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Invited Article

# Reverse pharmacology for developing an anti-malarial phytomedicine. The example of *Argemone mexicana*

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ABSTRACT

Classical pharmacology has been the basis for the discovery of new chemical entities with therapeutic effects for decades. In natural product research, compounds are generally tested *in vivo* only after full *in vitro* characterization. However drug screening using this methodology is expensive, time-consuming and very often inefficient.

Reverse pharmacology, also called bedside-to-bench, is a research approach based on the traditional knowledge and relates to reversing the classical laboratory to clinic pathway to a clinic to laboratory practice. It is a trans-disciplinary approach focused on traditional knowledge, experimental observations and clinical experiences. This paper is an overview of the reverse pharmacology approach applied to the decoction of *Argemone mexicana,* used as an antimalarial traditional medicine in Mali. *A. mexicana* appeared as the most effective traditional medicine for the treatment of uncomplicated *falciparum* malaria in Mali, and the clinical efficacy of the decoction was comparable to artesunate–amodiaquine as previously published. Four stages of the reverse pharmacology process will be described here with a special emphasis on the results for stage 4. Briefly, allocryptopine, protopine and berberine were isolated through bioguided fractionation, and had their identity confirmed by spectroscopic analysis. The three alkaloids showed antiparasitic activity *in vitro*, of which allocryptopine and protopine were selective towards *Plasmodium falciparum*. Furthermore, the amount of the three active alkaloids in the decoction was determined by quantitative NMR, and preliminary *in vivo* assays were conducted. On the basis of these results, the reverse pharmacology approach is discussed and further pharmacokinetic studies appear to be necessary in order to determine whether these alkaloids can be considered as phytochemical markers for quality control and standardization of an improved traditional medicine made with this plant.



## 1. Introduction

Every year, there are over 200 million cases of malaria around the globe, resulting in about 627,000 deaths. Most of them occur in children under the age of 5 (WHO, 2011). The parasite responsible for malaria is developing resistance which will increase as a result of drug pressure and the invasion of under-dosed fake medicines. The first signs of resistance to artemisinin derivatives are appearing on the Cambodia-Thailand border (Dondorp et al., 2010) and will continue to spread rapidly if artemisinin-based combination therapies (ACT) are delivered to patients who do not absolutely need them. Therefore, it is urgent to find new hits and lead compounds. But in the meantime, alternative processes are needed to slow down as much as possible the progression of resistance.

Most affected populations affected by malaria have little access to western medicine and therefore turn towards the use of traditional medicinal plants for their primary health care (WHO, 2013). In Africa, there are more traditional healers than medical doctors available for the population, especially in rural areas. Thus

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the use of herbal medicines constitutes the first line treatment (Abdullahi, 2011; WHO, 2013). Modern natural or synthetic antimalarial drugs have been mainly developed from only few plants: various *Cinchona* sp. from which quinine was isolated already in 1820, and more recently the aerial parts of *Artemisia annua* from which artemisinin was isolated in 1972 (Fig. 1). However, these two plants have been used for centuries by the local population as traditional medicine against fever in South America for *Cinchona* and in Asia for *Artemisia*.

In the conventional drug discovery approach of natural products, the classical laboratory to clinic process also called bench to bedside is used (Fig. 2). In this approach, numerous extracts, frequently selected through ethnobotanical studies, are screened and tested on targets which have been previously identified and validated. However clinical observations (follow up of human subjects) and experimental observations are not frequently involved. Yet they could help discover which treatment among others, has the best effect. The most promising extracts are analyzed, the active compounds isolated, and their chemical structures elucidated. Structure activity relationship and lead optimization by means of chemical synthesis, molecular modeling, and ADMET properties are carried out before the development phase, authority approval and product launch. However, this approach is time consuming and often very inefficient. The average time and cost of discovering, developing and launching a new drug is estimated to 10–15 years with a cost between 800 million and 1.5 billion US dollars. Why is it so expensive and why does it take so much time? Thousands of natural extracts have been screened and hundreds of compounds isolated during the last three decades. One major difficulty seems to be that a natural active molecule has very little chance to move to clinical studies because of poor absorption and pharmacokinetics and/or adverse-effect profile in animal studies. As a result, time and money are spent for compounds that ultimately cannot be further developed.

The concept of reverse pharmacology has been first introduced in the development of Ayurvedic medicines in India and suggested in 1950's for the development of a Chinese traditional medicine (Lei and Bodeker, 2004). Moreover, such an approach has been well reviewed in the context of natural product research by Wells (2011). Reverse pharmacology is a transdisciplinary approach integrating traditional knowledge, experimental observations and clinical experiences (Fig. 3) with the aim of reversing from the classical laboratory to clinic process to a clinic to laboratory approach.

We herein present an overview of the reverse pharmacology of *Argemone mexicana* L. (Papaveraceae). This plant is originated from the USA-Mexico border but has spread to tropical and subtropical areas around the world. It has a long history in traditional medicine for the treatment of several ailments including tumors, warts, skin diseases, inflammation, rheumatism, jaundice, leprosy, microbial infections, and malaria (Brahmachari and Rajiv Roy, 2013). It is used as an aqueous decoction in several African countries, including Mali where this study was undertaken. This plant appeared in a "retrospective treatment outcome" study as the most effective for the treatment of uncomplicated *falciparum* malaria in Mali (Diallo et al., 2006). Thus, this plant has been considered for a reverse pharmacology approach divided into 4 stages (Fig. 4): stage 1: Selection of the remedy, stage 2: Observational clinical trials, stage 3: Randomized control trials, and stage 4: Identification of active compounds (Willcox et al. 2011).

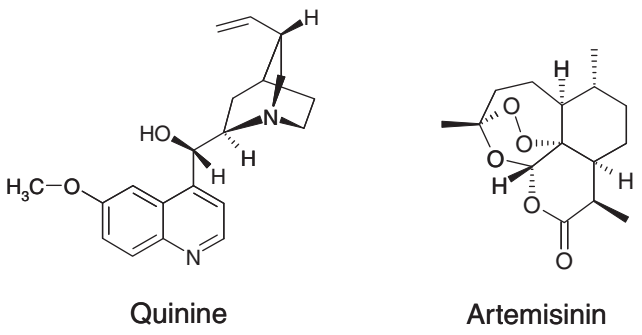


**Fig. 1.** Structures of quinine and artemisinin leading to current antimalarial drugs.

Results from stages 1–3 have been previously published (Willcox et al., 2007; Graz et al., 2010a,b) and will be summarized here in order to allow a better understanding of the topic. The results of the phytochemical and preliminary *in vivo* results retrieved during stage 4 are presented in detail within the Results and discussion section of this article.

### 1.1. Stage 1: Selection of a remedy

Stage 1 consists of the selection of the plants used by the local population, including the method of preparation, their administration and dosage. In the case of *A. mexicana* selection, this stage has been previously detailed in the literature (Graz et al., 2010a). Briefly, a representative sample of the population (952 persons) was interviewed in two rural districts. The recall period was kept short, two weeks for an uncomplicated malaria episode. The analysis of patient progress under various treatments was compared: modern and/or traditional medicine, treatment at home with traditional knowledge, in a health center or in the hospital. The plants or substances used were recorded and analyzed. If a traditional treatment, for example a plant preparation used alone, was systematically followed by a rapid and complete recovery, with no failure or important side effects, there was a good chance that the preparation deserved further scrutiny (Graz et al., 2010a). In that study, *A. mexicana* decoction appeared as the recipe associated with the best outcome among patients with presumed uncomplicated malaria. No reference to toxicity from an aqueous decoction of the aerial parts of this plant was reported by the population and nothing was found in the literature. Before proceeding to clinical studies, WHO guidelines state that if a product has been traditionally used without demonstrated harm, no specific restrictive regulatory action should be undertaken unless new evidence demands a revised risk-benefit assessment (WHO, 2004). Pre-clinical toxicity testing is only required for new medicinal herbal products which contain herbs with no established traditional history of use.

### 1.2. Stage 2: Observational study

As the population has been using the decoction for a very long time and the literature search did not reveal any toxicity, an observational clinical study was organized with patients who agreed to take the traditional preparation prescribed by the local traditional healer in Missidougou (Mali). The results of this stage have been published by Willcox et al. (2007). In that study, the diagnosis of uncomplicated malaria was confirmed for all patients according to pre-established criteria. Eighty patients were spread into 3 groups (A–C). Each group received a dose of decoction chosen by the healer. Group A received a dose lower than the one traditionally used. Group B and group C received the bottom and top of the usual dose range, respectively. The lowest dose (group A) consisted of 1 glass/day for 3 days, the mid-range of 1 glass 2 times daily for 7 days, and the highest dose of 1 glass 4 times daily for 4 days and then 1 glass twice a day for 7 days. Patients were followed up on days 1, 2, 3, 7, 14 and 28 (Willcox et al., 2007). According to the "Assessment and monitoring of antimalarial drug efficacy in low-transmission area for the treatment of uncomplicated *falciparum* malaria" established by WHO (2003), the parasite count by day 3 should have decreased to less than 25% of that on

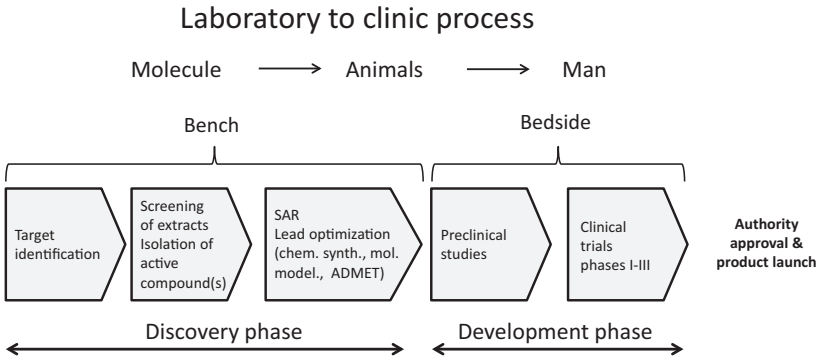


**Fig. 2.** Diagram of the conventional drug discovery approach (adapted from Patwardhan and Vaidya, 2010).

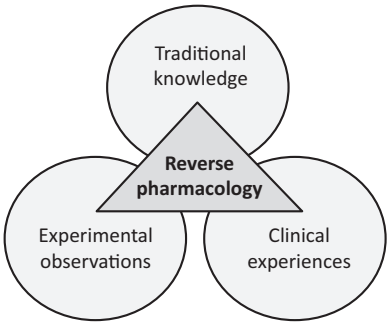


**Fig. 3.** Reverse pharmacology is a transdisciplinary approach integrating traditional knowledge, experimental observations and clinical experiences.

day 0 and the total parasite clearance should be completed by day 7 and maintained throughout day 28. In the case of *A. mexicana*, the number of parasites decreased dramatically in all groups by day 3. However, at day 14, the majority of patients still had a measurable parasitaemia, but at a lower level than at the start of the treatment and lower than 2000 parasites/μl (Fig. 5). Only 7 patients had complete parasite clearance at day 14 (Willcox et al., 2007). WHO also recommends total parasite clearance but this may not be applicable in high-transmission area like in Mali where the population develops partial immunity in early life and is rapidly re-infected even if parasite clearance is achieved. Although not required by WHO guidelines, chemical analysis was carried out and did not show any toxic compound in the decoction. In particular, sanguinarine, detected in the methanolic extract of the plant, was not found in the decoction (Willcox et al., 2007). Furthermore, no evidence of acute toxicity was detected at doses up to 3 g/kg of the freeze-dried decoction in mice and rats (Guissou, 2007).

### *1.3. Stage 3: Randomized controlled trial*

To test the safety and efficacy of *A. mexicana* decoction in the field, a study has been conducted by Graz et al. (2010b), including 301 patients, of which 197 received the decoction and 101 the ACT (artemisinin–amodiaquine) which is the nationally recommended first-line treatment against malaria in Mali. The objective of that study was to develop a phytomedicine for home-based management of malaria and to prevent severe malaria. It was important to inform local communities about the effects of the tested phytomedicine as first-line treatment for presumed malaria in semi-immune individuals and not to outperform the ACT. Patients were asked to take the treatment for one week, and if at the end of the first week they had largely improved but they were still experiencing few symptoms such as mild fever, they were advised to take the same treatment for a second week. For small children, sugar was added to the decoction to improve the taste. The dose of the ACT treatment was determined according to the patient's weight and given twice daily for 3 days. As a result, 89% of the patients in the *A. mexicana* group and 95% of the ACT group did not need a second-line treatment during the 28-day follow-up. No patient died in either group during the same period. No case of severe malaria was reported in patients aged over 5 years. In patients under 5 years, severe malaria was detected in less than 2% in both groups after 4 weeks. Among all severe malaria cases, none had coma or convulsions. A similar proportion of patients experienced side effects consisting mainly of cough and diarrhea for the *A. mexicana* group, and nausea and vomiting for the ACT group. These side effects started earlier in the ACT group than in the *A. mexicana* group and were described as mild (89%) or moderate (11%) for both groups. No severe adverse effect was reported in either group (Graz et al., 2010b).

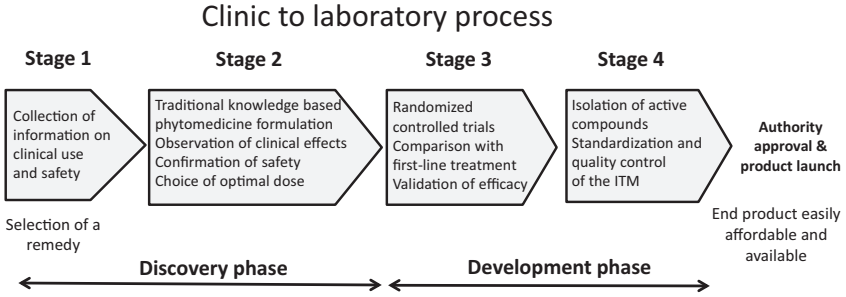


**Fig. 4.** Diagram of the reverse pharmacology approach (adapted from Patwardhan and Vaidya, 2010).

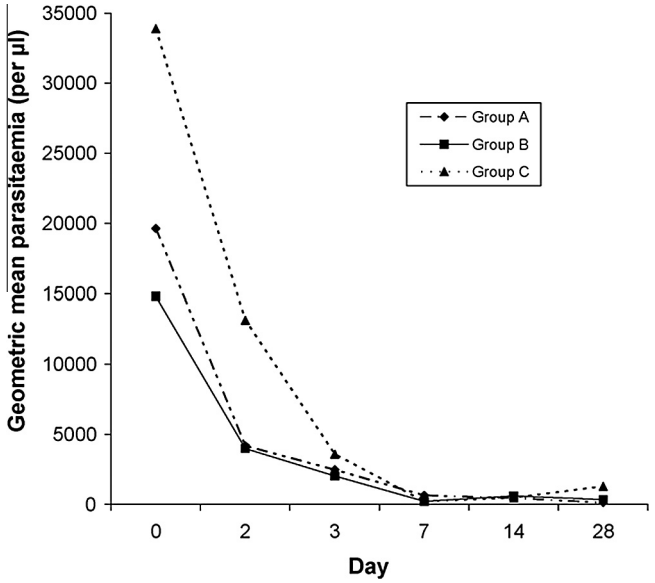


**Fig. 5.** Parasitaemia decreased in all patients treated with *A. mexicana* decoction during the observational stage 2 (Willcox et al., 2007; Copyright 2013, with permission from Oxford University Press).

## 2. Material and methods

### 2.1. Plant material and extract preparation

Two available *A. mexicana* leaf batches corresponding to clinical batches employed at stage 3 (Willcox et al., 2007) were considered in this study. Batch AM 8, used to treat patients 190–238 was collected in Missidougou, Mali in 2004, while batch AM 11, used to treat patients 239–301 was collected in Sikasso, Mali in 2006. For each batch, the plant was identified as *A. mexicana* L. (Papaveraceae) by Seydou Dembélé and a voucher specimen (number 873) was deposited at the Department of Traditional Medicine in Bamako, Mali. The extracts were prepared as described for the traditional preparation. The decoction was obtained by stirring 500 g of batch AM 8 in 2 L of boiling distilled water for 3 h. Then, the extract was freeze-dried to give 115 g of powder.

### 2.2. Vacuum liquid chromatography (VLC)

A VLC was conducted by filling a 250 mL Büchner funnel with the stationary $C_{18}$ phase LiChroprep® 40–63 µm (Merck, Darmstadt, Germany). The support was connected to a vacuum system. The stationary phase was first eluted with MeOH (5 × 250 mL) for conditioning followed by water (for equilibrating). The sample was diluted in 100 mL water and introduced into the support. The elution of the sample was first conducted with 600 mL water, followed by 600 mL MeOH.

### 2.3. Sephadex LH-20 size exclusion liquid chromatography

Size exclusion liquid chromatography was conducted using Sephadex LH-20 gel (GE Healthcare, Uppsala, Sweden) as stationary phase and MeOH as the mobile phase. Fraction was diluted in 1 mL MeOH to be applied onto the column and eluted with 100% MeOH.

### 2.4. Semi-preparative HPLC

Semi-preparative HPLC was used in order to purify the compounds from pre-purified fractions. The equipment was a Shimadzu LC-8A binary pump equipped with a SPD-10A VP Shimadzu UV–Vis detector (Kyoto, Japan). The flow rate was set to 10 mL/min. For *A. mexicana* fractions, an Xterra Prep MS $C_{18}$ OBDTM column (150 × 19 mm i.d., 5 µm) was employed for the separations and the solvent system was A) Water and B) MeOH. An isocratic mode at 5% B was applied for fraction AM 8_A_2, while an isocratic mode at 42% was chosen for fractions AM 8_D_1 and AM 8_D_2.

### 2.5. High resolution mass spectrometry (HRMS)

High resolution mass spectra were obtained on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Waters, MA, USA). ESI conditions: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2650 V, source temperature 120 °C, desolvation temperature 250 °C, cone gas flow 10 L/h, desolvation gas flow of 550 L/h. Detection was performed in positive and negative ion modes in the *m*/*z* range 100–1000 with a scan time of 0.25 s in centroid mode. For the dynamic range enhancement (DRE) lockmass, a solution of leucine–enkephalin (Sigma–Aldrich, Steinheim, Germany) at 5 µg/mL was infused through the lockmass probe at a flow rate of 20 µL/min with the help of a second LC pump (Shimadzu LC-10ADvp, Duisburg, Germany). The separations were carried out on Waters Acquity UPLC columns at 30 °C (BEH $C_{18}$: 50 mm × 1.0 mm, 1.7 µm) with the following solvent system: A) 0.1% formic acid–water, B) 0.1% formic acid–acetonitrile. The gradient elution was performed at a flow rate of 300 µL/min using: 5% B for 0.3 min, 5–98% B in 4 min and holding at 98% B for 2 min.

### 2.6. Nuclear magnetic resonance spectrometry (NMR)

Nuclear magnetic resonance spectrometry (NMR) was used as the main analytical method for the structural elucidation of the isolated compounds. The $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Varian Inova 500 MHz spectrometer (Palo Alto, CA, USA) at 500 and 125 MHz, respectively. The instrument was controlled by Varian VNMR software installed on a Sun workstation (Santa Clara, CA, USA). All NMR measurements were performed in deuterated solvents (Dr Glaser AG, Basel, Switzerland). The shifts are indicated in ppm relative to tetramethylsilane (TMS) as an internal standard for $^{1}H$ spectra, and the deuterated solvent shift as reference for $^{13}C$ spectra. In order to observe homo- and heteronuclear correlations between proton and carbon atoms of the analytes, complementary two dimensional (2D) experiments were performed. For advanced and 2D spectra including COSY, HSQC, HMBC and NOESY, standard pulse sequences provided in the original VNMR software were employed.

### 2.7. Spectroscopic data for allocryptopine

$^{1}H$ NMR, 500 MHz, pyridine-$d_5$, 70 °C, $\delta_H$: 1.95 (3H, s, N–$CH_3$), 2.52 (2H, brs, H-6), 2.91 (2H, brs, H-5), 3.68 (3H, s, O–$CH_3$), 3.79 (3H, s, O–$CH_3$), 3.80 (2H, s, H-8), 3.87 (2H, s, H-13), 6.69 (1H, s, H-4), 5.90 (2H, s, O–$CH_2$–O), 6.83 (1H, d, $J$ = 8.3 Hz, H-11), 7.03 (1H, d, $J$ = 8.3 Hz, H-12) 7.17 (1H, s, H-1).

$^{13}C$ NMR, 125 MHz, pyridine-$d_5$, 70 °C, $\delta_C$: 32.6 (C-5), 41.9 (N–$CH_3$), 47.3 (C-13), 51.6 (C-8), 56.3 (O–$CH_3$), 58.6 (C-6), 61.0 (O–$CH_3$), 102.1 (O–$CH_2$–O), 109.3 (C-1), 111.3 (C-4), 112.1 (C-11), 126.2 (C-12), 129.3 (C-8a), 130.1 (C-12a), 133.8 (C-14a), 137.5 (C-4a), 146.9 (C-2), 148.7 (C-9), 148.8 (C-3), 152.6 (C-10), 192.9 (C-14).

HRMS *m*/*z* 370.1635 $[M+H]^+$ (calculated for $C_{21}H_{24}NO_5$, 370.1654).

### 2.8. Spectroscopic data for protopine

$^1$H NMR, 500 MHz, pyridine-$d_5$, 70 °C, $\delta_H$: 1.94 (3H, s, N–$CH_3$), 2.46 (2H, brs, H-6), 2.89 (2H, brs, H-5), 3.63 (2H, s, H-8), 3.96 (2H, s, H-13), 5.86 (2H, s, O–$CH_2$–O), 5.90 (2H, s, O–$CH_2$–O), 6.71 (1H, s, H-4), 6.75 (1H, d, $J$ = 8.0 Hz, H-11), 6.77 (1H, d, $J$ = 8.0 Hz, H-12), 7.12 (1H, s, H-1).

$^{13}$C NMR, 125 MHz, pyridine-$d_5$, 70 °C, $\delta_C$: 31.7 (C-5), 41.7 (N–$CH_3$), 47.2 (C-13), 51.7 (C-8), 58.7 (C-6), 101.6 (O–$CH_2$–O), 102.1 (O–$CH_2$–O), 107.1 (C-11), 108.4 (C-1), 111.2 (C-4), 119.5 (C-8a), 126.2 (C-12), 130.4 (C-12a), 133.5 (C-4a), 137.5 (C-14a), 146.9 (C-2), 147.3 (C-9), 147.3 (C-10), 148.7 (C-3), 194.8 (C-14).

HRMS $m/z$ 354.1360 $[M+H]^+$ (calculated for $C_{20}H_{20}NO_5$, 354.1341).

### 2.9. Spectroscopic data for berberine

$^1$H NMR, 500 MHz, DMSO-$d_6$, 25 °C, $\delta_H$: 3.21 (2H, brs, H-5), 4.07 (3H, s, O–$CH_3$), 4.10 (3H, s, O–$CH_3$), 4.95 (2H, brs, H-6), 6.17 (2H, s, O–$CH_2$–O), 7.08 (1H, s, H-4), 7.80 (1H, s, H-10), 8.02 (1H, d, $J$ = 9.2 Hz, H-12), 8.21 (1H, d, $J$ = 9.2 Hz, H-11), 8.96 (1H, s, H-13), 9.90 (1H, s, H-8).

$^{13}$C NMR, 125 MHz, DMSO-$d_6$, 25 °C, $\delta_C$: 26.8 (C-5), 56.0 (C-6), 57.8 (O–$CH_3$), 63.2 (O–$CH_3$), 102.7 (O–$CH_2$–O), 106.5 (C-1), 106.5 (C-8), 109.3 (C-4), 121.0 (C-13), 121.0 (C-14a), 124.4 (C-12), 127.7 (C-11), 131.4 (C-4a), 133.8 (C-12a), 138.2 (C-14), 144.5 (C-9), 146.6 (C-8a), 148.5 (C-2), 150.6 (C-3), 151.2 (C-10).

HRESIMS $m/z$ 336.1222 $[M]^+$ (calculated for $C_{20}H_{18}NO_4$, 336.1236).

### 2.10. Quantification of alkaloids in the decoction by quantitative NMR

For assessing linearity and accuracy of the method, a calibration curve was established in triplicate for allocryptopine. A standard solution was first prepared with 9.0 mg of isolated allocryptopine and 9.0 mg of anthracene, used as internal standard, in 1800 μL of pyridine-$d_5$. Successive dilutions of the standard solution were conducted to get triplicates of five concentrations for allocryptopine (5.5, 2.5, 1.7, 0.85 and 0.6 mg/mL) and anthracene (5.3, 2.4, 1.6, 0.8, 0.5 mg/mL), according to the exactly weighed mass of standards. $^1$H NMR spectra were obtained using 600 μL of each diluted solution.

For the determination of alkaloid concentration in the traditional preparation, the decoction was prepared as described for the traditional preparation for two available clinical batches AM 8 and AM 11. The plant material was exactly weighed (10.0000 g) and added with 100.00 mL distilled water in a 500 mL Erlenmeyer flask. Flasks were weighted and allowed to boil for 3 h. After that, the flasks were weighed at room temperature in order to establish the loss of water. Batch AM 8 provided 42.31 mL of tea, while batch AM 11 provided 60.51 mL. The resulting tea was filtered and 3 aliquots of 10.00 mL of each batch were transferred to individual centrifuge tube. Each sample was added with 20 mL $CH_2Cl_2$, shaken for 5 min and centrifuged at 5000 rpm. The supernatant was transferred to another centrifuge tube and the operation was repeated 4 times. The $CH_2Cl_2$ layers of each sample were grouped in a 100 mL round flask and evaporated to dryness. Each sample was recovered in 600 μL pyridine-$d_5$ for NMR analysis.

The following $^1$H NMR parameters were used: spectra were recorded in the Varian Inova 500 MHz spectrometer previously described, at 42 °C. Each sample was recorded with the following parameters: 100 scans per sample 0.20 Hz/point, pulse width (PW) = 8.0 μs, and relaxation delay (RD) = 1.0 s. FID files were Fourier transformed with the 1D NMR processor of ACDlabs® 8.0. Sweep width = 6000.60 Hz, LB = 0.3 Hz. Signal to noise (S/N) ratio was higher than 20. Peak areas were expressed as absolute integrals and the start and end points of the integration of each peak were selected manually.

### 2.11. In vitro biological assays

The antiplasmodial assay on chloroquine-resistant strains of *Plasmodium falciparum* (*Pf*K1) and the cytotoxic effects on human fibroblasts were conducted exactly as previously described (Simões-Pires et al., 2009). *Trypanosoma cruzi* (epimastigote form of Y strain) and *Leishmania amazonensis* (promastigote form of MHOM/BR88/BA-125 Leila strain) were maintained at 25 °C in liver infusion tryptose medium (Difco, Detroit, MI, USA) supplemented with 10% FBS, 1% hemin (Sigma, St. Louis, MO, USA), 1% R9 147 medium (Hyclone) and 5% sterile human urine. Epimastigotes of *T. cruzi* and promastigotes of *L. amazonensis* were plated in 96-well plates at 1 × 107 and 5 × 106 parasites/well, respectively, with or without plant extracts, in non-toxic concentrations to mouse spleen cells. After incubation at 25 °C for 24 h, the number of viable parasites was evaluated by counting in Neubauer chamber using a light microscope. Mean values of the triplicates were calculated and growth inhibition was given as the percentage of control values.

### 2.12. In vivo antiparasitic assay

NMRI mice infected with *Plasmodium berghei* were given a single dose of the freeze-dried decoction of *A. mexicana*, berberine sulfate or vehicle (control). The decoction was administered orally at 3 different concentrations (375, 1125, and 3375 mg/kg). Berberine sulfate was administered orally (at 4, 12, and 40 mg/kg) and subcutaneously (at 3 and 10 mg/kg). Tested samples were solubilized in water or saline. Activity was determined comparing the number of parasitized red blood cells (%) between test groups ($n$ = 3) and control group ($n$ = 3). The pilot experiment was conducted under the rules of the Swiss Tropical and Public Health Institute (Basel, Switzerland).

## 3. Results and discussion

After validation of the efficacy of the decoction, the identification of the active compound(s) took place in order to provide the basis for the standardization and quality control of the improved traditional medicine, which corresponds to stage 4 of the reverse pharmacology process.

The freeze-dried aqueous extract obtained from leaves of *A. mexicana* (26 g) was fractionated by liquid-solid extraction in methanol providing a MeOH insoluble fraction (AM 8_A) and a MeOH soluble fraction (AM 8_B). AM 8_A was submitted to VLC on $C_{18}$ silica gel providing two major fractions: one from the aqueous elution and another one from the methanol elution (Fig. 6). This procedure resulted in a simplified fraction (AM 8 A_2) presenting an HPLC/UV profile similar to phenolic compounds. However, subsequent isolation steps resulted in degradation of the compounds from fraction AM 8_A_2 and their identification was not possible.

The MeOH soluble fraction (AM 8_B) presented a peak corresponding to berberine in an HPLC/UV analysis (Willcox et al., 2007), and it was then inferred that other alkaloids could be present. The dried fraction was then evaporated to dryness and recovered in water to be partitioned with $CH_2Cl_2$. The alkaloid enriched $CH_2Cl_2$ fraction (AM 8_D) was the only fraction considered active against *P. falciparum in vitro*, with an $IC_{50}$ value of 1.71 μg/mL. Interestingly, when the whole freeze-dried decoction was tested *in vitro*, no antiplasmodial activity could be observed (Table 1). The fractionation of the $CH_2Cl_2$ alkaloid-containing fraction AM 8_D led to the isolation of three active alkaloids by semi-preparative HPLC: protopine, allocryptopine and berberine (Fig. 7). These

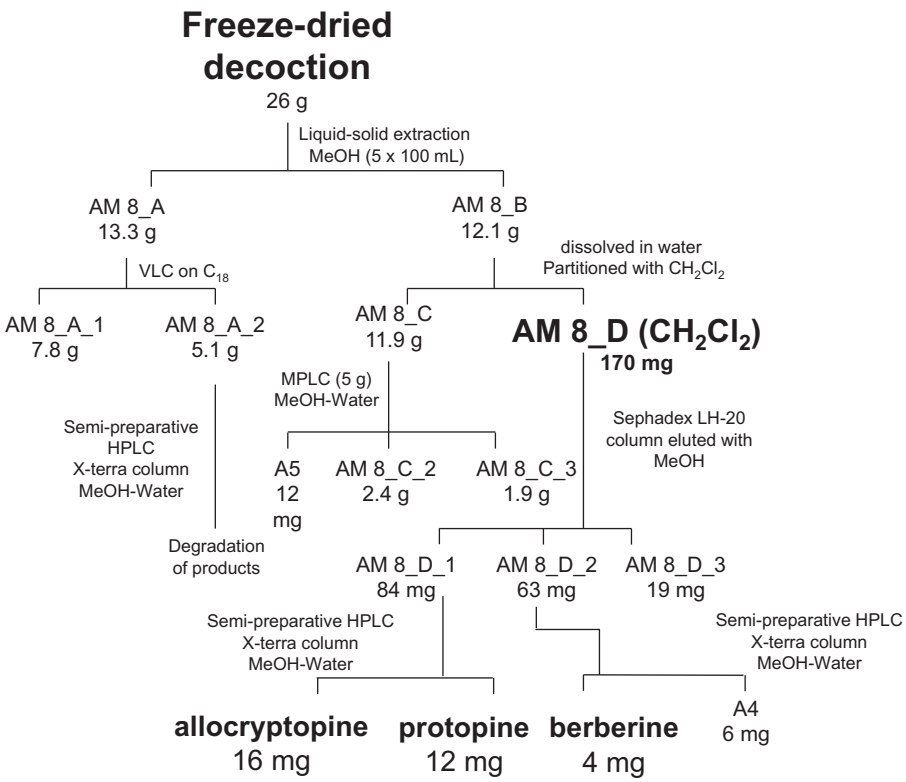


**Fig. 6.** General fractionation scheme for the freeze-dried *A. mexicana* decoction and isolation of the alkaloids allocryptopine, protopine and berberine.

alkaloids have been previously reported for *A. mexicana* extracts (Israilov and Yunusov, 1986; Bentley, 1998; Chang et al., 2003a,b); however, these 3 alkaloids together are first reported as the *in vitro* active ingredients in clinical decoction.

The isolated alkaloids were then tested not only for their antiplasmodial activity but also on parasites responsible for other parasitic diseases: American trypanosomiasis (Chagas disease) and African animal trypanosomiasis. Cytotoxicity was also determined on human fibroblasts. The alkaloids protopine, allocryptopine and berberine presented $IC_{50}$ values against *P. falciparum* of 0.32, 1.46 and 0.32 μg/mL, respectively. Berberine was also active against *T. cruzi* and *Trypanosoma brucei brucei* and showed a significant cytotoxicity (Table 1).

Based on these antiplasmodial *in vitro* results, the following questions needed to be answered: Are these alkaloids responsible for the clinical efficacy? Can we use these alkaloids as markers for quality control of a traditional medicine?

Despite the potent *in vitro* activity of berberine, previous studies showed that this compound was not able to decrease parasitaemia in *P. berghei*-infected mice (Vennerstrom and Klayman, 1988). In contrast, berberine significantly reduced parasitaemia in *Plasmodium chabaudi*-infected mice (McCall et al., 1994). According to some incomplete pharmacokinetic data from the literature, it has been suggested that berberine has very poor oral bioavailability *in vivo*. As a matter of fact, it was demonstrated that after a single oral dose of 400 mg, the amount of berberine in plasma is a very small part of what was administered (Gao et al., 2013). For protopine and allocryptopine very little pharmacokinetics data were available.

Given all the above, the next step was to conduct quantification of the active alkaloids directly in 3 clinical batches of *A. mexicana* decoction. A quantitative NMR analysis was chosen allowing the quantification of allocryptopine, berberine and protopine within a single spectrum acquisition. This is possible because the NMR response can be made the same for all components, contrary to HPLC/UV or MS methods. In fact, the strength of the NMR signal is proportional to the number of nuclei, thus the number of molecules, present in the analyzed sample. Moreover, in quantitative NMR analysis, a universal reference standard can be used for the analysis of most materials. As a consequence, quantitative determination of a specific compound does not require pure samples for calibration, which is of great interest for the determination of natural products not always available as standard compounds.

Preliminary analyses were conducted to find the best $^1$H NMR conditions (solvent and temperature) for allocryptopine, protopine and berberine. Allocryptopine and protopine are alkaloids of the protopine-type, presenting bad resolution in the region of the methylene protons of the ten-membered ring, especially at room temperature. For this reason, the chosen signals were those corresponding to the methylene dioxide protons of each molecule. A comparison of spectra for the three alkaloids in pyridine-$d_5$ at 42 °C is shown in Fig. 8. Under these conditions, one of the methylene dioxide signals of protopine overlaps with that of allocryptopine ($\delta_H$ 5.95). Since protopine has a second methylene dioxide signal at $\delta_H$ 5.90, this one could be considered for quantification and its integral value was subtracted from that at $\delta_H$ 5.95, for the quantification of allocryptopine. Spectra used for quantitative analysis had a signal/noise ratio higher than 20, and the signals for the three alkaloids found in the $CH_2Cl_2$-extracted decoction are depicted in Fig. 9.

The quantitative analysis showed that 1 L of decoction, which corresponds to a daily dose in the clinical assay, contains more than 300 mg of the three alkaloids considered as a whole (Table 2).

Moreover, we conducted a pilot *in vivo* experiment with the freeze-dried *A. mexicana* decoction and berberine sulfate. RPMI mice infected with *P. berghei* were given a single dose of samples either orally or subcutaneously, at different concentrations (highest oral dose of decoction = 3375 mg/kg). Unfortunately, no reduction of parasitaemia could be observed.

**Table 1**
Antiparasitic activity and cytotoxicity of fractions and compounds isolated from *A. mexicana* decoction.

| Compound | $IC_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | Anti-*P. falciparum*[a] | Anti-*T. cruzi*[b] | Anti-*T. b. brucei*[c] | Cytotoxicity[d] |
| Decoction | >64.00 | 39.24 | 0.57 | >64.00 |
| AM 8_A | >64.00 | 0.25 | >64.00 | >64.00 |
| AM 8_B | >64.00 | 51.42 | 10.08 | >64.00 |
| AM 8_C | >64.00 | >64.00 | 1.32 | >64.00 |
| AM 8_D | 1.71 | 0.25 | 0.25 | 24.98 |
| Protopine | 0.32 | >32.00 | 10.75 | >32.00 |
| Allocryptopine | 1.46 | >32.00 | 10.49 | >32.00 |
| Berberine | 0.32 | 0.32 | 1.66 | 3.20 |

[a] $IC_{50}$ > 16 μg/mL: inactive; 2 < $IC_{50}$ < 16 μg/mL: moderately active; $IC_{50}$ < 2 μg/mL: highly active.
[b] $IC_{50}$ > 30 μg/mL: inactive; 2 < $IC_{50}$ < 30 μg/mL: moderately active; $IC_{50}$ < 2 μg/mL: highly active.
[c] $IC_{50}$ > 5 μg/mL: inactive; 1 < $IC_{50}$ < 5 μg/mL: moderately active; $IC_{50}$ < 1 μg/mL: highly active.
[d] Tested on human fibroblasts (MRC-5 cell line); $IC_{50}$ > 30 μg/mL: not toxic; 10 < $IC_{50}$ < 30 μg/mL: moderately toxic; $IC_{50}$ < 10 μg/mL: highly toxic.

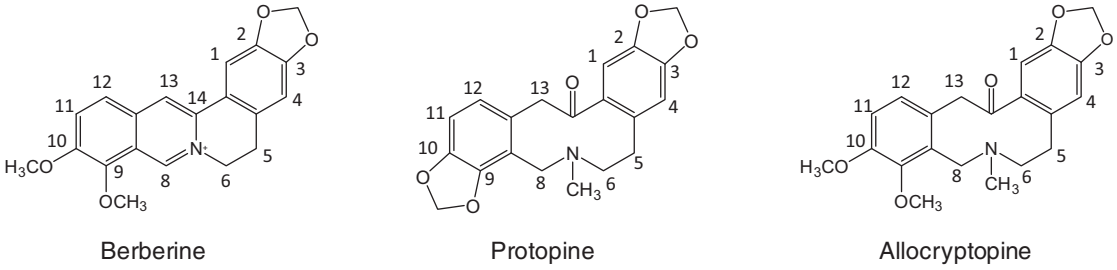


**Fig. 7.** Chemical structure of active alkaloids obtained from the bioguided fractionation of *A. mexicana* decoction.

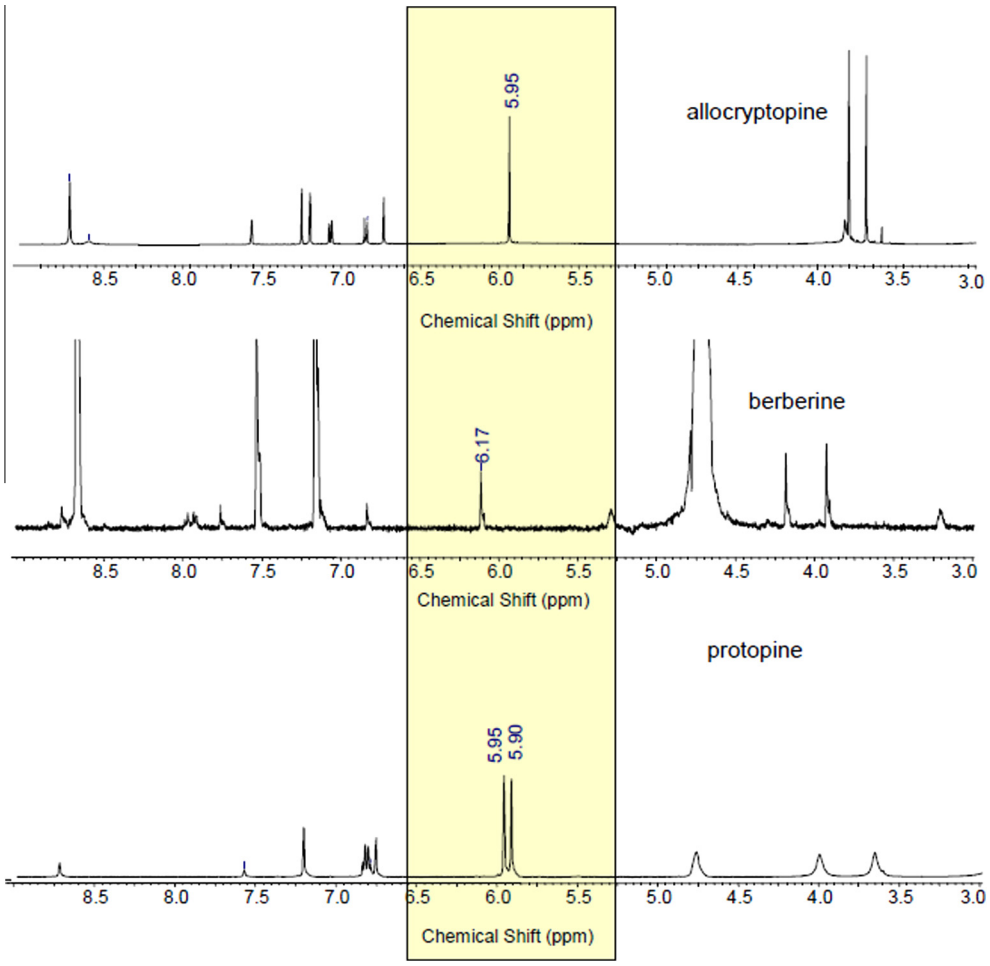


**Fig. 8.** Comparative $^1$H NMR spectra of allocryptopine, berberine and protopine (500 MHz, 42 °C, pyridine-$d_5$).

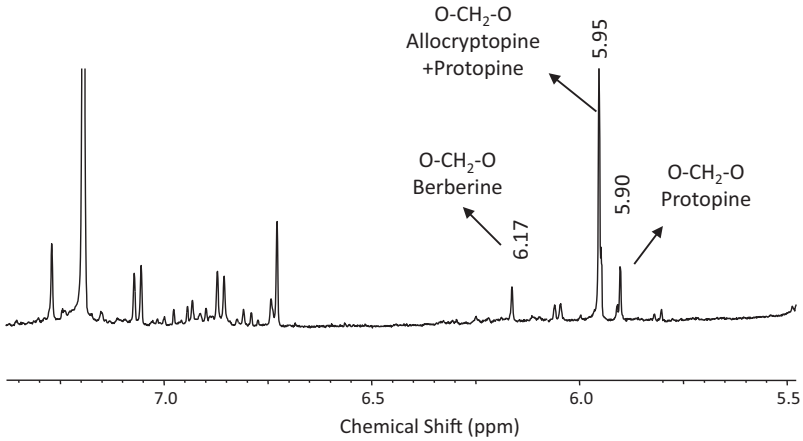


**Fig. 9.** [1]H NMR spectrum of an analytical sample obtained using the extraction method for the quantitative analysis of *A. mexicana* decoction (500 MHz, 42 °C, pyridine-$d_5$).

**Table 2**
Calculated concentrations of allocryptopine, protopine and berberine in the decoction of clinical batches of *A. mexicana* obtained by the quantitative NMR.

| *A. mexicana* clinical batch | Compound | Concentration in tea (mg/mL)[a] | % in plant (w/w)[a] |
|---|---|---|---|
| AM 8 | Allocryptopine | 0.11 ± 0.01 | 0.47 ± 0.03 |
| | Protopine | 0.12 ± 0.02 | 0.51 ± 0.02 |
| | Berberine | 0.11 ± 0.01 | 0.48 ± 0.03 |
| AM 11 | Allocryptopine | 0.18 ± 0.01 | 1.10 ± 0.07 |
| | Protopine | 0.06 ± 0.00 | 0.39 ± 0.01 |
| | Berberine | 0.07 ± 0.01 | 0.43 ± 0.04 |

[a] Average ± SD.

## 4. Conclusions

From an extensive research of the active substances of *A. mexicana*, we have not found an explanation for the clinical effectiveness of the decoction. This is not an exceptional situation: mechanisms of action of many medicinal plants remain a mystery, and this is true for many synthetic drugs as well. In the case of *A. mexicana*, from a molecular biology perspective, the reason why its use is associated with an excellent clinical outcome has not been elucidated so far. It is noteworthy that at stage 4, we started to apply a conventional method of phytochemical bioguided fractionation in order to find out the compounds responsible for the antimalarial efficacy of *A. mexicana* decoction. This highlights the very narrow spectrum of *in vitro* tests available for malaria: it is possible to detect direct toxicity on the parasite, but any other biological mechanism involved in the overall clinical effect cannot be studied by current *in vitro* methods.

On the other hand, the reverse pharmacology research process allowed us to avoid two mistakes: a) if we had started with animal studies, we would have discarded *A. mexicana* before conducting any human study, because animal studies did not show any antimalarial effects of the plant. b) if we would have studied *in vitro* active substances from *A. mexicana* without subsequent *in vivo* studies, we would have claimed that the active substances were discovered. Given the facility with which such claims might be established, we should re-visit claims of active substances of other plants and ask whether these substances are in sufficient quantity, and sufficiently absorbed, to explain the observed clinical effects.

Even if the active compounds from *A. mexicana* are not known for the moment, we think that "reverse pharmacology" is an interesting alternative for the development of a validated phytomedicine because its results have public health and health policy implications. The primary objective of this study was not to develop new drugs but to provide information on phytopreparations already in use locally by the population. It is a complement to existing strategies to be used in parallel with conventional drug development.

## Conflict of interest

The authors declare no conflict of interest.

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