Antimalarial Activity of *Bidens pilosa* Root Extract Co-spray Dried in the Presence of β-Cyclodextrin or Aerosil:Microcrystalline Cellulose Blend

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ABSTRACT

The purpose of this work was to evaluate if the antimalarial activity of *Bidens pilosa* L. root extract could be enhanced by co-spray drying with the pharmaceutical excipients blend of colloidal silicon dioxide:microcrystalline cellulose and β-cyclodextrin. The *in vivo* antimalarial activity of the products was evaluated in mice infected with *Plasmodium berghei*. Acute *in vivo* and *in vitro* toxicity in S5 HeLa cells were also carried out. *B. pilosa* L. root extract was lyophilized and used as a control. The spray-dried preparations enhanced the survival of the infected mice compared to the lyophilized crude root extract. The *Bidens* extract formulations were able to inhibit up to 71 % of the growth of the parasite in the lowest tested dose, being about five times more active than the crude extract, thus showing significant partial antiplasmodial activity. The dried preparations did not show signals of toxicity in both the *in vitro* and *in vivo* assays. The results showed strong evidence that the co-spray drying of *B. pilosa* root extract with the selected pharmaceutical excipients might stabilize the bioactive compounds and enhance its antimalarial activity compared with the lyophilized crude extract.

ABBREVIATIONS

Aerosil:MC	colloidal silicon dioxide and *microcrystalline* cellulose
Bp.Ro.At	*Bidens pilosa* root extract co-spray dried with a colloidal silicon dioxide:microcrystalline cellulose blend
Bp.Ro.Bc	*Bidens pilosa* root extract co-spray dried with *β*-cyclodextrin
Bp.Ro.Cru	lyophilized *Bidens pilosa* root extract
CC_{50}	50 % cytotoxic concentration
HPLC-DAD	high-performance liquid chromatography with diode array detection
HPLC/UV	high-performance liquid chromatography with ultraviolet detection
IAC	"Instituto Agronômico de Campinas"
MC	microcrystalline cellulose
OECD	Organization for Economic Cooperation and Development
TFC	total flavonoid content

Introduction

Despite notable progress in prevention and intervention policies, malaria remains a deadly disease. In 2018, nearly 228 million cases of malaria were reported worldwide with nearly 405 000 deaths, largely in Africa. Children aged under 5 years are the most vulnerable group and accounted for almost 67 % (272 000) of malaria deaths [1]. For centuries, quinine remained the major antimalarial drug, but in the 1930s, this natural product was largely replaced by synthetic substances, including primaquine, chloroquine, and mefloquine. However, by the early 1980s, several strains of *Plasmodium falciparum* have become multidrug resistant to the usual antimalarial drugs and there is not an effective vaccine [2–5]. Artemisinin, the active substance from *Artemisia annua* L., was identified in 1972 and is considered the most relevant and effective phytopharmaceutical antimalarial drug. However, its success has been threatened by the emerging of resistance of *P. falciparum* Southwest of Asia [6] and, more recently, in Rwanda [7], leading to the inefficacy of the antimalarial therapies based on artemisinin. Therefore, research and development into new antimalarial medicines is the best insurance policy against the risk of antimalarial resistance [8]. It is often argued that total plant extracts contain a mixture of substances that might act synergistically against diseases, although their exact mechanisms of action are not exactly understood [9]. For example, the presence of methoxylated flavonoids in *A. annua* artemisinin preparations contributed to a significant enhancement of its *in vitro* activity against *P. falciparum* of artemisinin [10–13], although the flavonoids do not exhibit a prominent activity alone. Products containing the whole components of the *A. annua* plant, as the dried tablets with leaves of *A. annua*, have been proven to be effective in cases of resistant malaria in Africa [14].

In the Amazon, where the disease is endemic [15–18], several plant species have been traditionally used to treat malaria, fever, and liver disorders [18]. Crude ethanol extracts (whole plant, leaves, and roots), and the chloroform and butanol fractions of *Bidens pilosa*, exhibited up to 90 % inhibition of *P. falciparum* growth (*in vitro*), while *in vivo* studies showed a 40–50 % reduction of *Plasmodium berghei* parasitemia in mice. The pharmacological activities of *B. pilosa*, such as antimalarial, anticancer, antimicrobial, immunosuppressive, anti-inflammatory, and antioxidant, have been linked to several of its phytochemicals, such as the polyacetylenes [e. g., 2-*β*-$_D$-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (cytopiloyne), 2-O-*β*-D-glucosyltrideca-11E-en-3,*5*,*7*,*9-tetrayn-1*,*2-diol*, phenyhepta-1,3,5-triyne, 1-phenyl-1,3-diyn-5-en-7-ol-acetate] and flavonoids (e. g., aurones, chalcones, or quercetin derivates) [19–23]. Some of these compounds are unstable and can easily decompose in the presence of light and heat [19, 24]. Therefore, the development of phytopharmaceutical preparations loaded with both groups of active compounds is highly relevant.

This work aimed to evaluate if the co-spray drying of *B. pilosa* root extract in the presence of Aerosil:MC or with *β*-cyclodextrin would enhance its *in vivo* antimalarial activity and stabilize the dried product.

Results

Total flavonoid content and chromatographic profile of the extractive solution

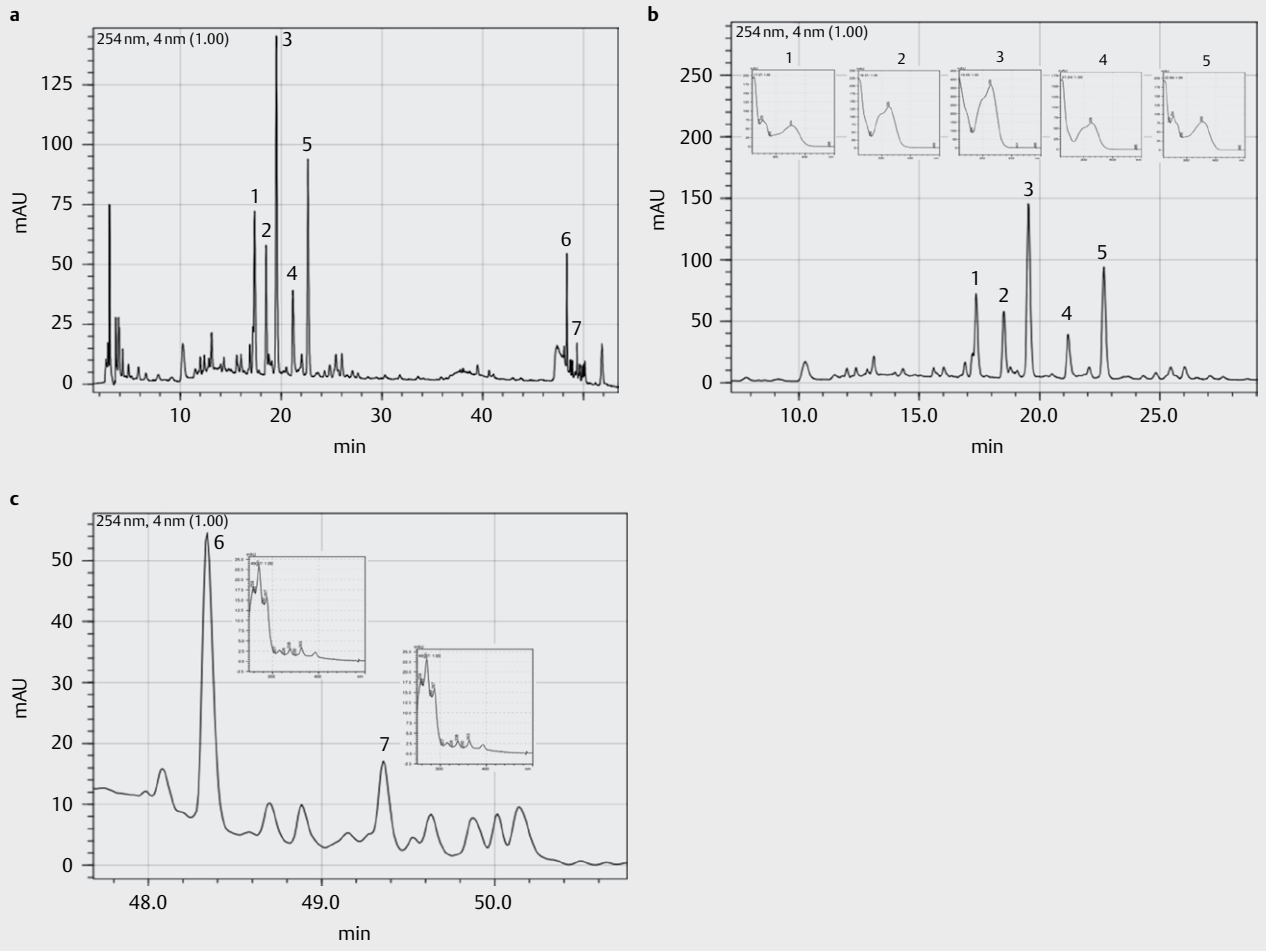
The roots used to prepare the extract presented a moisture content of 4.6 ± 0.2 % (w/w), and a TFC of 0.56 mg/g of herbal material. The TFC of the resulting extractive solution was 6.62 ± 0.04 mg/g (dry basis).

▶ **Fig. 1a-c** shows HPLC/UV chromatograms of the *B. pilosa* crude extract. Peaks for flavonoids and organic acids were detected showing an retention time (RT) between 10 and 22 min (peaks 1 to 5) and confirmed by their UV profiles, as can be seen in ▶ **Fig. 1b**. Polyacetylenes can be analyzed by HPLC with a characteristic UV profile, as shown in ▶ **Fig. 1c**, as previously reported by Brandão et al. [20]. HPLC-MS analysis of the root extract confirmed the presence of various flavonoid compounds, such as quercetin, methoxylated quercetin, dimethoxylated quercetin, hyperoside, and methoxylated hyperoside (data not shown). Both the flavonoids and polyacetylenes have been linked to *B. pilosa* antimalarial activity [19, 20].

▶ **Fig. 2** shows the qualitative HPLC profiles of the Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc dried preparations. It can be seen that the qualitative HPLC profiles of the dried extract preparations are almost similar, the differences being the heigh peaks caused by extract dilution due to the addition of the pharmaceutical excipients. Nevertheless, peak 6, presented in the HPLC profile of the concentrated crude extract (▶ **Fig. 1a**), is missing in the HPLC fingerprints of dried *B. pilosa* preparations. However, the Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc samples used in the actual analysis were prepared more than 2 years ago (maintained under storage at -20 °C), which can contribute to the loss or reduction of the concentration of the compound corresponding to peak 6.

Moisture content, water activity, and total flavonoid content of the co-spray dried products

Moisture content of the dried powders is linked to physicochemical properties such as solubility, morphology, and flowability. In this



▶ **Fig. 1** HPLC profiles of the crude extract of *B. pilosa* root at **a** 0 to 54 min, **b** 7 to 29 min, and **c** 47.8 to 50.8 min. UV profiles were paired with the corresponding peak number.

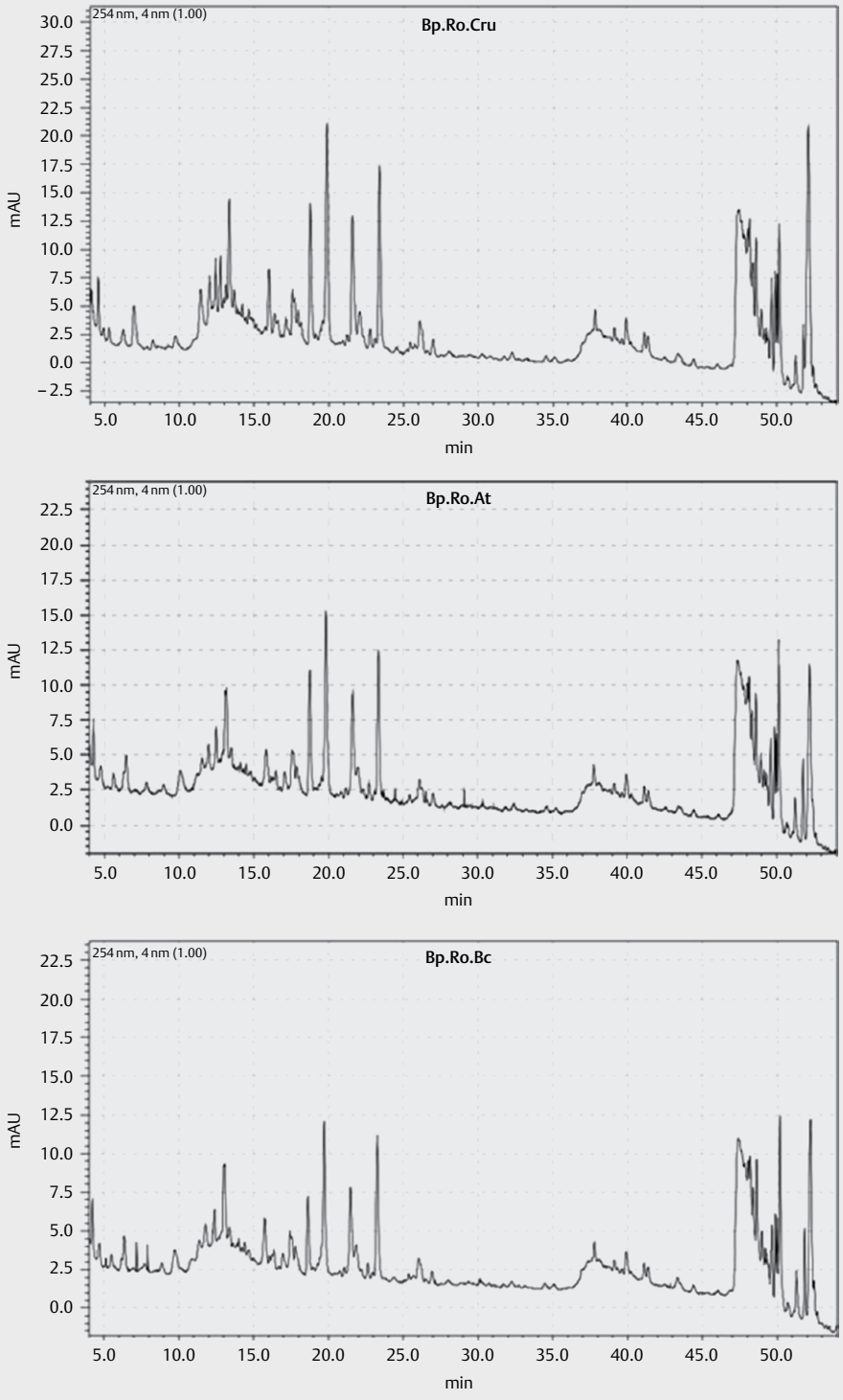
work, the moisture content of the dried products Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc were 2.7 ± 0.2, 5.6 ± 0.4, and 6.4 ± 0.1 % w/w, respectively. The water activity is a measure of the energy state of water present in a system, and is independent of the moisture content. The experimental results of water activities for Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc were, respectively, 0.1730 ± 0.0097, 0.2524 ± 0.0133, and 0.2353 ± 0.0041, *respectively*. Values of water activity lower than 0.5 minimize the rate of degradation reactions and inhibit microbial growth, improbing product stability during storage [25].

The TFC content of the lyophilized crude extract (Bp.Ro.Cru) and spray-dried products (Bp.Ro.At and Bp.Ro.Bc) was 6.21 ± 0.11, 3.85 ± 0.13, and 2.80 ± 0.09 mg/g (dry basis), respectively. The lower TFC values observed mainly for the spray-dried products Bp.Ro.At and Bp.Ro.Bc are mainly due to the dilution effect caused by the coprocessing excipients (Aerosil:MC or β-cyclodextrin) and product moisture. With the removal of these effects, it can be shown that the reduction of TFC contents for Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc caused by drying processing were only 3.6, 7.6, and 9.6 % of the value determined for the extractive solution (dry basis), respectively. These results are similar to those observed by Cortés-Rojas et al. in the spray drying of the extract of *B. pilosa* leaves in the presence of different excipients [26, 27].

Antimalarial activity

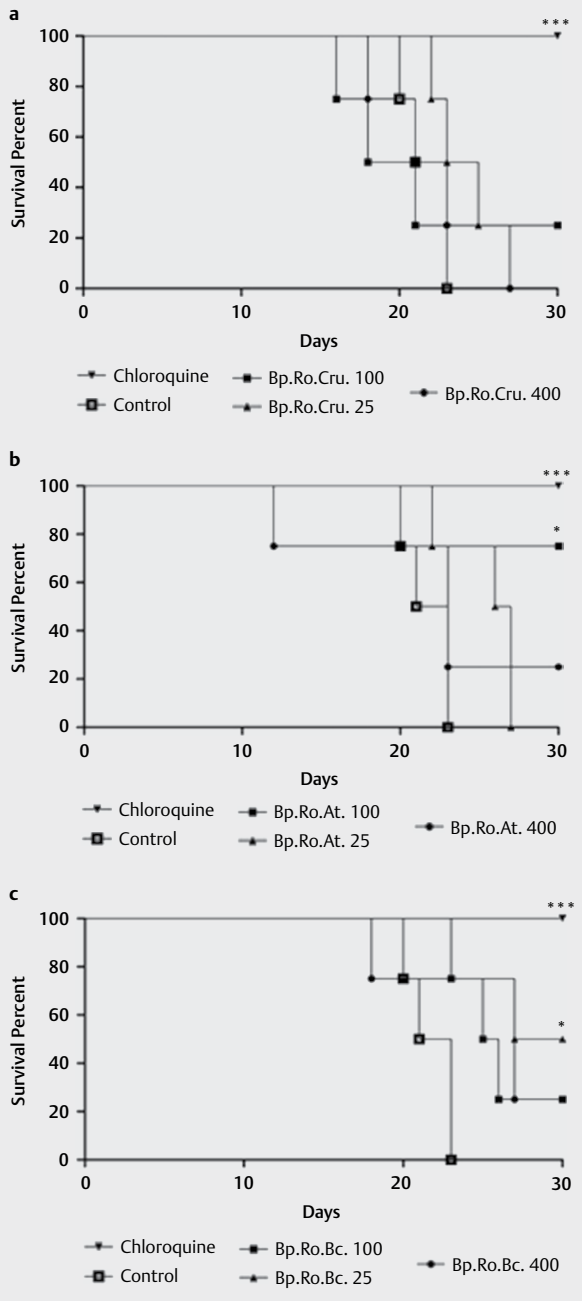
Bp.Ro.Cru at a dose of 100 mg/kg increased the survival of the animals compared to the control group that received only the vehicle (distilled water plus 0.1 % Tween-20, final volume) (▶ **Fig. 3a**). The co-spray dried *B. pilosa* root extracts (Bp.Ro.At and Bp.Ro.Bc) enhanced mice survival compared to Bp.Ro.Cru. The Bp.Ro.At increased animal survival at all doses tested, with 100 mg/kg being the most effective. At this dosage, the survival rate reached 75 % at the end of the experiment (▶ **Fig. 3b**). In the same way, the animals treated with Bp.Ro.Bc also showed an increased survival rate at all dosages tested. However, the most effective dosage was 25 mg/kg with a survival rate of 50 % at the end of the experiment (▶ **Fig. 3c**).

B. pilosa formulations were efficient to reduce the parasitemia in all treated groups (▶ **Table 1**). The animals treated with Bp.Ro.At caused a reduction of 61, 52, and 55.6 % in parasitemia on

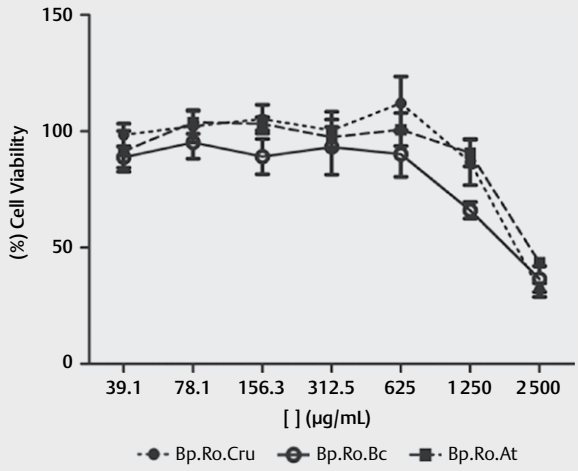


▶ **Fig. 2** Qualitative HPLC profiles of the Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc dried preparations.

day 5, while Bp.Ro.Bc saw a reduction of 71, 47, and 57.6 % for the doses of 25, 100, and 400 mg/kg, respectively. On the 7th day after infection, Bp.Ro.At at a dose of 25 mg/kg exhibited a significant reduction of parasitemia compared to the untreated group (the group that received only the vehicle). Bp.Ro.Bc, at concentrations of 25 and 100 mg/kg, maintained a similar and significant reduction of parasitemia of the animals when compared to what was observed on the 5th day after infection. The lyophilized *B. pilosa* root extract (Bp.Ro.Cru), showed less antimalarial activity than the dried co-spray *B. pilosa* root extracts, being about 1.4–5.0 times less effective at a dose of 25 mg/kg.



▶ **Fig. 3** Average mouse survival after oral treatment with chloroquine, vehicle (negative control, **a** Bp.Ro.Cru (the lyophilized *B. pilosa* root extract), **b** Bp.Ro.Bc (*β*-cyclodextrin), and **c** Bp.Ro.At (Aerosil:MC) in the Peters 4-day suppressive test.



▶ **Fig. 4** Cytotoxicity tests with S5 HeLa cells using dry extracts of *B. pilosa* roots. Bp.Ro.Cru (the lyophilized *B. pilosa* root extract), Bp. Ro.Bc (*β*-cyclodextrin), and Bp.Ro.At (Aerosil:MC). *Footnote:* Chloroquine, the positive control, was used in parallel. The *in vitro* CC_{50} of Bp.Ro.Bc was 1988 µg/mL, 2465 µg/mL for Bp.Ro.At, and 2104 µg/mL for Bp.Ro.Cru, while for chloroquine, it was 0.06 µg/mL.

▶ **Table 1** Antimalarial activity of the *B. pilosa* formulations against *P. berghei* in mice.

Products	Dose (mg/kg daily by oral route, 4×)	Inhibition of parasite growth (%)	
		5º day	7º day
Bp.Ro.Cru	0		
	25	43.4	13
	100	15.5	11
	400	56.2*	49.3*
Bp.Ro.At§	0		
	25	61**	48.4*
	100	52*	34.6
	400	55.6*	28.8
Bp.Ro.Bc§	0		
	25	71***	69.7***
	100	47*	46.6*
	400	57.6*	43.8

***P < 0.001, **p < 0.01, *p < 0.05. Bp.Ro.Cru (the lyophilized *B. pilosa* root extract), Bp.Ro. At (Aerosil:MC), Bp.Ro.Bc (*β*-cyclodextrin). §Doses of *Bidens'* formulations are based on the dried crude extract present. The zero dose (control) contains only the vehicle.

In vitro and *in vivo* toxicity

The *in vitro* CC_{50} of Bp.Ro.Bc was 1988 µg/mL, 2465 µg/mL for Bp.Ro.At, and 2104 µg/mL for Bp.Ro.Cru, while for chloroquine, it was 0.06 µg/mL. In the acute toxicity study, *B. pilosa* spray-dried formulations at doses of 300, 1000, and 2000 mg/kg did not cause any visible signs of toxicity or mortality in the 1st, 2nd, 4th, and 6th hours after administration nor at 14 days (monitored 1 h daily). The crude extract showed a slight piloerection at the highest dose tested (▶ **Table 2**).

Discussion

Today, significant efforts have been directed at developing pharmaceutical formulations of herbal products, which can improve

▶ **Table 2** Acute toxicity of *B. pilosa* root extracts.

Compounds (mg/kg)	Mice		Visible signs	
	Sex	D/T	SP	Symptoms
Bp.Ro.Cru				
0	Females	0/3	NS	NS
300	Females	0/3	NS	NS
1000	Females	0/3	NS	NS
2000	Females	0/3	NS	Mild piloerection
Bp.Ro.At				
0	Females	0/3	NS	NS
300	Females	0/3	NS	NS
1000	Females	0/3	NS	NS
2000	Females	0/3	NS	NS
Bp.Ro.Bc				
0	Females	0/3	NS	NS
300	Females	0/3	NS	NS
1000	Females	0/3	NS	NS
2000	Females	0/3	NS	NS

Bp.Ro.Cru (the lyophilized *B. pilosa* root extract), Bp.Ro.At (Aerosil:MC), Bp.Ro.Bc (*β*-cyclodextrin). 0 = only the vehicle (distilled water). D/T = dead mice/treated mice; SP = first day of symptom presentation; NS = no symptoms observed.

their biological activity and stability [28, 29]. The drying of plant extracts is a process used to reduce their moisture content and water activity to a very low level, consequently increasing the stability and shelf life of these products. Also, the use of dried products might facilitate the standardization and dosage of bioactive ingredients as well as reduce the risks of microbial spoilage and costs of transportation and storage [30, 31]. The addition of pharmaceutical excipients plays a key role in the final product properties and therefore should be accurately selected [32].

We have previously shown that the antimalarial activity of *B. pilosa* root extract is correlated with the presence of the lipophilic polyacetylenes together with methoxylated flavonoids [19, 20, 33]. A chloroform fraction enriched in acetylene, and other butanoic fractions enriched in flavonoids, for example, showed lower activity *in vivo* in comparison to the ethanol extract. By HPLC, the ethanol extract showed the presence of several peaks for polyacetylenes and flavonoids, while in the ether:ethanol and ether fraction, only flavonoids and acetylene were detected [19]. In *in vivo* tests, the ethanol extract caused a 36 % reduction of parasitemia on the 5th day and 29 % on the 7th day. The ether:methanol fraction caused a 38 % reduction on the 5th day but was inactive at day 7. The animal's survival was also higher in the animals that received the ethanol extract compared to the ether:ethanol and ether fractions. Of all the animals given the ethanol extract, 100, 60, 40, and 20 % survived 7, 11, 15, and 19 days after administration, while no animal that received the fractions survived from the 15th day after infection [19]. These results showed that similar to the artemisinin preparations, ethanol extracts administered via the oral route reduced parasitemia and mice mortality, and this activity is linked to the presence of both polyacetylenes and methoxylated flavonoids.

In previous studies, several conditions were tested to develop standardized dry extracts from *B. pilosa* leaves [26, 27]. In this study, we developed dried extracts from the roots of *B. pilosa* associated with pharmaceutical excipients with distinct physicochemical properties. The objective was to develop an innovative strategy to enhance its antimalarial activity. The system Aerosil:MC was proposed due to the high specific surface and adsorbent properties of colloidal silica [30]. MC has plasticity, which can be beneficial to tabletting [34]. Cyclodextrin is used as a pharmaceutical excipient due to its ability to form inclusion complexes with nonpolar molecules [35]. The *β*-cyclodextrin molecule possesses a characteristic cage-like supramolecular structure composed of 6–8 glucopyranose subunits. The lipophilic cavity of cyclodextrins provides a microenvironment for nonpolar compounds, protecting them from external conditions [36]. Acetylenes present in *B. pilosa* extracts are highly hydrophobic compounds and possess a high propensity to form inclusion complexes with cyclodextrins, which might protect them from the harmful effects of temperature, light, and oxidation [35, 36]. Qualitative HPLC chromatograms obtained for Bp.Ro.Cru, Bp.Ro.At, and Bp.Ro.Bc did not show evidence of significant differences in the chemical profiles of the herbal preparations caused by drying or excipient addition.

The *B. pilosa* root extract co-spray dried with Aerosil:MC or *β*-cyclodextrin showed enhanced antimalarial activity compared to the lyophilized crude extract, reducing mortality and the parasitemia of mice infected with *P. berghei*. However, there was no direct correlation between increased extract concentration and antimalarial activity, since in the highest doses tested, *Plasmodium* growth inhibition was only 34.6–57.6 % on days 5 and 7 after inoculation. This result may be a consequence of an immunosuppressive activity provided by constituents of the herbal preparations. Actually, the immunomodulation effect of the crude extracts of *B. pilosa* and polyacetylene compounds has been demonstrated in previous studies [37–39]. Analyzing from this perspective, it seems plausible that when administered in smaller doses, the concentration of the constituent responsible for immunomodulation is possibly not sufficient to trigger an immunosuppressive effect additive to malaria. Otherwise, the *β*-cyclodextrin composition was able to maintain the antimalarial activity for a longer time compared to the lyophilized crude extract, as evidenced by the reduced persistence of parasitemia. *In vitro* and *in vivo* assays with the dried preparations did not show any toxicity signal, which was ranked in category five of the OECD and considered nontoxic or of very low toxicity.

Taken together, the results reported here demonstrated that the *B. pilosa* root extract co-spray-dried with Aerosil:MC or *β*-cyclodextrin exhibited a higher parasitic reduction of *P. berghei* in mice compared to the lyophilized crude extract of *B. pilosa* roots, which correlated with the increased survival of infected animals. The positive effects on the antimalarial activity caused by the technological processing of the *B. pilosa* root extract with pharmaceutical excipients are very promising and deserve further studies to improve their pharmacokinetic properties aiming at the development of a phytomedicine that can be used in combination with the antimalarial drugs.

Material and Methods

Bidens pilosa roots

B. pilosa individuals in the flowering phenophase, at least 1.5 m in height, were collected at the Experimental Farm of IAC (Agronomic Institute of Campinas) in Monte Alegre do Sul, São Paulo, Brazil (S.22°41.57.91'5, W.46°40'32.70.0). The soil analysis showed an organic content of 34 % and a pH of 5.2. The plant species was identified by Prof. Dr. Milton Groppo Jr., Department of Botany of the Faculty of Philosophy, Science and Letters at Ribeirão Preto/USP. A voucher specimen was deposited at the herbarium of the University of São Paulo (SPFR 12751). *B. pilosa* roots were manually separated and washed with distilled water and submitted to air drying at 37 °C for 24 h. Once dried, the roots were milled in a knife mill until passing through a 20-mesh sieve, and hermetically stored in sealed containers protected from humidity and light until use.

Preparation of *Bidens pilosa* roots extract

B. pilosa root extract was produced by dynamic maceration, according to the conditions proposed by Cortés-Rojas et al. [26], using ethanol 62.7 % (v/v), an extraction temperature of 66.2 °C, and a plant-to-solvent ratio of 1/10 (w/v). The extractive mixture was vacuum filtered using filter paper (80 g/m^2, 14 µm pore, 205 µm thickness; J. Prolab). Then, the filtered extractive solution was concentrated in a rotary evaporator at a vacuum pressure of 600 mmHg and maximum temperature of 55 °C until reaching a solid concentration of 4.0 ± 0.1 % w/w. The solid concentration was determined in an infrared moisture analyzer (MA-35; Sartorius). The result expresses the mean and average error of triplicate measurements. The extractive solution was characterized through the determination of the TFC and by HPLC fingerprints [27]. TFC values are expressed as quercetin equivalent.

Chromatographic profiles of the *Bidens pilosa* root extract

The characterization of the *B. Pilosa* root extract and dried extract preparations were carried out by HPLC-DAD. Analyses were performed in a Shimadzu LC-20A series and an LC-6A double pump using a C-18 column [Shimadzu Shim-Pack CLC (M) 4.6 mm × 25 cm, 5 µm, 100 Å] at 30 °C. Chromatograms were recorded at 254 nm [27]. The mobile phase was a gradient of acetonitrile-acidified water (pH 2.8). The acetonitrile concentration was gradually increased as follows: 0–5 min 10 %, 5–7 min 20 %, 7–31 min 31 %, 32–44 min 40 %, 44–50 min 100 %, and 55–58 min 10 %. The samples were exactly weighed in an analytic balance, placed in glass containers together with methanol 80 % (concentration of 7 mg/mL for root extract, and 1 mg/mL for dried extract preparations), and maintained under magnetic agitation (Marte, Brazil model mag-multi, 500 rpm) for 12 h for complete extraction of bioactive compounds. Finally, samples were filtered through a 0.45-µm Millipore membrane and 10 µL were injected into the chromatograph.

Co-spray drying of *Bidens pilosa* root extract

The co-spray drying of *B. pilosa* root extract was carried out by combining the concentrated extract (solids content of 4.0 ± 0.1 %) with (i) a blend of colloidal silicon dioxide (Aerosil 200; Evonik Degussa) plus microcrystalline cellulose MC-102 and (ii) β-cyclodextrin (Roquette Ref.795872). For product (i), the extract was concentrated to a solid content of 8.7 % and a blend of Aerosil:MC (13:37) was added at a of 2:1 ratio. This product was named Bp.Ro.At. For product (ii), β-cyclodextrin was added at a proportion of 1:1 and stirred for 15 min at 200 rpm (Bp.Ro.Bc product). Afterwards, the formulations were concentrated in a rotary evaporator (vacuum pressure of 600 mmHg at 55 °C) until a solids content of 11.41 ± 0.03. This product was named Bp.Ro.Bc. For products (i) and (ii), the concentrations were calculated concerning solid content (dry basis). The crude extract, without any excipient, was lyophilized, named Bp.Ro.Cru, and used as a reference material.

The spray drying runs of the products Bp.Ro.At and Bp.Ro.Bc were performed in a bench-top Lab-Plant SD-05 spray dryer with a concurrent flow regime. Briefly, it consists of a drying chamber of 215 mm diameter and 500 mm height, equipped with a peristaltic pump, a two-fluid atomizer (inlet orifice diameter of 0.5 mm) connected to an air compressor, and a feed system of the drying air composed by a compressor, filter, and temperature control. The dried product was collected in a Lapple cyclone (column diameter of 0.085 m, cut diameter 3.9 microns.). Atomization pressure and airflow rate were maintained constant at 2 bar and 15 L/min, respectively. The feed rate was 9.0 ± 0.3 g/min and the inlet drying temperature was 155.2 °C [27].

Lyophilization

A portion of the crude concentrated extract (without pharmaceutical excipient) was lyophilized and the product was used as a reference sample since dehydration occurred at a low temperature. The liquid crude extract of *B. pilosa* was placed in 50 mL Falcon centrifuge tubes and frozen at -20 °C in the horizontal position. For the lyophilization process, the cap was removed and replaced by a perforated plastic film. The lyophilization was carried out in a Thermo Fisher Scientific freeze dryer, model SNL 108, containing a Micromodulyo 1.5 L freeze-drying unit. The drying process lasted 24 h. This product was named Bp.Ro.Cru.

Spray dried and lyophilized products (Bp.Ro.At, Bp.Ro.Bc, and Bp.Ro.Cru) were characterized by determination of the moisture content by the Karl-Fischer method (water activity using an Aqua Lab 4Tev • water activity meter; Decagon Devices) and by quantification of TFC [26]. The results are expressed as a mean of three determinations.

Antimalarial activities

All antimalarial assays were performed according to the Guidelines for Ethical Conduct in The Care and Use of Animals (CEUA) from the Federal University of Rio Grande do Norte (CEUA, Protocol number 046/2013; approved on January 31, 2014). The antimalarial suppressive test described by Peters [40] and modified by Carvalho et al. [41] was performed in mice (female, 22–25 g), injected intraperitoneally with 1×10^5 erythrocytes parasitized with *P. berghei* strain NK-65. The herbal preparations were suspended in distilled water plus 0.1 % Tween-20 (final volume) immediately before use, then diluted with distilled water so that doses of 25, 100, or 400 mg/kg could be administered in a 0.2-mL volume per animal via gavage. Doses were standardized based on the amount of lyophilized crude extract (Bp.Ro.Cru). Hence, the quantities used for extract preparations differ by the presence of the pharmaceutical

excipients added at a ratio of 1:1 in Bp.Ro.At and 1:2 (carrier:dried extract) in Bp.Ro.Bc. The animals were randomly separated into groups of four for each drug test and treatment was carried out daily via the oral route 24 h after infection for 4 consecutive days. A group of animals received the standard chloroquine (25 mg/kg) and another group received the vehicle (distilled water plus 0.1 % Tween-20) used as positive and negative controls, respectively.

Antimalarial activity was evaluated by counting parasitemia in blood smears, taken on days 5 and 7, by microscopy (methanol fixation and staining with Giemsa). Inhibition of parasite growth in the drug-treated groups was calculated concerning the negative control (vehicle) group. Results are expressed as a percentage of parasitemia reduction. Products that reduced parasite growth by ≥ 30 % were considered active [41]. Animal mortality was monitored daily for 4 weeks.

In vitro and *in vivo* acute toxicity

The *in vitro* cytotoxicity of the encapsulated products was evaluated by the MTT assay based on Oliveira et al. [42]. The compounds to be tested were diluted with a 0.02 % final concentration of Tween-20 in culture medium. Concentrations of 39.1 up to 2500 µg/mL (2 × serial dilution) were then incubated with the HeLa S5 cell line (human uterine carcinoma cells) in a 96-flat-bottomed-well microtiter plate (1×10^4 cells/well/200 µL). Control wells were incubated with medium plus 0.02 % Tween-20, the same medium used to dilute the compounds tested *in vitro*. After 24 and 48 h incubation (37 °C, 5 % CO_2), the culture medium was replaced with 200 µL of fresh medium, with or without the test samples and positive control (chloroquine), followed by the addition of 20 µL of MTT solution (5 mg of thiazolyl blue salt) in RPMI 1640, without phenol red to each well, for another 3 h incubation. After this period, the medium was removed and 200 µL of DMSO were added to each well to solubilize the formazan crystals. The culture plates were read in a spectrophotometer with a filter of 570 ŋm and a background of 630 ŋm. The CC_{50} of the drug was determined by an estimate of the relative CC_{50} in linear interpolation analysis. Compounds with CC_{50} values below 30 µg /mL were considered to have high cytotoxicity.

The acute *in vivo* toxicity assay was conducted according to OECD [43]. Products at doses of 300, 1000, and 2000 mg/kg were administered to Swiss mice (female, 8–10 weeks, 25–30 g) via the oral way. Animals were observed continuously for 1 h each day to detect any symptoms of toxicity (piloerection, weight loss, and postural abnormalities) or death for a period of 4 weeks.

Statistical analysis

Analysis of variance (ANOVA) followed by Bonferroni's post hoc test (when needed) was used to evaluate the statistical significance of the data. The Mantel-Cox log-rank test was performed for survival time analysis using GraphPad Prism version 5.0 for Windows.

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Conflict of Interest

The authors declare they have no conflict of interest.

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