Densitometric HPTLC Quantification of 2-Azaanthraquinone Isolated from *Mitracarpus scaber* and Antimicrobial Activity against *Dermatophilus congolensis*

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Key Words:

HPTLC
Mitracarpus scaber
Dermatophilus congolensis
2-Azaanthraquinone

Summary

2-Azaanthraquinone (benzo[*g*]isoquinoline) isolated from *Mitracarpus scaber* has been found to have in-vitro antimicrobial activity against *Dermatophilus congolensis* (MIC 7.5 µg mL^{-1}), the causative agent of bovine dermatophilosis. To quantify this compound in *Mitracarpus scaber*, a new, simple and rapid high-performance thin-layer chromatographic (HPTLC) method was developed and validated for selectivity, recovery, and repeatability. Compounds were separated on silica gel 60F$_{254}$ plates with toluene–ethyl acetate–methanol, 80 + 18 + 2, as mobile phase. Detection was performed by densitometric scanning at λ = 310 nm and calibration plots showed the response was linearly dependent on concentration in the range 10–100 µg mL^{-1}, with good values of R^2. The method was repeatable and precise with relative standard deviations between 0.98 and 1.59% intra-day and between 3.41 and 5.56% inter-day for concentrations from 10 to 100 µg mL^{-1}. Limits of detection and quantification were 3 and 6 µg mL^{-1}, respectively (corresponding to 15 and 30 ng, respectively, on the plate). Concentrations of azaanthraquinone were found to be 0.009% in *Mitracarpus scaber* aerial parts, 0.057% in the alcoholic extract, and 1.95% in the alkaloid extract. Azaanthraquinone may explain the antimicrobial activity of this last extract and part of the effect of the alcoholic extract.

1 Introduction

Bovine dermatophilosis is an enzootic skin infection of cattle in tropical and subtropical countries caused by the Gram-positive bacterium *Dermatophilus congolensis*. Infection may lead to death of the animal in severe acute cases and is difficult to cure, even by parenteral use of antibiotics [1]. In Benin and the neighboring countries of West Africa, *Mitracarpus scaber* Zucc. ex Schult + Scult.f. (Rubiaceae) is currently used in traditional medicine to treat a variety of skin diseases such as eczema [2] or ringworm [3]. Recent studies have shown that alcoholic extracts of the aerial parts of *Mitracarpus scaber* had in-vitro antimicrobial activity against *Dermatophilus congolensis* [1]. Other experiments have proved that ointments containing alcoholic extracts of *Mitracarpus scaber* used topically had a high efficiency against bovine dermatophilosis and cured tested animals without recurrence [4]. In addition to 2-azaanthraquinone (AAQ) [5], phytochemical studies have provided evidence of the presence of gallic acid, 3,4,5-trimethoxybenzoic acid, 4-methoxyacetophenone, 3,4,5-trimethoxyacetophenone, rutin, psoralen, kampferol-3-*O*-rutinoside [6], and oleanolic and ursolic acids [7] in the aerial parts of *Mitracarpus scaber*. AAQ (**Figure 1**) has been shown to have many properties – antiviral [8, 9], antimicrobial against several organisms [5, 8], and antiprotozoal against *Trypanosoma congolense* [10, 11] and against chloroquine-resistant *Plasmodium falciparum* [12].

The purpose of this work was to verify the presence of AAQ in extracts and samples of *M. scaber* grown in Benin, to evaluate its antimicrobial activity against *D. congolensis*, and to establish an HPTLC–densitometric detection method for quantification of AAQ in the plant, to determine the extent of its involvement in the activity of extracts on *D. congolensis*.

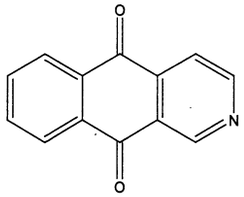

Figure 1

The structure of 2-azaanthraquinone (benzo[*g*]isoquinoline).

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2 Experimental

2.1 Chemicals and Reagents, Plant Material, and Preparation of Extracts

All chemicals and solvents were of analytical grade.

The aerial parts of the plant were collected in the area of Cotonou, Abomey-Calavi and were identified and authenticated by the National Herbarium of the University of Abomey–Calavi in the Republic of Benin, where a voucher specimen (nr: AA.6272/HNB) was deposited. The plant was first dried at room temperature for 5 days, during which time it was turned over every day, then dried in an oven at 50°C for 48 h and subsequently reduced to coarse powder [13], by use of a grinder, and stored at room temperature. The powder (500 g) was macerated with ethanol (4 L; Merck) for 72 h, with constant shaking, and the extract was filtered and concentrated (15.9% yield) by vacuum evaporation under reduced pressure. This extract (5 g) was dissolved in H_2SO_4 (0.5 M, 100 mL; final pH of solution 2.5) and extracted with *n*-hexane (3 × 100 mL). The aqueous solution was made alkaline (1 M NaOH) to pH 10 and extracted with CH_2Cl_2 (3 × 100 mL). The organic phase (total alkaloid extract) was concentrated (2.9% yield) and stored under refrigeration.

2.2 Purification of Azaanthraquinone

AAQ from the alkaloid extract was purified by preparative TLC on silica gel with toluene–ethyl acetate–methanol, 80 + 18 + 2, as mobile phase. Final purification was achieved by VLC (vacuum liquid chromatography) on silica gel (0.062–0.2 mm, 10 cm × 5 cm) eluted with diethyl ether (150 mL) and dichloromethane (100 mL).

2.3 Antimicrobial Test In Vitro

The agar dilution method [7] was used to determine the minimum inhibitory concentration (MIC – the minimum concentration completely inhibiting growth of the microorganism) of *Mitracarpus scaber* extracts, with tetracycline (Sigma) as positive control. The microorganisms were grown overnight on tryptone soya broth (Oxoid, UK). Inoculates of 10^3–10^4 colony-forming units (CFU) were spotted with a Denley (UK) A400 multipoint inoculator on Muller-Hinton agar supplemented with the extract or antibiotic at concentrations ranging from 1000 to 2 μg mL^{-1} for the extracts and from 64 to 0.5 μg mL^{-1} for the antibiotic and AAQ. Blank (DMSO used to dissolve extracts) was included. The plates were incubated for 4 days at 37°C. Tests were performed in duplicate, at least.

2.4 Instrumentation

2.4.1 MS System and NMR Analysis

Mass spectra in positive-ion mode were acquired with an LCQ mass spectrometer, equipped with an APCI source. Data acquisition and processing were performed with Xcalibur software. ^1H and ^{13}C NMR spectra in $CDCl_3$ were obtained on a Bucker 300.

2.4.2 HPTLC System and Analysis

Chromatography was performed on 10 cm × 20 cm silica gel $60F_{254S}$ HPTLC plates (Merck). Extracts and standard solutions (5 μL) were spotted on the plates by use of a Camag automatic TLC Sampler III. The plates were developed in a saturated vertical development chamber containing toluene–ethyl acetate–methanol, 80 + 18 + 2, as mobile phase, then dried at room temperature (30 min). Densitometry at λ = 310 nm was performed with a Camag TLC Scanner 3 in absorption mode at a scanning speed of 20 mm s^{-1}. The slit dimensions were 6.00 mm × 0.45 mm and the data resolution 100 μm per step. Data acquisition and processing were performed with Wincats software V. 1.2.6.

2.5 Quantification

2.5.1 Calibration Plots

A series of AAQ (Sigma) standards were prepared by diluting a stock solution of AAQ (1 mg mL^{-1} in toluene–ethyl acetate–methanol, 80:18:2) with the same solvent mixture to furnish solutions containing 10, 20, 25, 50, and 100 μg mL^{-1}.

2.5.2 Extract Preparation

From the total alkaloid extract (Section 2.1) a primary solution (2 mg mL^{-1}) in the mobile phase was prepared and filtered through a hydrophobic filter (PTFE Fluoropor). This primary solution was diluted with the mobile phase to furnish a solution containing 1000 μg mL^{-1}.

2.5.3 Repeatability

Interday and intraday precision were determined by analyzing five standards, concentrations from 10 to 100 μg mL^{-1}, several times (n = 5) on the same day and on different (n = 5) days.

2.5.4 Recovery

AAQ (1 mg) was added to the initial powdered plant material (50 g; Section 2.1). The total alkaloid mixture was extracted as in Section 2.1 (adapting the solvents proportionally). The same extraction procedure was used on 50 g powdered plant material without AAQ. Both extracts were analyzed by HPTLC and the recovery was calculated by use of the equation:

$$\text{Recovery} = [(A - B)/C] \times 100$$

where A is the quantity of AAQ in the spiked powder, B is the quantity of AAQ in the powder without added standard, and C is the quantity of AAQ added.

3 Results and Discussion

Table 1 shows the MIC of the alcoholic and the total alkaloid extracts of AAQ and tetracycline from *M. scaber*. All were effective against *D. congolensis* in vitro. The presence of AAQ

in the total alkaloid extract of *M. scaber* collected in Benin, after purification, was confirmed by MS and NMR analysis [5].

An HPLTC method was established to quantify AAQ in the plant. A typical chromatogram obtained from the total alkaloid extract is shown in **Figure 2**. The presence of AAQ was verified by comparison of R_F (0.50 ± 0.02), by co-elution, and by comparison of the UV spectra obtained from the sample and the standard. The limit of detection (taken as a peak height 3× the noise level) was determined as 3 µg mL^{-1} (equivalent to 15 ng on the plate) and the quantification limit (taken as a peak height 10× the noise level) was calculated as 6 µg mL^{-1} (30 ng on the plate).

There was a linear relationship between peak area and concentration in the range 10 to 100 µg mL^{-1} (50 to 500 ng on the plate) for the standard. Analysis of AAQ standard on five different days yielded the mean calibration plot $y = 36.77x + 128.83$ ($R^2 = 0.9954$) with a confidence interval of 95%. Interday and intraday ($n = 5$) variation for determination of AAQ were less than 6% for all the concentrations analyzed (**Table 2**).

The recovery of the method was 99.5 ± 0.36% ($n = 5$). The concentration of AAQ in 1 mg total alkaloid extract was calculated as 19.5 ± 0.26 µg or 1.95%; this was equivalent to 0.057% in the crude alcoholic extract which corresponded to 0.009% in the drug (powdered plant material).

4 Conclusion

Our study showed that alcoholic and alkaloid extracts of *Mitracarpus scaber*, and AAQ, have antimicrobial activity in vitro against *Dermatophilus congolensis*. We also confirmed the presence of AAQ in Beninese *Mitracarpus scaber* samples. We developed a new HPTLC method for quantification of AAQ in *Mitracarpus scaber* and validated it for selectivity, recovery, and repeatability. We also determined LOD and LOQ. This validated method enabled us to determine the quantity of AAQ – 0.009% in *Mitracarpus scaber* aerial parts, 0.057% in its alcoholic extract and 1.95% in its alkaloid extract. This concentration of AAQ explains the antimicrobial activity of the alkaloid extract against *D. congolensis* and part of the effect of the alcoholic extract of the plant.

Table 1

MIC values for AAQ, alcoholic, and total alkaloid extracts of *Mitracarpus scaber* against *D. congolensis*.

Sample	MIC [µg mL^{-1}]
DMSO	No activity
Alcoholic extract	1000
Alkaloid extract	750
Azaanthraquinone	7.5
Tetracycline	1

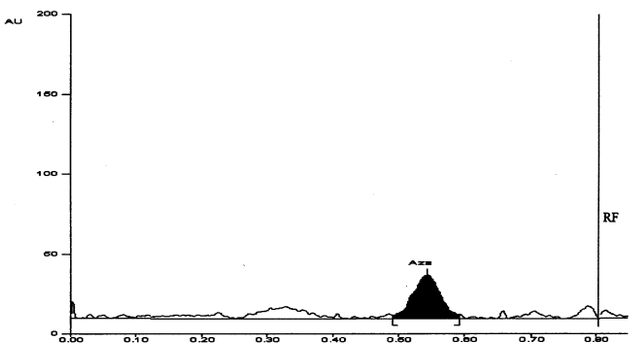

Figure 2

Typical HPTLC chromatogram obtained from *M. scaber* alkaloid extract.

Table 2

Inter- and intra-day precision for quantification of azaanthraquinone in plant material.

Concentration [µg mL^{-1}]	RSD [%]	
	Intra-day ($n = 5$)	Inter-day ($n = 5$)
100	1.93	5.65
50	1.13	3.41
25	0.98	3.59
20	1.72	3.43
10	1.59	3.96

Acknowledgment

We wish to thank the members of the microbiology laboratory at UCL, and *M.C. Crutzen*. This work was supported by the CGRI (Commissariat Général des Relations Internationales: Belgique-francophone), CIUF (Coopération Institutionnelle Universitaire francophone), UCL (Université Catholique de Louvain) and UAC (Université d'Abomey-Calavi: Bénin).

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Ms received: June 14, 2005
Accepted by ER: June 14, 2005