Journal of Chromatography B, 879 (2011) 1886–1892

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Flaubert Mbeunkui[a], Mary H. Grace[a], Carmen Lategan[b], Peter J. Smith[b], Ilya Raskin[c], Mary Ann Lila[a,*]

[a] *Plants for Human Health Institute, North Carolina State University, North Carolina Research Campus, 600 Laureate Way, Kannapolis, NC 28081, USA*
[b] *Division of Pharmacology, University of Cape Town, Observatory 7925, Cape Town, South Africa*
[c] *SEBS, Rutgers University, New Brunswick, NJ 08901, USA*

ARTICLE INFO

Article history:
Received 1 December 2010
Accepted 8 May 2011
Available online 14 May 2011

Keywords:
Centrifugal partition chromatography
N-alkylamides
Antiplasmodial activity

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.

© 2011 Elsevier B.V. All rights reserved.

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.

* Corresponding author. Tel.: +1 704 250 5407; fax: +1 704 250 5409.
E-mail addresses: mlila@ncsu.edu, fmbeunk@ncsu.edu (M.A. Lila).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.05.013

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.

2.5. CPC experimental conditions

A two-phase solvent system consisting of heptanes–ethyl acetate–methanol–water (3:2:3:2, v/v/v/v) was selected to separate *N*-alkylamides from the crude extract of *S. acmella* flowers. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and was separated into two phases before use. The entire column was first filled with the upper stationary phase at 100 mL/min with columns rotating at 500 rpm. The columns speed was then increased to 1800 rpm and the lower mobile phase was pumped into the inlet of the column at a flow rate of 10 mL/min. Equilibration was reached when only the mobile phase was eluted from the column while the displaced volume of stationary phase remained constant. After equilibration, the dried sample (1.5 g) dissolved in 20 mL of 1:1 (v/v) mixture of each phase was injected through the sample loop. The UV detection was performed at 254 nm from the outlet of the column and the fractions were collected in 15-mL glass tubes. The mobile phase was pumped into the inlet of the column at 10 mL/min for 150 min and the stationary phase at the same flow rate for another 150 min. Reproducible results were obtained by three repeated CPC experiments. Collected fractions were concentrated and dried using a Buchi rotary evaporator.

2.6. HPLC-DAD analysis of CPC fractions

HPLC-DAD analyses were carried out using an integrated Agilent 1200 series Rapid Resolution LC System equipped with an autosampler, a column oven, a binary pump, a DAD detector and a degasser. HPLC separation was performed on a reversed-phase column Eclipse XDB-C18 (4.6 mm × 250 mm, 5 μm) thermostatted at 30 °C using a mixture of A (0.1% formic acid in water) and B (0.1% formic acid in methanol) as mobile phase. Sample injections corresponded to 5 μg dried material from CPC fractions in 5 μL methanol. After each injection, samples were eluted at a flow rate of 1 mL/min with the following gradient: 5–95% B over 15 min, isocratic at 95% B for 2 min, return to 5% B over 1 min, and isocratic at 5% B over 4 min. Eluent was monitored using DAD at 254 nm.

2.7. Microscale flash column chromatography for compound purification

Non-pure fractions from CPC separation were purified using a glass column packed with silica gel. CPC fractions (50–150 mg) were loaded on the top of the column (20 mm × 200 mm) and were step gradient eluted with a combination of hexane–ethyl acetate with an initial of 95:5 (v/v) after a previous column wash with 100% hexane. The stepwise gradient elution was performed by increasing the amount of ethyl acetate by 5%. The volume of the solvent mixture used in each step was 250 mL and collected in fractions of 50 mL. Air pressure was applied onto the top of the column to increase the flow of the mobile phase. TLC was run on each fraction collected to monitor the elution of the target compound, and similar fractions were pooled together. Purified compounds were identified by ESI-IT-TOF-MS and confirmed by NMR analysis.

2.8. Mass spectrometry analysis of N-alkylamides

N-alkylamides analysis was performed by ESI-IT-TOF-MS on a Shimadzu LC-MS-IT-TOF instrument equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A diode array detector). On this system, purified compounds were injected into the mass spectrometer via a C18 guard column (7.5 mm × 4.6 mm, 5 μm; Grace Davidson Discovery Sciences, Deerfield, IL). The mobile phase was a mixture of 60% A (0.1% formic acid in water) and 40% B (0.2% formic acid in methanol) at a flow rate of 0.2 mL/min. The heat block and curved desolvation line (CDL) were maintained at 200 °C. Nitrogen gas was used as nebulizer and drying gas with the flow rate set at 1.5 L/min and 10 L/min, respectively. The ESI source voltage was set at 4.5 kV and the detector was set at 1.49 V. The instrument was calibrated to <5 ppm error in mass accuracy with an external standard of sodium TFA solution. Ionization was performed using a conventional ESI source in positive ionization mode. Shimadzu's LCMS Solution software was used for data analysis. The formula predictor function of LCMS Solution was used in identification and confirmation of unknown signals

2.9. In vitro assays

2.9.1. Antiplasmodial activity

Samples were tested in triplicate against chloroquine sensitive strain of *P. falciparum* (D10). Continuous in vitro cultures of asexual erythrocyte stages of *P. falciparum* was maintained using a modified method of Trager and Jensen [22]. Quantitative assessment of antiplasmodial activity in vitro was determined via the parasite lactate dehydrogenase assay using a modified method described by Makler et al. [23].

The test samples were prepared to a 2 mg/mL stock solution in 10% DMSO and sonicated to enhance solubility. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at −20 °C. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) (89% pure) was used as the reference drug in all experiments. A full dose–response was performed for pure compounds to determine the concentration inhibiting 50% of parasite growth (IC_{50}-value). Test samples were tested at a starting concentration of 100 μg/mL, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 μg/mL. The same dilution technique was used for all samples. CQ was tested at a starting concentration of 100 ng/mL. CPC fractions and purified compounds were tested at three concentrations: 20, 10, and 5 μg/mL. The IC_{50}-values were obtained using a non-linear dose–response curve fitting analysis via Graph Pad Prism v.4.0 software.

2.9.2. Cytotoxicity

Chinese Hamster Ovarian (CHO) cell line was used to assess the cytotoxicity using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide assay [24]. Purified compounds were tested with the antiplasmodial assay at three concentrations: 20, 10, and 5 μg/mL and emetine hydrochloride (99% pure) was used as a positive control. The initial concentration of emetine hydrochloride was 10 μg/mL, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 10^{-4} μg/mL. IC_{50}-values were obtained from full dose–response curves, using a non-linear dose–response curve fitting analysis via GraphPad Prism v.4 software.

3. Results and discussion

3.1. Solvent system selection for CPC separations

The critical point for a successful separation by CPC is the choice of an appropriate solvent system [19]. The basic requirement for such a solvent system is that the target compounds have different partition coefficients in the two immiscible phases. The partition coefficient is the concentration of an analyte in the upper phase divided by the concentration of the same analyte in the lower phase of an equilibrated biphasic solvent system. Therefore, the characteristics of the target compounds or class of compounds should be known before starting a CPC experiment. Three most abundant *N*-alkylamides in *S. acmella* (an *N*-isobutylamide, precursor *m*/*z*: 204; spilanthol, precursor *m*/*z*: 222; a 2-methylbutylamide,

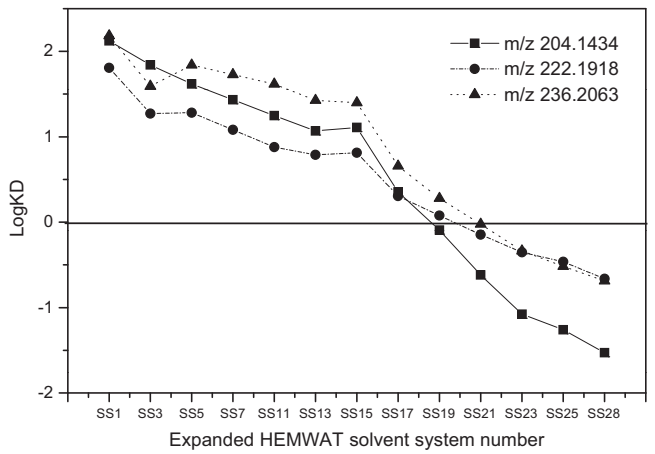

Fig. 1. Determination of the suitable solvent system for CPC experiment. Log K_D of three *N*-alkylamides (*m*/*z* 204, 222, 236) is plotted against the HEMWat solvent systems. For solvent system 19 (SS19), the K_D values are close to 1 while log K_D values are close to 0.

precursor *m*/*z*: 236) [11] were selected for the determination of a suitable solvent system for this class of compounds. The distribution coefficient (K_D) of these 3 *N*-alkylamides was investigated in all 28 solvent systems (see Table 1) of the expanded HEMWat family [21]. Equal amounts of sample were completely solubilized in each solvent system and the system was allowed to equilibrate. After equilibration, equal volumes of sample from each phase were removed and analyzed using an Accurate-Mass TOF LC/MS. The log K_D plot of the 3 *N*-alkylamides in the expanded HEMWat family solvent system is shown in Fig. 1. The log K_D values of these 3 compounds decreased from SS1 (most polar) to SS28 (least polar) in the same pattern, demonstrating that these 3 compounds account for the *N*-alkylamides class of compounds. Ideally, the partition coefficient K_D has to be close to 1, usually in the range of 0.5–2 ($-0.3 \leq \log K_D \leq 0.3$) [25] and the solvent system 19 (SS19) was the most appropriated as shown in Fig. 1. In the case of higher K_D values ($\log K_D > 0.3$), compounds will give long retention times and considerable peak broadening, whereas in lower K_D values ($\log K_D < -0.3$) compounds will elute too quickly and not well separated. The K_D values of the three *N*-alkylamides investigated were 0.81, 1.20, and 1.90 corresponding to log K_D values of −0.09, 0.08, and 0.28, respectively. The base peak chromatograms (BPCs) of sample from solvent system SS19 (upper and lower phases) with the extracted ions chromatograms (EICs) of the three selected compounds, are shown as Supplementary data (Fig. A1). Even though the BPCs look quite different, the EICs look roughly the same demonstrating the selectivity of the solvent system for this class of compounds. This method is more accurate than the traditional shake flask partition method [25], where a small amount of sample in the biphasic solvent system is shaken, allowed to equilibrate and the concentration of the analytes in each phase is then determined by spectrophotometry, HPLC [26,27], GC or TLC [28]. There are at least three practical considerations that come into play, however, when comparing shake flask partition method and the present method. First, shake flask *K* values are typically determined by UV absorption ratios while the K_D values of the present method are determined by a selected peak position for the retention time of the analyte corresponding to its accurate mass in order to determine the peak area ratio K_D. Secondly, the influence of other compounds present in the mixture may significantly affect the *K* values of the shake flask partition. Shake flask partition coefficients are usually determined with a single compound in a biphasic system, whereas the partition coefficients of more than one compound can be determined by screening all the expanded HEMWat solvent system family concurrently.

3.2. CPC separation of N-alkylamides

A successful separation by CPC depends on the suitable selection of a two-phase solvent system, which should have good stability and solubility of the target compounds, short settling time (<30 s) and satisfactory stationary phase retention [28]. The settling time of the selected solvent system HEMWat SS19 [heptanes–ethyl acetate–methanol–water (3:2:3:2, v/v/v/v)] was 10 s. The revolution speed of the column was 1800 rpm while the flow rate of mobile phase was 10 mL/min with 80% stationary phase retained in the column after equilibration. This is consistent with the retention of the stationary phase's rule stating that "the higher the retention of the stationary phase, the better the peak resolution" [25]. The total volume of the sample loop was 30 mL and 20 mL sample was injected into the CPC columns.

The result of CPC separation is illustrated by the UV chromatogram measured at 254 nm as shown in Fig. 2. Fractions were collected in 15-mL glass tubes at mobile phase flow rate 10 mL/min over 300 min. The collected fractions were analyzed by TLC to assess the efficiency of CPC separation and fractions containing identical major compound(s) were merged, concentrated and dried using a rotary evaporator. Nine major fractions resulted from this combination (Fig. 2), and fraction 1 contained about 60% of the total sample injected. All nine fractions were submitted to the in vitro bioassay to evaluate the antiplasmodial activity. As expected, no activity was detected in fraction 1 and it was therefore discarded. Through TLC and LC/MS analysis, it was determined that fraction 1 consisted of very polar compounds, essentially sugars and high polymeric compounds. Fractions 2–9 showed good antiplasmodial activity and were further analyzed (Table 2). Fractions 6–9 essentially contained non-polar compounds like chlorophyll pigments that do not belong to the *N*-alkylamides. However, some *N*-alkylamides were detected in minor quantity in those fractions. Fractions 2–5 contained intermediate polar compounds; they are enriched in compounds with a good separation between the two phases of the solvent system. According to HPLC and LC/MS/MS analysis, these four fractions were enriched in major *N*-alkylamides in *S. acmella* [11] and were effectively separated by CPC as shown on HPLC chromatograms in Fig. 3. Compounds **1**, **2**, **3** and **4** were purified from CPC fractions 2, 3, 4 and 5; and their relative quantification by peak area ratio on the chromatogram indicated 51%, 95%, 65% and 46%, respectively. Fraction 3 represented the most abundant *N*-alkylamide in *S. acmella*, and was purified by one CPC run (Fig. 3B). The other *N*-alkylamides represent less than 12% of the total *N*-alkylamides and the relatively abundant ones were successfully isolated by CPC. In order to assess the antiplasmodial activity of each compound individually, fractions 2–5 were further purified by flash column chromatography and the yield was 20 mg, 105 mg, 15 mg and 54 mg corresponding to compounds **1**–**4**, respectively. These recoveries are relatively low compared to the amount of sample injected (1.5 g) into the CPC columns, and this is due to the complex mixture of the methanolic extract. The in vitro antiplasmodial activity of the purified compounds was performed and the results are shown in Table 3. The antiplasmodial activity of the fractions was higher than the antiplasmodial activity of pure compounds, thus demonstrating possible interactions between *N*-alkylamides for potential antiplasmodial activity.

3.3. Compounds analysis and identification

The identification of *N*-alkylamides in *S. acmella* starts with the demonstration that the target compound is in fact a *N*-alkylamide. Various *N*-isobutylamides, 2-methylbutylamides

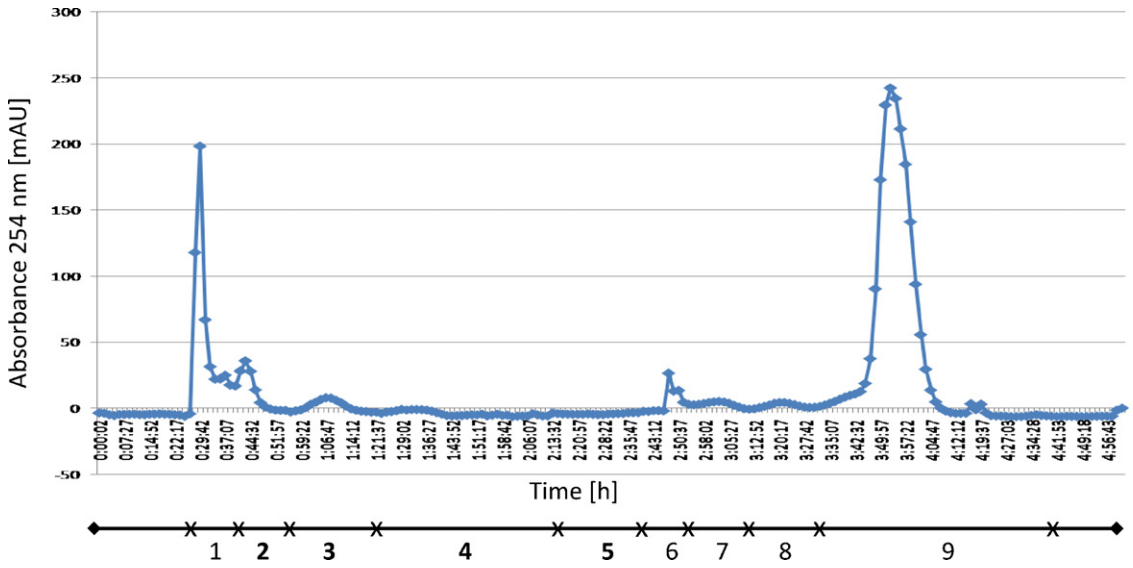

Fig. 2. CPC-UV chromatogram of the methanol crude extract of *S. acmella* flowers. Experimental conditions: solvent system: heptanes–ethyl acetate–methanol–water (3:2:3:2, v/v/v/v), mobile phase: lower layer, revolution speed: 1800 rpm, flow rate: 10 mL/min, detection: 254 nm. Fractions 2–5 correspond to major *N*-alkylamides.

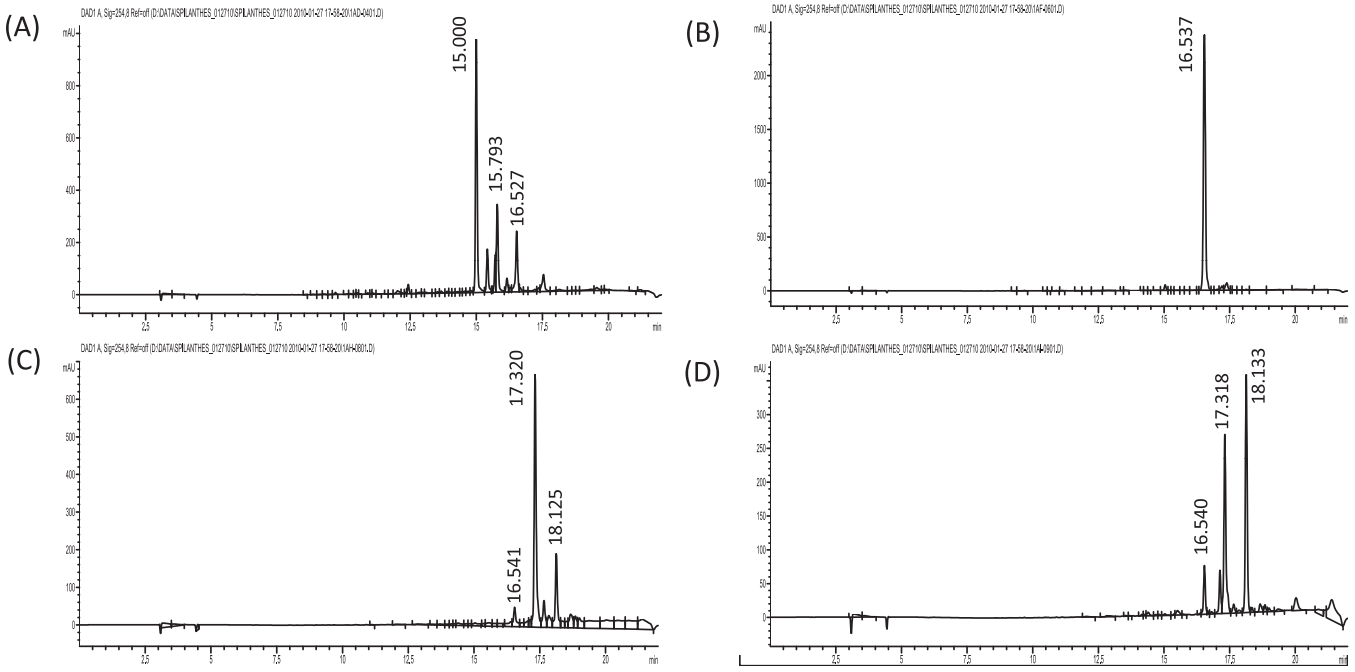

Fig. 3. HPLC-DAD chromatograms of CPC fractions. Fraction 2 (A), fraction 3 (B), fraction 4 (C) and fraction 5 (D). Wavelength of detection: 254 nm.

Table 2
Antiplasmodial activity of CPC fractions from the methanol crude extract of *S. acmella* flowers against the chloroquine sensitive strain (D10) of *Plasmodium falciparum*.

CPC fraction number	1	2	3	4	5	6	7	8	9
D10 (IC_{50} μg/mL)	>100	14.91	22.04	26.17	12.21	18.76	14.46	13.46	37.35

Table 3
Antiplasmodial activity and cytotoxicity of isolated and identified *N*-alkylamides from *S. acmella* against the chloroquine sensitive strain (D10) of *Plasmodium falciparum*.

Compound number	Compound (MW)	Compound name	D10 IC_{50} (μg/mL) $n = 3$	CHO IC_{50} (μg/mL) $n = 3$
1	203	(z)-Non-2-en-6,8-diynoic acid isobutylamide	54.03	>100
2	221	Spilanthol	26.43	>100
3	231	(2E)-*N*-isobutylundeca-2-ene-8, 10-diynamide	29.34	>100
4	235	Spilanthic acid 2-methylbutylamide	33.73	>100

D10, *P. falciparum* strain D10; CHO, Chinese Hamster Ovarian cell line; *n*, number of replicates.

and 2-phenylethylamides revealing different characteristic *N*-isobutylamide and 2-methylbutylamide fragmentation ions by CID [29–31], have been identified [11]. *N*-alkylamide fragmentation by CID often forms an acyllium ion, with a m/z value indicative for the amount of carbon atoms in the alkyl chain. Mass spectra of the precursor ion (MS^1) and the corresponding fragment ions (MS^2) of each *N*-alkylamide purified are shown as Supplementary data (Fig. A2). The structure of *N*-alkylamides could be identified based upon fragmentation information combined with the molecular weight of previously identified *N*-alkylamides in *S. acmella* [11], the formula prediction provided by the LC/MS solution software and the search in Beilstein/Gmelien database. Compound **1**, an *N*-alkylamide with $m/z = 204$ with major fragments ions 103 [C_8H_6(+H)] and 148 [C_9H_9NO(+H)] and formula $C_{13}H_{17}NO$, was identified as (2*Z*)-*N*-isobutyl-2-nonene-6,8-diynamide [32]. This compound shares the same fragmentation mechanism with its corresponding (2*E*)-*N*-isobutyl-2-undecene-8,10-diynamde [33] (compound **3**, $m/z = 232$, $C_{15}H_{21}NO$) (see Supplementary data, Fig. A2). Compound **2**, the major *N*-alkylamide in *S. acmella* with formula $C_{14}H_{23}NO$ ($m/z = 222$), was identified as (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide or spilanthol [34]. It also shares the same fragmentation pattern with its corresponding (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide [32] (compound **4**, $m/z = 236$, $C_{15}H_{25}NO$). According to the literature [11,29,30], these compounds were previously identified and characterized but this is the first study to report their individual antiplasmodial activity. ^1H and ^{13}C NMR spectra for purified compounds **1**–**4** were acquired to validate the mass spectral results (Supplementary data, Table A). A total of 11 *N*-alkylamides can be detected in *S. acmella* [11] and only the most abundant ones could be quantitatively isolated for in vitro bioassay. The four compounds isolated in this work represent the major *N*-alkylamides in *S. acmella* [11].

3.4. Interactions between N-alkylamides potentiate antiplasmodial activity

The antiplasmodial activity of all semi-pure fractions collected from CPC separation is reported in Table 2. The antiplasmodial activity of compounds resulting from the purification of these fractions is reported in Table 3. Compounds **1**, **2**, **3** and **4** were the result of further purification of fractions 2, 3, 4 and 5, respectively. Results showed that the antiplasmodial activity of semi-pure compounds was consistently significantly higher than the activity from pure compounds (see Tables 2 and 3). The IC_{50} values of fractions 2 and 5 were more than 3 times lower than the IC_{50} values of the corresponding pure compounds. These data demonstrated that the higher antiplasmodial activity of the CPC fractions is likely due to the interactions between compounds in the mixture as shown on the HPLC chromatograms of the fractions (Fig. 3). The IC_{50} value of fraction 3 (22.04 μg/mL) and the corresponding pure compound (compound **2**, $IC_{50} = 26.43$ μg/mL) did not show much difference compared to other fractions and the corresponding pure compounds. Fraction 3 was about 95% pure as shown on the HPLC chromatogram (Fig. 3B) and the clean-up step did not result in enhanced activity of the compound. Spilanthol, the purified compound from fraction 3, is the most abundant *N*-alkylamide in *S. acmella* and represents about 88.8% of the total *N*-alkylamides [11]. All purified compounds showed no detectable cytoxicity as shown in Table 3. Hence, they may be candidates in the discovery and development of new combinations of drugs against malaria. The mechanism of antiplasmodial interaction between these compounds is still unknown and the investigation of this mechanism is underway in our laboratories.

Antiplasmodial activity of various compounds has been previously demonstrated in vivo and in vitro. The potent synergistic antiplasmodial interaction between the structurally similar compounds, rufigallol and exifone in vitro, in *P. falciparum* was reported by Winter et al. [35]. To account for this synergism, the authors proposed that rufigallol, which possesses a pro-oxidant activity, transforms exifone, inside the parasitized erythrocytes into a tricyclic xanthone derivative, 2,3,4,5,6-pentahydroxyxanthone, with potent antiplasmodial properties. Likewise, the antiplasmodial effect of the combination of exifone with ascorbic acid (vitamin C), known to exert oxidative stress on plasmodium-infected erythrocytes, was later investigated by the same group [36]. From these studies, the authors concluded that, ascorbic acid, like rufigallol, also augments antiplasmodial activity of exifone, in vitro against a number of *P. falciparum* via the formation of a tricyclic xanthone derivative, 2,3,4,5,6-pentahydroxyxanthone. More recently, Mahajan et al. [37] conducted a similar investigation on benzophenone derivatives, individually and in combination with rufigallol, in mice infected with *Plasmodium berghei*. Benzophephenone derivatives showed good antiplasmodial activity, in vivo, when tested in combination with rufigallol, indicating the synergism between them. de Monbrison et al. [38] also investigated the antiplasmodial interaction of two flavonoids, dehydrosilibin and dimethylallyl campferide, on several chloroquine-resistant strains of *P. falciparum* in vitro. The IC_{50} values from this investigation ranged from 0.8 to 11.5 μg/mL. However, the naturally occurring flavonoid silybin had no antiplasmodial activity reported. The mode of action of silybin derivatives is unknown and although they had no synergistic effect with chloroquine; they reversed chloroquine resistance or affected *Plasmodium* p-glycoproteins.

4. Conclusions

Bioactive compounds in plant extracts commonly represent a very small proportion. Hence, isolating these minor compounds from a complex crude extract is always a challenge. The potential of CPC for large-scale isolation of major *N*-alkylamides from *S. acmella* has been demonstrated. Using *N*-alkylamides as an example, a method for a rapid screening of a suitable solvent system for a class of bioactives separation by CPC has been developed. Four *N*-alkylamides were isolated and purified using a solvent system heptanes–ethyl acetate–methanol–water (3:2:3:2, v/v/v/v). Results comparing the antiplasmodial activity of the CPC fractions and corresponding pure compounds showed that the CPC fractions (semi-pure) were more active than the purified compounds. Finally, these findings highlighted a possible potentiating interaction in antiplasmodial activity of *N*-alkylamides.

Acknowledgments

This work was supported by funds from the Medicines for Malaria Venture. The authors thank Rocky Graziose, Ph.D. candidate, Rutgers University, for collecting the plant material.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.05.013.

References

[1] C.F. Spencer, F.R. Koniuszy, E.F. Rogers, J. Shavel, N.R. Easton, E.A. Kaczka, F.A. Kuehl, R.F. Phillips, A. Walti, K. Folkers, C. Malanga, A.O. Seeler, Lloydia 10 (1947) 145.
[2] V. Pandey, V. Agrawal, K. Raghavendra, A.P. Dash, Parasitol. Res. 102 (2007) 171.
[3] W. Fabry, P. Okemo, R. Ansorg, Mycoses 39 (1996) 67.
[4] M.K. Rai, A. Varma, A.K. Pandey, Mycoses 47 (2004) 479.
[5] R.S. Ramsewak, A.J. Erickson, M.G. Nair, Phytochemistry 51 (1999) 729.
[6] World Health Organization, World Malaria Report, 2008.

[7] J. Marfurt, T.A. Smith, I.M. Hastings, I. Muller, A. Sie, O. Oa, M. Baisor, J.C. Reeder, H.P. Beck, B. Genton, Malar. J. 9 (2010) 8.
[8] S.V. Dlamini, K. Beshir, C.J. Sutherland, Malar. J. 9 (2010) 68.
[9] M.H. Kinzer, K. Chand, H. Basri, E.R. Lederman, A.I. Susanti, I. Elyazar, G. Taleo, W.O. Rogers, M.J. Bangs, J.D. Maguire, Malar. J. 9 (2010) 89.
[10] S. Prachayasittikul, S. Suphapong, A. Worachartcheewan, R. Lawung, S. Ruchirawat, V. Prachayasittikul, Molecules 14 (2009) 850.
[11] J. Boonen, B. Baert, C. Burvenich, P. Blondeel, S. De Saeger, B. De Spiegeleer, J. Pharm. Biomed. Anal. 53 (2010) 243.
[12] S.S. Bae, B.M. Ehrmann, K.A. Ettefagh, N.B. Cech, Phytochem. Anal. 21 (2010) 438.
[13] Y. Ito, I. Aoki, E. Kimura, K. Nunogaki, Y. Nunogaki, Anal. Chem. 41 (1969) 1579.
[14] F. Yang, J. Quan, T.Y. Zhang, Y. Ito, J. Chromatogr. A 803 (1998) 298.
[15] A. Berthod, M.J. Ruiz-Angel, S. Carda-Broch, Anal. Chem. 75 (2003) 5886.
[16] Y. Lu, C. Sun, Y. Wang, Y. Pan, J. Chromatogr. A 1151 (2007) 31.
[17] H. Ye, L. Chen, Y. Li, A. Peng, A. Fu, H. Song, M. Tang, H. Luo, Y. Luo, Y. Xu, J. Shi, Y. Wei, J. Chromatogr. A 1178 (2008) 101.
[18] R.J. Case, Y. Wang, S.G. Franzblau, D.D. Soejarto, L. Matainaho, P. Piskaut, G.F. Pauli, J. Chromatogr. A 1151 (2007) 169.
[19] H.B. Li, F. Chen, J. Sep. Sci. 28 (2005) 268.
[20] H. Li, L. Wan, Y. Hashi, S. Chen, Rapid Commun. Mass Spectrom. 21 (2007) 2497.
[21] I.J. Garrard, L. Janaway, D. Fisher, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 151.
[22] W. Trager, J.B. Jensen, Science 193 (1976) 673.
[23] M.T. Makler, J.M. Ries, J.A. Williams, J.E. Bancroft, R.C. Piper, B.L. Gibbins, D.J. Hinrichs, Am. J. Trop. Med. Hyg. 48 (1993) 739.
[24] T. Mosmann, J. Immunol. Methods 65 (1983) 55.
[25] Y. Ito, J. Chromatogr. A 1065 (2005) 145.
[26] H. Ye, S. Ignatova, H. Luo, Y. Li, A. Peng, L. Chen, I. Sutherland, J. Chromatogr. A 1213 (2008) 145.
[27] C.Y. Kim, H.J. Lee, M.K. Lee, M.J. Ahn, J. Kim, J. Sep. Sci. 30 (2007) 2693.
[28] C. Roullier, M. Chollet-Krugler, A. Bernard, J. Boustie, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 2067.
[29] N.B. Cech, M.S. Eleazer, L.T. Shoffner, M.R. Crosswhite, A.C. Davis, A.M. Mortenson, J. Chromatogr. A 1103 (2006) 219.
[30] K. Spelman, M.H. Wetschler, N.B. Cech, J. Pharm. Biomed. Anal. 49 (2009) 1141.
[31] R.D. Hiserodt, B.M. Pope, M. Cossette, M.L. Dewis, J. Am. Soc. Mass Spectrom. 15 (2004) 1462.
[32] H. Greger, O. Hofer, A. Werner, Monatsh. Chem. 116 (1985).
[33] R. Bauer, P. Remiger, H. Wagner, Phytochemistry 28 (1989) 505.
[34] N. Nakatani, M. Nagashima, Biosci. Biotechnol. Biochem. 56 (1992) 759.
[35] R.W. Winter, K.A. Cornell, L.L. Johnson, M. Ignatushchenko, D.J. Hinrichs, M.K. Riscoe, Antimicrob. Agents Chemother. 40 (1996) 1408.
[36] R.W. Winter, M. Ignatushchenko, O.A. Ogundahunsi, K.A. Cornell, A.M. Oduola, D.J. Hinrichs, M.K. Riscoe, Antimicrob. Agents Chemother. 41 (1997) 1449.
[37] S.S. Mahajan, V.R. Kamath, S.S. Ghatpande, Parasitology 131 (2005) 459.
[38] F. de Monbrison, M. Maitrejean, C. Latour, F. Bugnazet, F. Peyron, D. Barron, S. Picot, Acta Trop. 97 (2006) 102.