Scientific Research Publishing

Advances in Biological Chemistry, 2025, 15(2), 31-39
https://www.scirp.org/journal/abc
ISSN Online: 2162-2191
ISSN Print: 2162-2183

Phytochemical Analysis and Antibacterial Activity of Trunk Bark of *Erythrina excelsa* Baker

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How to cite this paper: Djouatsa, Y.N.N., Tematio, E.L., Fouokeng, Y., Nkouayeb, B.M.N., Kamdem, A.F.W., Martínez, E.J.L., Tsopgni, W.D.T. and Azebaze, A.G.B. (2025) Phytochemical Analysis and Antibacterial Activity of Trunk Bark of Erythrina excelsa Baker. *Advances in Biological Chemistry*, **15**, 31-39.
https://doi.org/10.4236/abc.2025.152003

Received: February 25, 2025
Accepted: April 14, 2025
Published: April 17, 2025

Abstract

Pseudomonas agarici is a bacterial strain responsible for at least three diseases affecting cultivated mushrooms: drippy gill, brown discoloration and yellow blotch. The present study aimed to investigate the trunk barks extract of *Erythrina excelsa* Baker, to find bioactive constituents against *Pseudomonas agarici*. The bioactivity guided fractionation of the methanolic extract yielded ten known compounds: neorautenol (**1**), calopocarpin (**2**), abyssinone-IV-4′-O-methyl ether (**3**), abyssinone-V-24′-O-methyl ether (**4**), abyssinone V (**5**), (-)-sigmoidin E (**6**), 4′-O-methylderrone (**7**), erycaffra A (**8**), genistein (**9**) and 5,4′-dihydroxy-2″-hydroxyisopropyldihydrofurano[4,5:7,8]isoflavone (**10**). Their structures have been established using one-dimensional (1D) and two-dimensional (2D) Nuclear Magnetic Resonance (NMR) experiments in combination with Infared (IR) and Mass Spectrometry (MS). The broth microdilution method was used to assess the antibacterial activity of the crude extract against *Pseudomonas agarici* and three others strains namely *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus warneri*. The crude extract and *n*-Hexane/ethyl acetate (7:3) fraction exhibited significant activity against *P. agarici*, with Minimum Inhibitory Concentration (MIC) values of 36.9 and 27.1 µg/mL, respectively. From the isolates, only calopocarpin (**2**) showed significant activity against *P. agarici*, with MIC value of 12.8 µg/mL, comparable to gentamycin as reference.

Keywords

Erythrina excelsa, Antimicrobial Activities, Calopocarpin, *Pseudomonas agarici*

1. Introduction

Different species of cultivated mushrooms faced numerous diseases caused by fluorescent pseudomonas among which *Pseudomonas agarici*, which is responsible for at least three reported diseases affecting mushrooms crop. The first, a disease called drippy gill was reported in 1970 [1]. The pathogen damages the gills, after the inner veil has been broken, and the mushrooms do not develop, or their growth is delayed and they become distorted [2]. *P. agarici* has also been reported to cause brown discoloration, a disease affecting *Agaricus bisporus* and yellow blotch, a disease which damages *Pleurotus ostreatus*. The impact of *Pseudomonas agarici* on mushroom cultivation goes beyond just yield and quality reduction. Infected mushrooms are unsuitable for sale resulting in enormous economic losses and reduced income for producers [3]. The disease's occurrence varies from country to country and from year to year. In Western Europe, it has been reported that the incidence can vary from 8% to 15% of crop weight. In dramatic years, harvests can be reduced by 40% - 50% [4]-[6]. Mushroom growers in Africa are frequently faced with bacterial and fungal diseases with the same consequences, but no economic data or statistics are available. The presence of this pathogen can also lead to secondary infections from other pathogens, further compromising the crop. Control against these diseases is based on environmental control (relative humidity, temperature and CO_2 levels in the growing room) chemical and biological control. These methods are not accessible to growers [7] [8]. Thus alternative control methods must therefore be considered.

The pharmacological and phytochemical studies of plants used in pharmacopeia could lead not only to the discovery of new antibacterial compounds, but also to a biological solution. This study focuses on biological activities of *Erythrina excelsa* also know as *Erythrina bagshawei*, a plant belonging to family of Fabaceae. Various species of the Erythrina genus are used in traditional medicine to treat microbial infections and inflammation [9] [10] dizziness, amenorrhea, headache, eye disorders [11], female sterility [12], liver dysfunctions, asthma, epilepsy, malaria [13] [14], and injuries [15]. Previous chemical studies report the presence of alkaloids and flavonoids as the main bioactive compounds [16] [17]. Triterpenes, stilbenes, sterols, coumarins, phenolic esters and pterocarpans have also been reported [18]. The aim of this work was to investigate the trunk barks of *Erythrina excelsa* following a bioactivity guided approach, in order to find active compounds against *Pseudomonas agarici*.

2. Materials and Methods

2.1. Plant Materials

The trunk bark of *E. excelsa* was collected on January 2017, in Bangangté, West region of Cameroon, more precisely at Bandiangseu, located between latitude 5˚8'39.6816"N and longitude 10˚31'26.3532"E, altitude above sea level 1350 m. The plant was identified by Mr. Victor Nana of the National Herbarium of Cameroon, Yaoundé, where a voucher specimen (N˚61487/HNC) has been deposited.

2.2. Extraction and Isolation

The trunk barks were sliced into pieces and dried in the open air in the shade at room temperature, then grounded into powder. Then, 4.50 kg of the powdered plant material was extracted with MeOH (15 L) (48 h then 24 h) at room temperature. After filtration using Whatman filter paper N˚1, the solution was dried under reduced pressure at low temperature to give 350.00 g of a red crude extract. A portion of 300.00 g of the extract was submitted to open column chromatography (length: 70 cm and inner diameter 4 cm) eluting with *n*-Hexane, mixtures of *n*-Hexane/EtOAc and EtOAc of increasing polarities. A total of 100 fractions of 250 mL each were collected and combined on the basis of the Thin Layer Chromatography (TLC) profiles into 06 main fractions as follow: F1 (Hex/EtOAc (9:1)), F2 (Hex//EtOAc (4:1)), F3 (Hex//EtOAc (7:3)), F4 (Hex//EtOAc (2:3)), F5 (Hex//EtOAc (1:4)) and F6 (EtOAc). Fraction F3 which displayed a good antibacterial effect against *Pseudomonas agarici*, was selected for further steps. The other fractions were found to be either inactive. F3 (1000.50 mg, Hex-EtOAc (3:2, v/v)) was sub-fractionated on silica gel column chromatography (length: 70 cm and inner diameter 1.5 cm) with an isocratic solvent system of Hex-EtOAc (13:7, v/v) to give 100 sub-fractions of 25 mL each, which were combined on the basis of the Thin Layer Chromatography (TLC) profiles into 03 subfractions F3A (250.15 mg), F3B (300.50 mg) and F3C (400.50 mg). Sub-fraction F3A was then suggested to Sephadex LH-20 column chromatography (length: 70 cm and inner diameter 1.5 cm), eluted with methanol to give compounds 3 (10.15 mg), 4 (7.10 mg) and 5 (5.10 mg), while F3B gave compounds 6 (10.15 mg), and 7 (5.10 mg). By the same means, F3C was further chromatographed on Sephadex LH-20 column chromatography (length: 70 cm and inner diameter 1.5 cm), eluted with methanol to give compounds 1 (6.50 mg), 2 (4.30 mg), 9 (8.50 mg) and 10 (4.05 mg).

2.3. Experimental Procedures

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 500 MHz NMR spectrometer equipped with a 5 mm cryogenic DCH $^{13}C/^{1}H$ probe head. Chemical shifts (δ) were reported in parts per million (ppm). Coupling constants (J) were reported in Hz. MS data were obtained on a quadrupole-orbitrap mass spectrometer (Thermo Scientific, Waltham) using electro-spray ionization in both modes. The spray voltage was fixed at 3.5 kV; the sheath gas flow rate (N_2) at 50 units; the capillary temperature was set at 320˚C; the S-lens RF level at 50; and the probe heater temperature was set at 425˚C. Column chromatography was carried out on silica gel (70 - 230 mesh, Merck), Sephadex LH-20 and flash silica gel (230 - 400 mesh, Merck). TLC was performed on Merck precoated silica gel 60 F254 aluminium foil, using sulfuric acid spray reagent for visualization. All reagents used were of analytical grade.

3. Antibacterial Assay

The antimicrobial activity of the isolated compounds was determined using mi-

cro-dilution method with some modification [13] [19]. In this study, *Escherichia coli* (DSMZ1058), *Bacillus subtilis* (DSMZ704), *Pseudomonas agarici* (DSMZ11810) and *Staphylococcus warneri* (DSMZ20036) bacterial strains were grown on agar medium (3 $g{\cdot}L^{-1}$ beef extract, 10 $g{\cdot}L^{-1}$ peptone and 20 $g{\cdot}L^{-1}$ agar) and the pH was adjusted to 7.2. The test was undertaken by inoculating a suspension of the overnight tested microorganism (DO_{600} = 0.05 - 0.1) on the nutrient agar medium. The compounds tested were diluted with 100 µL of broth in the first well of a 96-well microtiter plate. The compounds tested were diluted with 100 µL of broth in the first well of a 96-well microtiter plate. The final concentrations of the compounds were between 250 $mg{\cdot}mL^{-1}$ to 0.01 $mg{\cdot}mL^{-1}$. Microtiter plates containing *Escherichia coli* and *Staphylococcus warneri* were incubated at 37˚C and those containing *Bacillus subtilis* and *Pseudomonas agarici* were incubated at 30˚C for 24 h. The minimum inhibition concentration (MIC) was calculated as a sigmoidal dose response curve using GraphPad Prism 4.03.3.

4. Results and Discussion

The methanol extract of trunk barks of *E. excelsa* including fractions F1 (Hex/EtOAc (9:1)), F2 (Hex//EtOAc (4:1)), F3 (Hex//EtOAc (7:3)), F4 (Hex//EtOAc (2:3)), F5 (Hex//EtOAc (1:4)) and F6 (EtOAc) were evaluated for antimicrobial activity against *Pseudomonas agarici* DSMZ11810. From the results (**Table 1**) the crude extract displayed a significant activity against *Pseudomonas agarici* with a MIC value of 36.9 µg/mL.

Table 1. MIC (µg/mL) of the trunk barks extract, fractions and isolates against tested bacterial stains.

	Microbial organisms			
Samples	**Psa**	**Ecoli**	**Bs**	**Stw**
Crude extract	36.9	>50	>50	>50
F3	27.1	>50	>50	>50
F1, F2, F4, F5 and F6	>50	>50	>50	>50
1, 3 - 10	>50	>50	>50	>50
2	12.8	>50	6.1	9.2
4	>50	>50	>50	8.5
Gentamycin	5.5	3.7	2.6	2.1

Psa = *Pseudomonas agarici* (DSMZ11810); Ecoli = *Escherichia coli* (DSMZ1058); Bs = *Bacillus subtilis* (DSMZ704); Stw = *Staphylococcus warneri* (DSMZ20036); F1 to F6 = fractions from crude extract; 1 to 10 = Isolated compounds from fraction F3; MIC = Minimum Inhibitory Concentration. Samples with MICs > 50 are inactive according to the applied methodology.

After fractionation, fraction F3 (Hex/EtOAc (7:3)) appeared to be the only active fraction with a MIC value of 27.1 µg/mL, indicating that it could contain com-

pounds responsible for this activity. Fraction F3 was then submitted to separations and purification using column chromatography. This resulted in the isolation of 10 compounds whose structures were determined on the basis of NMR and mass analysis, and confirmed by comparison with previously reported data (**Figure 1**).

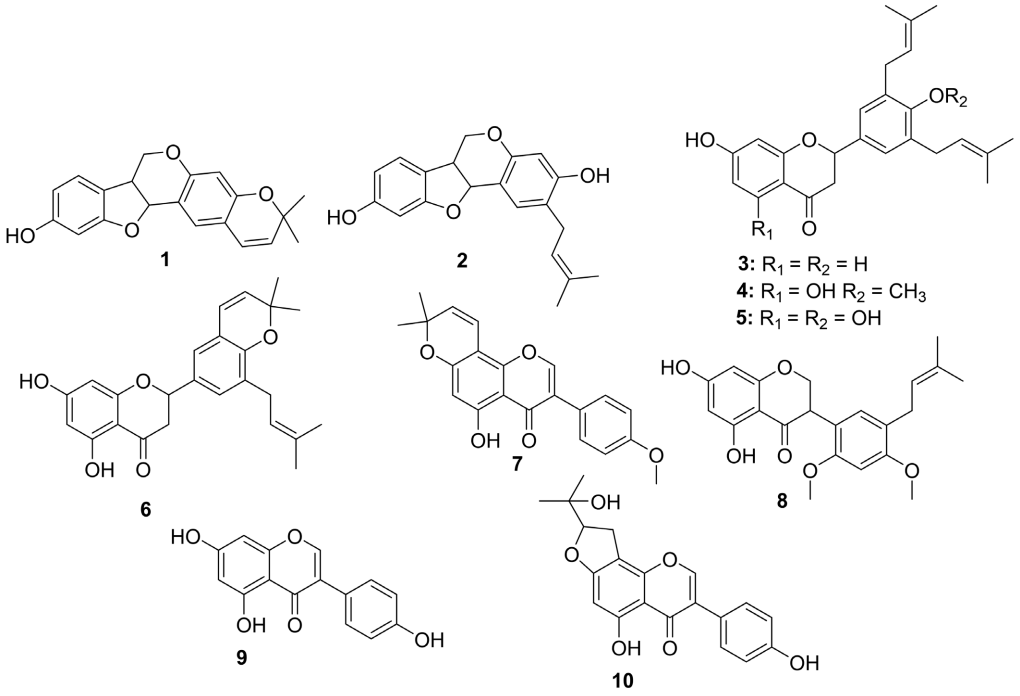

Figure 1. Structures of isolated compounds 1 - 10 from *E. excelsa.*

They were identified as two pterocarpans: neorautenol (**1**) [20], calopocarpin (**2**) [21], four flavanones: abyssinone-IV-4′-*O*-methyl ether (**3**) [21], abyssinone-V-24′-*O*-methyl ether (**4**), abyssinone V (**5**) [20], (-)-sigmoidin E (**6**) [22], four isoflavanones: 4′-*O*-methylderrone (**7**) [23], erycaffra A (**8**) [24], genistein (**9**) [25], and 5,4′-dihydroxy-2″-hydroxyisopropyldihydrofurano[4,5:7,8]isoflavone (**10**) [26], The antibacterial activity of these then compounds on *Pseudomonas agarici* has been also evaluated (**Table 1**). Only calopocarpin (**2**) appeared to be active on this strain. It showed a significant activity close to those of gentamycin (reference compound) with a MIC value of 12.8 µg/mL. It could be responsible for the activity observed with the fraction F3 and the extract.

Antibacterial activity of all samples (extract, fractions and isolated compounds) has been also evaluated on other pathogen strains *Escherichia coli* DSMZ1058 (Gram negative), *Bacillus subtilis* DSMZ704 and, *Staphylococcus warneri* DSMZ20036 (gram positive) (**Table 1**). Only calopocarpin (**2**) and a0.

byssinone-V-24′-*O*-methyl ether (**4**) displayed significant activities. The former (**2**) was active against *Bacillus subtilis* and *Staphylococcus warneri* with MIC values of 6.1 and 9.2 µg/mL respectively, while the latter (**4**) was only active against *Staphylococcus warneri* with an MIC value of 8.5 µg/mL. Within the group of flavonoids found in the genus *Erythrina*, pterocarpan has been identified as the

most active class with antibacterial activity, particularly against *Staphylococcus aureus*. The study of the structure-activity relationship highlighted the essential contribution of the prenyl functional group to improving the antibacterial activity of flavonoids. It has been reported that increasing the number of prenyl groups increased antibacterial efficacy, while decreasing the number of prenyl groups reduced antibacterial activity [27]. The antibacterial activity of calopocarpin may be due to the presence of a prenyl group in its structure. It has been reported that the antibacterial mechanisms of the flavonoid group found in the genus *Erythrina* include suppression of nucleic acid synthesis, disruption of cytoplasmic membrane function, inhibition of ATP synthase and modulation of energy metabolism [27]. In addition to its activity on the Staphylococcus strain, pterocarpan has shown antibacterial activity on the Streptococcus strain as well as on *Clostridium perfringens* and *Vibrio cholerae* [28], On the last-mentioned pathogens, this molecule acts as a neuraminidase inhibitor, reducing the adhesion of *V. cholerae* to the host cell [29]. Further work is currently required to precisely determine the mechanism of action on the strains tested where the molecule showed activity.

This result contributes to acknowledging calopocarpin as a potential agent for the development of new antibiotics.

Moreover, the methanolic extract of *E. excelsa* and particularly the fraction at Hex/EtOAc (7:3) could be used as bioagents to fight against *Pseudomonas agarici*. It could be very useful since the use of fungicides and antibiotics is restricted in many countries. Further studies need to be carried out on this plant and this compound in order to assess their efficacy on other pathogenic bacteria strains, as well as their toxicity.

5. Conclusion

In the quest for discovery of bioactive constituents against *Pseudomonas agarici* which is responsible for various diseases affecting cultivated mushrooms. Methanolic extract of trunk barks of *Erythrina excelsa* showed a significant activity against *P. agarici*. Bioguided study of crude extract indicated that the hexane/ethyl acetate (7:3) fraction appeared to be active. From this fraction, ten compounds have been isolated and only one, calopocarpin, showed a significant activity comparable to the reference gentamycin. This compound could be responsible for the activity of the extract. These preliminary results from the *in vitro* assays are in accordance with the uses of the plant in local pharmacopeia and allow us to position the plant as an effective alternative in the fight against *P. agarici*.

Availability of Data and Materials

The data used and/or analyzed during the current study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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