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Isolation and identification of antiplasmodial *N*-alkylamides from *Spilanthes acmella* flowers using centrifugal partition chromatography and ESI-IT-TOF-MS

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ABSTRACT

The development of new antiplasmodial drugs is of primary importance due to the growing problem of multi-drug resistance of malaria parasites. *Spilanthes acmella*, a plant traditionally used for the treatment of toothache, was targeted as a lead for its potential antiplasmodial activity. A systematic approach for investigating a suitable centrifugal partition chromatography (CPC) solvent system for *N*-alkylamides separation was reported. The partition behavior of three *N*-alkylamides has been studied using several biphasic solvent mixtures in search of an adequate CPC solvent system for this class of compounds. Major *N*-alkylamides in *S. acmella* were isolated from a methanolic crude extract of flowers by CPC with the solvent system heptanes–ethyl acetate–methanol–water (3:2:3:2, v/v/v/v). Four *N*-alkylamides were purified and the structures were illustrated by electrospray ionization-ion trap-time of flight-mass spectrometry (ESI-IT-TOF-MS), ^{1}H nuclear magnetic resonance (^{1}H NMR) and ^{13}C nuclear magnetic resonance (^{13}C NMR). The CPC fractions, which contained natural mixtures of phytochemicals, demonstrated significantly higher antiplasmodial activity compared to corresponding purified *N*-alkylamides, thus suggesting that interactions between these *N*-alkylamides may potentiate antiplasmodial bioactivity.

1. Introduction

During World War II, a U.S. government-directed program focused on the identification of quinine replacements for the treatment of malaria. As a result, about 600 plant species were screened with in vivo bioassays by Merck and Co. scientists [1]. Unfortunately, none of these plant species were exhaustively characterized before the early termination of the antiplasmodial screening program in 1947. With the support of the Medicines for Malaria Venture organization, our laboratories joined forces in 2009 to follow up on these significant and largely forgotten anti-malarial screening efforts.

Spilanthes acmella is accredited with numerous medicinal properties, e.g. larvicidal [2], antimicrobial [3,4] and insecticidal [5] because of the presence of a number of bioactive compounds, including spilanthol and other *N*-isobutylamides. Recently, *S. acmella* was investigated for its potential for the treatment and prevention of malaria [2]. Antiplasmodial activity is particularly pertinent in light of the World Health Organization's estimation that nearly five million people are infected with malaria worldwide and more than one million die each year from the disease [6]. The emergence and spread of drug-resistant malarial parasites [7–9] highlights the need for novel or improved approaches for novel antiplasmodial compound isolation and purification.

S. acmella contains several bioactive compounds [10] of which the most studied group has been the *N*-alkylamides, which are abundant in this plant. A number of studies have demonstrated techniques for qualitative analysis of *N*-alkylamides from *Spilanthes* [11,12], but none of these have provided an efficient approach for quantitative isolation of these compounds in one step. Counter current chromatography is a liquid–liquid based separation method, using a liquid stationary phase of a two-phase solvent system, and was first invented by Ito et al. in the late 1960s [13]. With the expansion and operational strategy of CPC technology [14,15], research on the separation of natural products [16–18] has been streamlined due to the advantages of the technology such as the elimination of irreversible absorption, the high recovery of target compounds and the high throughput compared with other traditional separation methods such as thin-layer chromatography and column chromatography. Despite the advancement of CPC tech-

Abbreviations: BPC, base peak chromatogram; CID, collision induced dissociation; CPC, centrifugal partition chromatography; EIC, extracted ions chromatogram; ESI-IT-TOF-MS, electrospray ionization-ion trap-time-of-flight-mass spectrometer; HPLC, high-performance liquid chromatography; HPLC-DAD, high-performance liquid chromatography-diode array detector; LC/MS, liquid chromatography/mass spectrometry; NMR, nuclear magnetic resonance; SS, solvent system; TLC, thin layer chromatography.

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nology, the choice of a suitable solvent system is fundamental and can require a significant time investment, which can occupy up to 90% of the time devoted to CPC experimental design [19]. The mixture of heptanes, ethyl acetate, methanol and water (termed the HEMWat family) works well for many classes of natural compounds, but there is no widely accepted convention on how the proportions were listed. The results of shake-flask experiments are expressed in terms of the partition coefficient for each analyte of interest in each solvent system, which become more laborious when isolating a group of compounds. LC/MS/MS is considered a good technique for complete analysis of natural compounds in mixtures [11,12]. Bioactives identification and characterization may be enhanced by high mass resolution and multiple fragmentations (up to MS^7) using electrospray ionization-ion trap-time of flight-mass spectrometry [ESI-IT-TOF-MS] [20].

The aim of this study was to optimize the isolation and purification conditions for *N*-alkylamides in *S. acmella* and to assess their antiplasmodial activity against the chloroquine sensitive strain (D10) of *Plasmodium falciparum*. A method to determine a suitable CPC solvent system for a particular group of bioactive compounds was described using a combination of Accurate-Mass TOF LC/MS and CPC techniques. The isolated and purified compounds were identified using ESI-IT-TOF-MS and validated by NMR analysis. The comparison of the antiplasmodial activity of CPC collected fractions and the pure compounds was relevant in light of possible interactions between phytochemicals that potentiate antiplasmodial activity of *N*-alkylamides.

Table 1
Proportions of test solvents in the extended HEMWat solvent system. The highlighted solvent system 19 was the suitable one selected for *N*-alkylamides separation.

No	Heptanes	Ethyl acetate	Methanol	Butanol	Water
1	0.0	0.0	0.0	5.0	5.0
2	0.0	1.0	0.0	4.0	5.0
3	0.0	2.0	0.0	3.0	5.0
4	0.0	3.0	0.0	2.0	5.0
5	0.0	4.0	0.0	1.0	5.0
6	0.0	5.0	0.0	0.0	5.0
7	0.3	4.8	0.3	0.0	4.8
8	0.5	4.5	0.5	0.0	4.5
9	0.7	4.3	0.7	0.0	4.3
10	0.8	4.2	0.8	0.0	4.2
11	1.0	4.0	1.0	0.0	4.0
12	1.3	3.8	1.3	0.0	3.8
13	1.4	3.6	1.4	0.0	3.6
14	1.7	3.3	1.7	0.0	3.3
15	2.0	3.0	2.0	0.0	3.0
16	2.3	2.7	2.3	0.0	2.7
17	2.5	2.5	2.5	0.0	2.5
18	2.7	2.3	2.7	0.0	2.3
19	3.0	2.0	3.0	0.0	2.0
20	3.3	1.7	3.3	0.0	1.7
21	3.6	1.4	3.6	0.0	1.4
22	3.8	1.3	3.8	0.0	1.3
23	4.0	1.0	4.0	0.0	1.0
24	4.2	0.8	4.2	0.0	0.8
25	4.3	0.7	4.3	0.0	0.7
26	4.5	0.5	4.5	0.0	0.5
27	4.8	0.3	4.8	0.0	0.3
28	5.0	0.0	5.0	0.0	0.0

2. Materials and methods

2.1. Chemicals

Solvents for extraction (HPLC grade) and formic acid (ACS reagent grade) were purchased from Fisher Scientific (Waltham, MA). LC–MS grade solvents were obtained from Honeywell Burdick & Jackson (Muskegon, MI). Purasil silica gel (70–230 mesh) for flash chromatography was purchased from Whatman Inc. (Piscataway, NJ). Chloroquine diphosphate and emetine hydrochloride were obtained from Sigma–Aldrich (St Louis, MO).

2.2. Instrumentation

Centrifugal partition chromatography was performed on an Armen fully integrated CPC spot instrument SCPC 2 × 500 (Armen instrument, St-Ave, France). This instrument is a fully automated system consisted of a CPC column compartment, a pump, an injector, a UV/Vis detector, a fraction collector, a digital screen and a flat PC, and Armen Glider CPC software (Armen instrument, St-Ave, France). Total column volume of this model is 1000 mL (2× 500 mL). HPLC was performed on an integrated Agilent 1200 series Rapid Resolution LC System and the separation was performed using Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) (Agilent Technologies, Wilmington, DE). Agilent 6220 series Accurate-Mass TOF LC/MS was used for CPC solvent system determination and the separation was performed using Eclipse XDB-C18 (3 mm × 250 mm, 5 μm) (Agilent Technologies, Wilmington, DE). Electrospray ionization-ion trap-time of flight-mass spectrometry was used for compound analysis and formula determination (Shimadzu Scientific Instruments, Columbia, MD). NMR spectra were recorded on Bruker Avance 950 MHz spectrophotometer (Bruker BioSpin Corporation, Billerica, MA) located in the DHMRI Core Laboratory facility at North Carolina Research Campus.

2.3. Plant material and preparation of crude extract

Flowers from *S. acmella* were collected from a specimen growing in a greenhouse at Rutgers University, New Brunswick, NJ. Air-dried flowers were ground into a fine powder using a Waring stainless steel blender (Waring Commercial, Torrington, CT). The powdered material (300 g, particle size <500 μm) was extracted at room temperature three times each with 3 L methanol for 3 days. Methanol was selected as solvent for *N*-alkylamides extraction because of its higher ability to break the amide–amide interactions compared to hexane. The filtered extract was concentrated using a rotary evaporator (BUCHI Corporation, New Castle, DE). The dried crude extract (15 g) was kept at −20 °C until use for *N*-alkylamides isolation.

2.4. CPC solvent system screening

Different solvent systems from the expanded HEMWat family (28 solvent systems) [21] (Table 1) were prepared and evaluated using Accurate-Mass TOF LC/MS. From each solvent system, 500 μL of both phases was mixed and 1 mg of dried methanolic crude extract was dissolved in the two phases mixture. The test tubes were shaken vigorously until equilibrium had been established in both phases and then left overnight at 4 °C. Equal volumes (200 μL) of upper and lower phases were then transferred into separate HPLC vials and evaporated to dryness under vacuum. Finally, the residues were dissolved in 200 μL methanol and analyzed by LC/MS. The distribution ratio/partition coefficient (k_D) of three major *N*-alkylamides in *S. acmella* was evaluated in all 28 solvent systems screened. The partition coefficient was calculated as the ratio of the peak area of the compound's selected ion chromatogram in the upper (stationary) phase to the lower (mobile) phase. A plot of the HEMWat solvent system number against $\log k_D$ was used to determine the appropriate proportions of solvent for *N*-alkylamides isolation.