense and uniform coloration, indicating that lower mordant concentrations were more effective for the interaction between the *A. cupreus* T8 pigment and cellulose fibers. The pigment demonstrated notable pH–dependent chromatic variability. Cotton yarns mordanted with 5% alum and dyed using pigment solutions adjusted from pH 3 to 10 displayed distinct color shifts (Fig. 3B). Acidic conditions (pH 3–4) yielded reddish-brown tones, while near-neutral conditions (pH 5–7) produced purple to deep violet shades, with the most intense purple observed at pH 6. In alkaline conditions (pH 8–10), the pigment shifted to brown-yellow hues. These reversible, pH–responsive color changes suggest structural modifications of the pigment chromophore and highlight its potential as a multifunctional natural colorant. These findings also demonstrate that the crude pigment possesses good dyeing capability, favorable mordant affinity, and tunable color expression, making it suitable for sustainable textile dyeing applications.

Antibacterial activity of T8–dyed fabrics

In this study, the antibacterial activity of cotton fabrics dyed with the *A. cupreus* T8 pigment was evaluated against two clinically relevant pathogens, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. The agar

diffusion assay (Fig. 4 and Table S4) demonstrated that antibacterial activity was selective toward Gram–positive *S. aureus*, with no inhibition observed against Gram–negative *E. coli*. Both unmordanted fabrics and those mordanted with 5% alum produced clear zones of inhibition measuring 16.3 ± 0.5 mm and 12.1 ± 0.8 mm, respectively, whereas fabrics mordanted with 10% or 15% alum showed no detectable activity. These findings suggest that mordant concentration influences pigment–fiber interactions in ways that may affect the release or availability of bioactive compounds. Quantitative assessment using the AATCC 100–2004 method (Table S5) further supported these results. The unmordanted dyed fabric showed the highest antibacterial performance, with a 33.33% reduction in *S. aureus*, followed by fabrics mordanted with 5% (31.88%), 10% (4.35%), and 15% alum (2.90%). The diminished activity at higher alum concentrations may be attributed to stronger pigment–fiber fixation, reducing the diffusion of active molecules responsible for bacterial inhibition. This demonstrates the pigment's potential as a natural antimicrobial colorant suitable for applications in medical textiles, baby products, and garments designed for individuals with sensitivities to chemical dyes.

Chemical profiling of T8 crude pigment by GC–MS

GC–MS profiling of the crude pigment extract from *A. cupreus* T8 revealed a chemically diverse mixture of low-molecular weight metabolites, reflecting the fungus's biosynthetic versatility and its potential to produce multiple biologically active compounds. A total of 66 compounds were detected, nine of which represented major constituents based on peak area contributions. The chemical structures of the major identified compounds are shown in Fig. 5A. Detailed information on each compound, including molecular weight, retention time, peak area percentage, and reported biological activities, is summarized in Table 3. The dominant compound, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (17.97%), is a known pyranone derivative frequently reported in fungi and associated with antioxidant and antimicrobial activities. Other prominent metabolites included 4-cyclopentene-1,3-dione (8.76%), maleic hydrazide (5.09%), and several furanone derivatives, all of which have documented antibacterial, antifungal, or antioxidant properties. Notably, the detection of compounds such as acetic acid, 6-morpholin-4-yl-9-oxobicyclo[3.3.1]non-3-yl ester (5.70%) and triazole-containing indole derivatives (5.32%) suggest the presence of secondary metabolites with potential antidiabetic or pharmacological relevance.

Taken together, the chemical profile supports the biological properties observed in earlier assays, including antimicrobial, antioxidant, and low-cytotoxicity outcomes. Many of the detected compounds are recognized food-grade flavoring agents or natural antioxidants commonly found in edible fungi, reinforcing the pigment's suitability for applied uses[14]. The presence of multiple bioactive molecules indicates that the biological effects of the *A. cupreus* T8 crude pigment are likely the result of synergistic interactions among structurally diverse metabolites, rather than a single dominant compound. These findings highlight the potential of *A. cupreus* T8 as a valuable source of natural pigments enriched with multifunctional bioactive compounds suitable for safe, eco-friendly textile dyeing and other biotechnological applications.

Table 3. Major compounds identified in the crude pigment extract of *A. cupreus* T8 by GC–MS analysis.

No.	RT	Compound Name	Formula	MW (g/mol)	Peak Area (%)	Biological activity
1.	5.6574	4-Cyclopentene-1,3-dione	$C_5H_4O_2$	96.021	8.76	Antibacterial, antifungal[15]
2.	7.9598	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	144.042	3.60	Antimicrobial, antioxidant[16]
3.	9.5429	1-Butanamine, 2-methyl-N-(2-methylbutylidene)-	$C_6H_8O_3$	155.000	1.93	Antibacterial, antifungal[17]
4.	10.1609	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	$C_6H_8O_3$	128.000	2.23	Antibacterial, antifungal[18]
5.	10.7258	Acetic acid, 6-morpholin-4-yl-9-oxobicyclo[3.3.1] non-3-yl ester	$C_{15}H_{23}NO_4$	281.163	5.70	Antimicrobial, antidiabetic[19]
6.	12.2684	2-Acetyl-2-hydroxy-.gamma.-butyrolactone	$C_6H_8O_4$	144.000	2.38	Antibacterial, antifungal[20]
7.	12.514	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.000	17.97	Antimicrobial,antioxidant[21]
8.	13.046	Maleic hydrazide	$C_4H_4N_2O_2$	112.027	5.09	Antibacterial activity[22]
9.	13.2892	(1H-indol-3-ylmethylene)-[1,2,4]Triazol-4-YL-amine	$C_{11}H_9N_5$	211.000	5.32	Antibacterial activity[23]

RT, retention time; MW, molecular weight.

LC–MS/MS analysis of T8 crude pigment

The chemical composition of the T8 crude pigment was investigated using LC–MS/MS in both negative and positive ionization modes. The total ion chromatograms revealed multiple detected compounds, predominantly eluting at early retention times (1–2 min), along with a prominent peak at approximately 6.2 min (Fig. 5B and 5C). In the negative ion mode, several primary metabolites were identified, including sugars (sucrose and trehalose), amino acids (glutamic acid, phenylalanine, and tryptophan), organic acids, and nucleotide derivatives. These compounds, detected between 1.19 and 5.01 min with high library matching scores, are commonly associated with potato dextrose broth (PDB) culture media. Comparison with the PDA medium control confirmed that these early-eluting compounds originated from the culture

medium and were therefore excluded from consideration as pigment-related metabolites (Table S6). This distinction is essential, as PDB-derived compounds are frequently co-extracted during pigment recovery and may interfere with accurate metabolite profiling. By eliminating these background signals, the analysis confirms that oosporein is a true fungal-derived metabolite rather than an artifact of the cultivation substrate.

After excluding these background components, a dominant compound was consistently observed at a retention time of 6.20 min and identified as oosporein based on accurate mass measurement and MS/MS spectral matching, with a high library score (98%). This compound also exhibited the highest peak intensity among all detected analytes. In the positive ion mode, oosporein was similarly detected at 6.18 min with strong signal intensity and reliable spectral confirmation. Other compounds detected in this mode, including amino acids, vitamins, and small organic molecules, were likewise attributed to residual medium components or primary metabolism. These results confirm that oosporein is the major secondary metabolite responsible for the observed pigmentation in the crude extract.

Discussion

This study demonstrates that the endophytic fungus *Arcopilus cupreus* T8, isolated from *Rhodomyrtus tomentosa*, is a promising producer of a red extracellular pigment with significant biotechnological applications. Natural microbial pigments are increasingly sought after as sustainable alternatives to synthetic dyes due to environmental concerns and toxicity associated with petrochemical dyes[4,5]. The findings of this study contribute novel insights into pigment-producing endophytes and expand the known functional capacity of the genus *Arcopilus*. The T8 isolate produced an intense red pigment on PDA and in submerged culture. Morphological traits, including ostiolate ascomata with characteristic hairs, were consistent with *Chaetomiaceae* descriptions[24]. ITS rDNA sequencing confirmed 100% similarity to *A. cupreus*, supporting species-level identification. Pigment yields (1.47 g pigment per g dry biomass) were high compared with yields reported for established pigment-producing fungi such as *Monascus purpureus*, *Talaromyces purpureogenus*, and *Penicillium oxalicum*[25]. These results suggest that *A. cupreus* T8 is inherently robust in pigment biosynthesis, even without medium optimization.

Water solubility, which is a desirable trait for eco-friendly textile dyeing, was excellent. Many fungal pigments (e.g., carotenoids from *Blakeslea trispora* and *Rhodosporidium* spp.) require organic extraction and demonstrate low aqueous solubility[26,27]. In contrast, the T8 pigment behaves similarly to hydrophilic pigments from *Monascus* and *Talaromyces* species[28,29], enhancing its industrial relevance. The pigment exhibited strong thermal and photostability, maintaining activity at temperatures up to 120 °C and under UV or sunlight exposure. These are characteristics reported for only a subset of fungal pigments, such as those from *Epicoccum* and *Monascus*[30]. pH-dependent color shifts (from red to violet to brown) indicate chromophore sensitivity to protonation states, a phenomenon also observed in polyketide pigments from *Talaromyces* spp. This property supports application in pH-responsive or multifunctional textiles. pH-induced color variation produced reddish-brown shades under acidic conditions and vibrant purple tones at pH 6, which is the deepest shades recorded. Comparable chromatic diversity has been reported in

furanone- and polyketide-based fungal pigments[31], highlighting the potential for customizable natural dye palettes.

T8-dyed cotton fabrics demonstrated selective antibacterial activity against *Staphylococcus aureus* but not *Escherichia coli*. Selective inhibition of Gram-positive bacteria is common among fungal pigments due to differences in cell wall permeability. The unmordanted fabrics exhibited the highest inhibition, while higher mordant concentrations reduced activity, which is likely due to stronger pigment fixation reducing diffusion of active compounds, a pattern also noted in textile applications of *Monascus* and *Talaromyces* pigments[29,32]. Given the growing demand for antimicrobial textiles in healthcare and hygiene applications, the T8 pigment's activity against *S. aureus* is particularly valuable.

The crude pigment showed moderate antioxidant activity (IC_{50} = 59.45 µg/mL), comparable with values reported for *Talaromyces purpureogenus* and *Fusarium proliferatum* pigments[33,34]. Although less potent than L-ascorbic acid, the T8 pigment's scavenging ability suggests the presence of phenolic, pyranone, or furanone metabolites capable of donating electrons or hydrogen atoms. Remarkably, antioxidant properties of the T8 pigment increase its value for cosmetics, packaging, and functional textiles. The pigment exhibited no cytotoxic effects toward human dermal fibroblasts, with viability remaining above 70% at all concentrations, meeting ISO 10993-5 standards for non-cytotoxic materials[13]. Similar safety profiles have been documented for pigments from *Talaromyces albobiverticillius* and *Penicillium oxalicum*[29]. Importantly, unlike *Monascus* pigments, which may co-produce the mycotoxin citrinin[35], the T8 pigment showed no toxicity, supporting its safety for topical and textile applications.

GC–MS profiling revealed 66 metabolites, with nine major compounds contributing to the pigment's bioactivity. The dominant metabolite, a pyranone derivative (17.97%), is well recognized for antimicrobial and antioxidant activities and is commonly found in edible fungi[14]. Furanones such as 4-hydroxy-2,5-dimethyl-3(2H)-furanone, widely used as food flavor compounds, were also detected and are known for strong antioxidant properties[36,37]. Compounds such as maleic hydrazide and indole–triazole derivatives have documented antibacterial or pharmacological effects[22,23]. The presence of diverse metabolites indicates that the pigment's biological activities likely arise from synergistic effects rather than a single active compound, a pattern consistent with reports from *Epicoccum nigrum* and *Talaromyces* spp.[38].

The LC–MS/MS analysis demonstrated that oosporein is the predominant secondary metabolite in the crude pigment extract after excluding primary metabolites derived from the culture medium. Oosporein is a quinone-based pigment widely reported in filamentous fungi and is known for its strong chromophoric properties[39], which facilitate effective light absorption and interaction with textile fibers. Its chemical structure enables hydrogen bonding and π–π interactions with protein-based fibers such as silk, suggesting favorable dye–fiber affinity and potential for good color strength and fastness[40]. These characteristics highlight the potential of the pigment as a sustainable alternative to synthetic dyes, aligning with the growing demand for eco-friendly and biodegradable colorants[41].

Despite these promising properties, oosporein has also been associated with various biological activities, including antimicrobial and cytotoxic effects[39,42]. This raises important safety considerations, particularly for applications involving direct skin contact. Interestingly, the present study demonstrated that the crude pigment extract from *A. cupreus* T8 exhibited no cytotoxicity toward normal human dermal fibroblasts (NHDFs) under the tested conditions. Cell viability remained above 85% across all concentrations and exceeded the 70% threshold defined by ISO 10993–5, indicating that the extract can be classified as non-cytotoxic[13]. Furthermore, the slight increase in cell viability at lower concentrations suggests a possible hormetic response, indicating mild stimulation of cellular metabolic activity rather than toxicity[43]. The discrepancy between previously reported cytotoxic effects of oosporein and the non-cytotoxic behavior observed in this study can be attributed to several factors. First, the use of a crude extract rather than purified oosporein means that the observed biological response reflects the combined effects of multiple co-existing metabolites. LC–MS/MS analysis revealed the presence of additional compounds, including medium-derived constituents and metabolic byproducts, which may attenuate or modulate the biological activity of oosporein[44]. Second, differences in cell type sensitivity are likely to contribute, as NHDFs are normal skin cells that may exhibit greater tolerance compared to other cell models. Additionally, the effective concentration of oosporein within the crude extract may not have reached a threshold sufficient to induce measurable cytotoxic effects.

From a textile application perspective, the combination of strong coloration and low cytotoxicity observed in the crude extract is particularly encouraging. The demonstrated biocompatibility suggests its potential suitability for dyeing fabrics intended for direct skin contact. However, further evaluation of dyeing performance is required, including assessments of color fastness to washing and rubbing, as well as performance across different fabric types. To further enhance the applicability of this pigment, strategies such as partial purification, optimization of fermentation conditions, and encapsulation techniques may be explored to balance color performance with safety. In addition, comprehensive toxicological assessments, including long-term exposure studies and mechanistic investigations (e.g., oxidative stress, apoptosis, and genotoxicity), will be essential to fully establish the safety profile of the pigment. In summary, this study highlights both the potential and the challenges associated with fungal-derived pigments. The identification of oosporein as the major pigment, combined with the demonstrated biocompatibility of the crude extract, positions *A. cupreus* T8 as a promising candidate for sustainable textile dye development. These findings further support the potential of endophytic fungi as valuable resources for eco-friendly, non-toxic, and multifunctional natural colorants.

Methods

Isolation, maintenance, and pigment observation of endophytic fungi

Endophytic fungi were isolated from healthy leaves of *Rhodomyrtus tomentosa* (Aiton) Hassk. collected in Songkhla Province, Thailand. The plant material used in this study was obtained from cultivated specimens maintained in Thailand, which were originally collected in Songkhla Province by Assoc. Prof. Dr. Lakkhana Kanhayuwa Wingfield (Division of Biological Science, Faculty of Science, Prince of Songkla

University). The collection was conducted in accordance with applicable local regulations, and no specific permits were required as the material was derived from cultivated sources. The plant species was identified by J. Passamon, and a voucher specimen (KW. Lakkhana 001; voucher no. 0014620) has been deposited in the herbarium of the Division of Biological Science, Faculty of Science, Prince of Songkla University, Thailand. Leaf samples were surface-sterilized by immersion in 70% ethanol (1 min), followed by 3% sodium hypochlorite (3 min), and rinsed three times with sterile distilled water. Sterilized leaf sections (5 × 5 mm) were placed on potato dextrose agar (PDA) supplemented with 50 mg/L chloramphenicol and incubated at 28 °C for 5–7 days. Emerging fungal hyphae were transferred to fresh PDA plates to obtain pure cultures. All isolates were maintained on PDA slants at 4 °C and routinely subcultured on PDA at 28 °C for 7 days before characterization. Pigment production was assessed by observing extracellular pigment diffusion into the agar. All fungal strains were stored and maintained at the Mycology Laboratory, Division of Biological Science, Prince of Songkla University, for use in subsequent experiments.

Identification of fungal strain T8

For morphological identification, strain T8 was identified to the genus and species levels based on its macroscopic and microscopic characteristics following the taxonomic keys. The strain was cultured on PDA and incubated at 28 °C for 7 days, after which colony characteristics were recorded. Microscopic features of vegetative and reproductive structures were examined using a compound microscope (Olympus CX23, Japan), and detailed morphological observations were performed using the slide culture technique. Molecular identification was conducted using the internal transcribed spacer (ITS) region. Genomic DNA was extracted from fresh mycelia using the DNeasy® Plant Mini Kit (QIAGEN, UK) following the manufacturer's protocol. PCR amplification was carried out using the primer set and cycling conditions described by Wingfield et al[45]. Amplicons were verified by agarose gel electrophoresis, purified using the MinElute® Gel Extraction Kit (QIAGEN), and sequenced on a 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA) by Macrogen (Seoul, Korea). The resulting ITS sequences were queried using BLASTn against the NCBI GenBank database to determine the closest matching taxa. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 12.1[46], where sequences were aligned with MUSCLE and manually refined prior to constructing a maximum–likelihood tree. Branch support was assessed using 1,000 bootstrap replicates.

Fungal fermentation

Four mycelial agar plugs from a 7-day-old culture of strain T8 were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth (PDB), prepared in triplicate, and incubated at 25 °C for 3 weeks. After incubation, the culture broth was filtered to separate the mycelium. The filtrate was collected and freeze-dried for 48 h, while the harvested mycelium was washed three times with distilled water and dried at 50 °C for 16 h. The dry weight (DW) of both the mycelium and the crude fungal pigment was recorded. Pigment yield was expressed as mg of crude pigment per g of mycelial DW per liter of PDB and calculated using the following formula: Biomass estimation = (DW of crude fungal pigment / DW of mycelium) × 1000

Characterization of crude T8 pigment

Solubility test

The solubility of the crude pigment was evaluated in various solvents, including distilled water, absolute methanol, 95% ethanol, ethyl acetate, and hexane. The assay was conducted following the method of Ahmad et al[47] with minor modifications. One milligram of crude pigment was transferred into test tubes containing 3 mL of each solvent. The mixtures were vortexed and kept in darkness at room temperature (25 °C). After 12 h, the absorption spectrum (400–700 nm) of each solution was measured using a spectrophotometer, and the pigment intensity at 500 nm was recorded. All treatments were conducted in triplicate.

Stability test

The stability of the pigment toward pH, temperature, light, and autoclaving conditions was assessed according to Velmurugan et al[48] and Ahmad et al[47] with modifications. Pigment solutions (0.1 mg/mL in distilled water; 3 mL total volume) were adjusted to pH 4–11 using 1 M NaOH or 1 M HCl. Samples were incubated at room temperature for 2 h, and the effect of pH on pigment stability was recorded. For thermal stability, pigment samples were incubated in a water bath at 40, 60, 80, 100 and 120 °C for 2 h. Light stability was assessed by exposing samples to sunlight (11:00 a.m.–13:00 p.m.) and to UV light for 2 h. Autoclave stability was evaluated under standard sterilization conditions. Following each treatment, the absorption spectrum was scanned, and pigment intensity at 500 nm was compared with the untreated control. The percentage stability of the pigment was calculated using the formula: Stability (%) = (As/Ao) x 100, where As is the absorbance after treatment and Ao is the initial absorbance before treatment. All analyses were performed in triplicate.

Antioxidant assay

The *in vitro* antioxidant activity of the crude T8 pigment was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay. Crude pigment was diluted in distilled water to obtain six concentrations (3.125–100 µg/mL). A 200 µM DPPH solution in methanol served as the radical source, and L–ascorbic acid, prepared in six concentrations (0.3125–10 µg/mL), was used as the positive control. Equal volumes of sample and DPPH solution were mixed, transferred to a 96–well plate, and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a microplate reader (SpectraMax iD5). Radical scavenging activity (% inhibition) was calculated as: % inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$, where $A_{control}$ and A_{sample} are the absorbance values of DPPH free radical solution without the test sample, and with the test sample, respectively. The IC_{50} value was determined from plots of percentage inhibition versus concentration. Results represent mean ± SD from three independent experiments.

In vitro cytotoxicity assay

The in vitro cytotoxicity of the crude fungal pigment was evaluated using the MTT assay in accordance with ISO 10993-5 guidelines. Normal human dermal fibroblasts (NHDFs), representing human skin cells, were used to assess the safety of the pigment for potential topical applications. NHDFs were purchased from ATCC (PCS-201-012, Manassas, VA, USA). Cells were cultured under standard conditions (37 °C, 5% CO_2) for 24 h prior to treatment. The non-treated control (NT) consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM GlutaMAX™, and 1% penicillin–streptomycin, while the positive control (PC) was prepared by adding 1.5 mM organotin to NT medium. The crude pigment stock solution (10 mg/mL in NT) was serially diluted to obtain final concentrations ranging from 0.0156 to 2.0000 mg/mL. Following 24 h exposure to the pigment, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 3 h at 37 °C. The resulting formazan crystals were solubilized with 200 µL dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated relative to the NT control, and the half-maximal inhibitory concentration (IC_{50}) was determined from dose–response curves. Cytotoxicity was interpreted based on ISO 10993-5 criteria, where cell viability ≥ 70% at the highest tested concentration is considered non-cytotoxic. All results are presented as the mean of four independent experiments.

Characterization of T8–dyed textile

Cotton dyeing procedure

Raw cotton fabric was cut into 5 × 5 cm pieces, pre-soaked, and boiled in a 1 g/L dishwashing solution at 70 °C for 1 h. The fabric was then rinsed thoroughly with distilled water and air-dried. Pre-treated cotton pieces were subsequently mordanted with alum at concentrations of 5%, 10%, and 15% (w/v) at 70 °C for 45 min, then rinsed with distilled water and air-dried. One hundred milligrams of dried crude pigment were dissolved in 500 mL distilled water. Both un-mordanted and mordanted cotton fabrics were dyed in the pigment solution at 70 °C for 45 min. Following dyeing, the fabrics were treated with 1% acetic acid, washed three times with distilled water, and dried overnight.

pH variation dyeing method

To assess pH–dependent color variation, 100 mg of crude pigment were dissolved in 500 mL of distilled water and the solution pH was adjusted to between 4 and 10 using 1 M NaOH or 1 M HCl. Dyeing experiments were conducted on pre-treated cotton yarn mordanted with 5% alum. The yarn was dyed at a material-liquor ratio (MLR) of 1:50 at 70 °C for 45 min. After dyeing, yarns were treated with 1% acetic acid, rinsed three times with distilled water, and dried overnight.

Antibacterial activity of dyed fabrics

The antibacterial activity of T8–dyed fabrics was evaluated against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Fabric samples (1 × 1 cm) were sterilized by autoclaving prior to testing. Bacterial suspensions were prepared in Mueller–Hinton broth (MHB), incubated at 35 °C for 3 h, and adjusted to approximately 1.5×10^8 CFU/mL. Sterile fabrics, including untreated controls, un-mordanted dyed samples, and those mordanted with 5–15% alum, were placed on Mueller–Hinton agar (MHA) plates

swabbed with bacterial suspensions and incubated at 35 °C for 24 h; inhibition zones were measured. Fabrics were also assessed using AATCC 100–2004 method[49] by immersing samples in 5 mL of bacterial suspension (10^3 CFU/mL) for 24 h, followed by plating aliquots to determine surviving cells. Antibacterial activity was expressed as percent reduction (%R) relative to the untreated control using the equation: %R = [(B – A)/B] x 100, where A is the colony forming unit (CFU)/mL recovered from bacteria exposed to dyed fabrics, and B is the CFU/mL from bacteria exposed to undyed control fabrics.

Gas chromatography–mass spectrometry (GC–MS) profiling of crude pigment

GC–MS analysis of crude T8 pigment was performed using an Agilent 7890B GC system coupled to a 5977A Mass Selective Detector. Separation was achieved on a VF–WAXms capillary column (30 m × 250 µm × 0.25 µm) with helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 60 °C, ramped to 160 °C at 10 °C/min, then increased to 250 °C at 2.5 °C/min and held for 15 min. The mass spectrometer operated in electron ionization mode at 70 eV with a source temperature of 230 °C, scanning from m/z 35–500. Chemical constituents were identified by comparing mass spectra with entries in the Wiley spectral library.

Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) analysis of crude pigment

The fungal pigment extract was subjected to LC–MS/MS for compound profiling. Prior to analysis, the extract was reconstituted in ethanol and filtered through a 0.22 µm nylon membrane filter before injection. LC–MS/MS analysis was carried out using a SCIEX X500 QTOF mass spectrometer operated in both negative and positive electrospray ionization (ESI) modes. Data acquisition was performed separately in each ionization mode, and chromatographic data were recorded as total ion chromatograms (TICs). MS/MS acquisition was conducted using a collision energy of approximately 35 ± 15 eV. Compound identification was performed using SCIEX OS software (version 2.0.0) based on accurate mass measurement, retention time, MS/MS fragmentation patterns, and library matching via the Smart Confirmation workflow. Putative compound annotation was assigned based on library match score, precursor ion mass, observed mass, and fragmentation pattern. A control extraction of uninoculated potato dextrose broth (PDB) medium was analyzed under identical LC–MS/MS conditions. Chromatographic separation was achieved using a ZORBAX Eclipse Plus C18 column (150 mm × 2.1 mm i.d., 1.8 µm) maintained at 35°C. The mobile phase consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile, delivered at a flow rate of 0.2 mL/min with the following gradient program: 0.00 min, 35% B; 14.00 min, 35% B; 22.00 min, 60% B; 28.00 min, 80% B; 34.00 min, 100% B; 40.00 min, 100% B; 45.00 min, 10% B. The injection volume was 5 µL, and the total run time was 45 min.

Statistical analysis

All experiments were performed in triplicate. Data is presented as a mean standard deviation (SD). Statistical significance was assessed using either Student’s t-test or one-way analysis of variance (ANOVA), followed by Tukey’s post hoc multiple comparison test to identify differences among group

means. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analyses were conducted using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA).

Declarations

Data availability

The sequence data of *Arcopilus cupreus* generated in this study are available in the NCBI GenBank repository under accession number PZ292733 (https://www.ncbi.nlm.nih.gov/). All other data generated or analyzed during this study are included in this published article and its supplementary information files.

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Author contributions statement

N.C. and L.K.W. conceived and designed the experiments, N.C., N.J. and L.K.W. conducted the experiments, N.C. and L.K.W. analyzed the results. L.K.W. drafted the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests

Additional information

Supplementary Information

Supplementary Information

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Figures

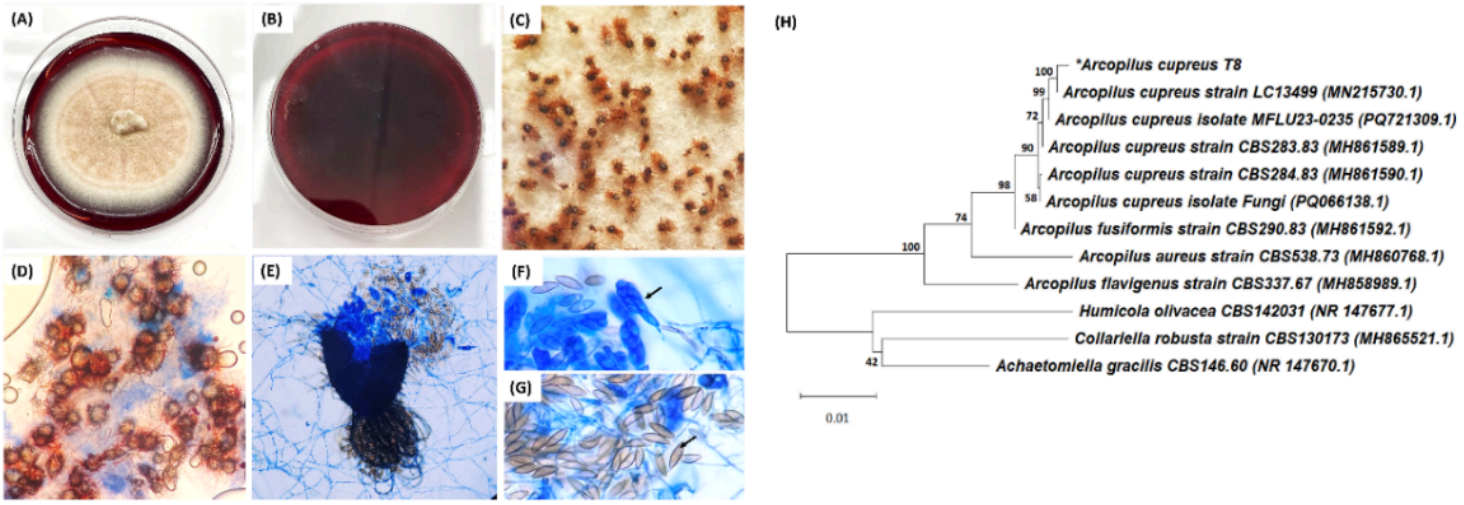

Figure 1

Morphological and molecular identification of the pigment-producing endophytic fungus *Arcopilus cupreus* T8. (A–B) Colony morphology of strain T8 on PDA after 7 days at 28 °C showing a brown central region,

white peripheral mycelia, and secretion of a diffusible red pigment into the medium. (C–E) Microscopic features of T8 displaying rounded to ovoid ostiolate ascomata covered with characteristic hairs. (F) Ascus. (G) Ascospores. (H) Phylogenetic placement of strain T8 based on ITS rDNA sequence analysis, showing 100% sequence similarity to *Arcopilus cupreus*.

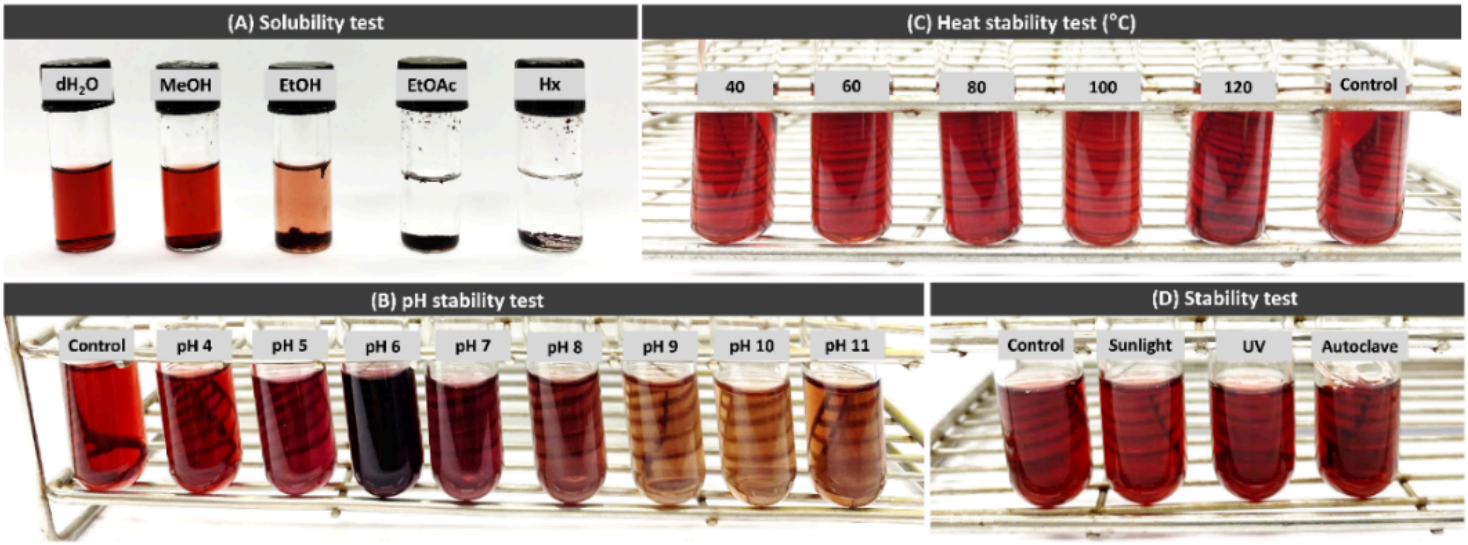

Figure 2

Characterization of the crude fungal pigment produced by *A. cupreus* T8. (A) Solubility of the crude pigment in various solvent systems. (B) pH stability profile across different pH values. (C) Thermal stability of the pigment at different temperatures. (D) Stability of the pigment under diverse environmental conditions.

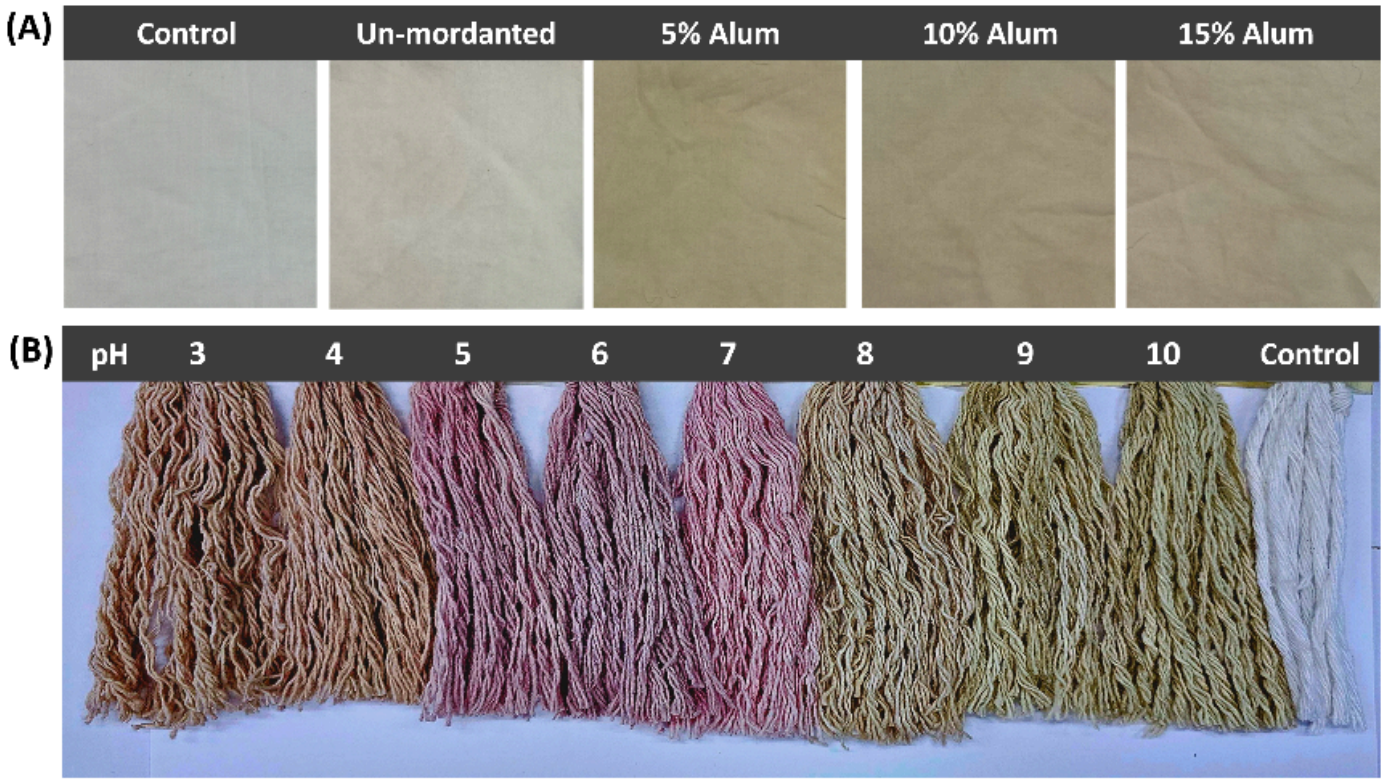

Figure 3

Effect of mordanting and pH on cotton dyeing using crude pigment from *A. cupreus* T8. (A) Cotton fabrics dyed with crude pigment from *A. cupreus* T8 under different mordant conditions. (B) Color variation of cotton yarns dyed with *A. cupreus* T8 crude pigment under different pH conditions.

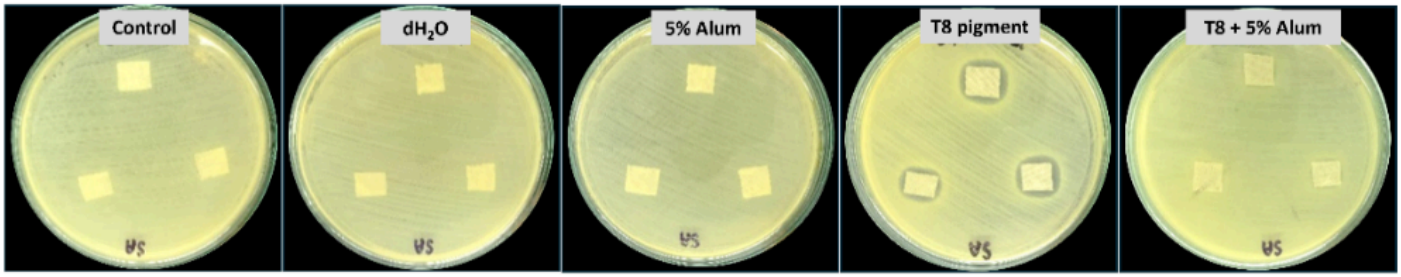

Figure 4

Antibacterial activity of *A. cupreus*T8–dyed cotton fabrics against *S. aureus* using the agar diffusion plate test.

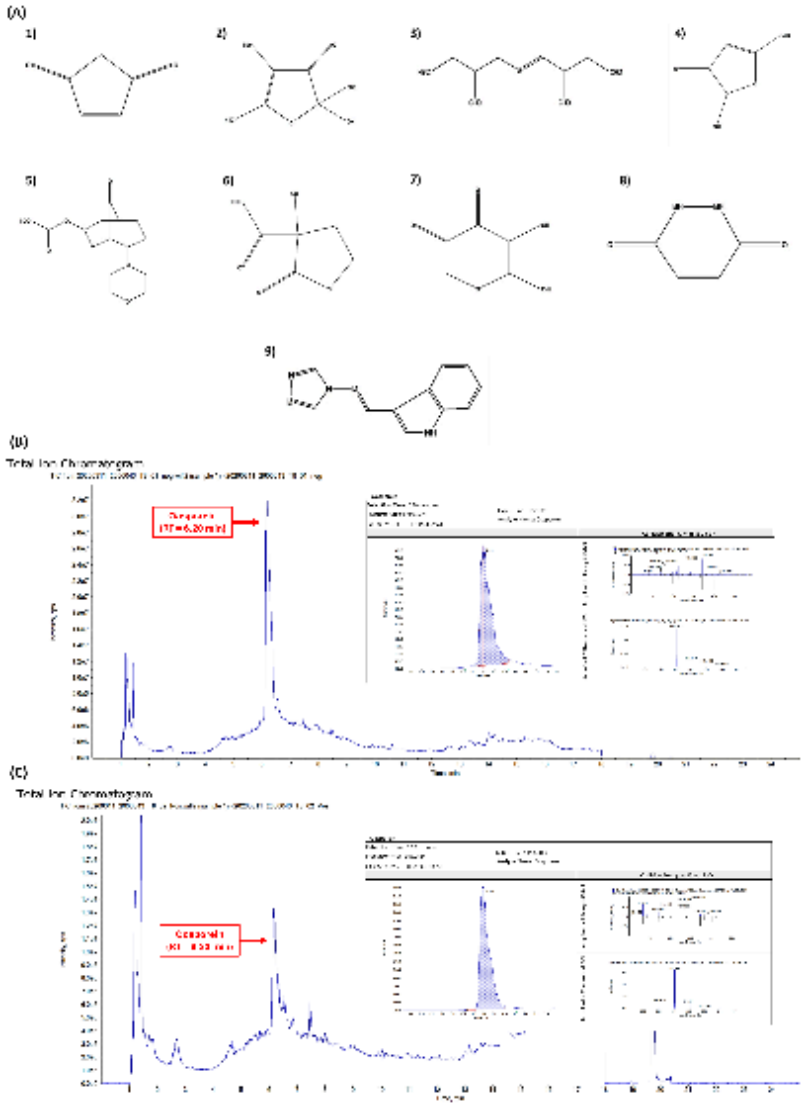

Figure 5

Chemical structures of nine major metabolites tentatively identified from the crude pigment extract of *A. cupreus* T8 based on GC–MS analysis (A), together with total ion chromatograms (TIC) obtained in (B) negative and (C) positive ionization modes. A prominent peak corresponding to oosporein is observed at approximately 6.2 min in both modes. The identified metabolites represent the dominant constituents contributing to the pigment's bioactive profile.

Supplementary Files

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- TableSUPP.docx