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Article

Enhanced Antimalarial Activity of Extracts of *Artemisia annua* L. Achieved with Aqueous Solutions of Salicylate Salts and Ionic Liquids

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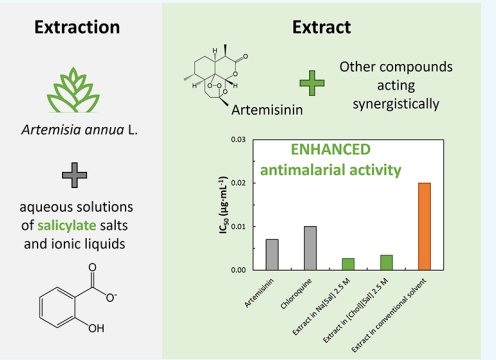
ABSTRACT: Artemisinin, a drug used to treat malaria, can be chemically synthesized or extracted from *Artemisia annua* L. However, the extraction method for artemisinin from biomass needs to be more sustainable while maintaining or enhancing its bioactivity. This work investigates the use of aqueous solutions of salts and ionic liquids with hydrotropic properties as alternative solvents for artemisinin extraction from *Artemisia annua* L. Among the investigated solvents, aqueous solutions of cholinium salicylate and sodium salicylate were found to be the most promising. To optimize the extraction process, a response surface method was further applied, in which the extraction time, hydrotrope concentration, and temperature were optimized. The optimized conditions resulted in extraction yields of up to 6.50 and 6.44 mg·g^{-1}, obtained with aqueous solutions of sodium salicylate and cholinium salicylate, respectively. The extracts obtained were tested for their antimalarial activity, showing a higher efficacy against the *Plasmodium falciparum* strain compared with pure (synthetic) artemisinin or extracts obtained with conventional organic solvents. Characterization of the extracts revealed the presence of artemisinin together with other compounds, such as artemitin, chrysosplenol D, arteannuin B, and arteannuin J. These compounds act synergistically with artemisinin and enhance the antimalarial activity of the obtained extracts. Given the growing concern about artemisinin resistance, the results here obtained pave the way for the development of sustainable and biobased antimalarial drugs.



KEYWORDS: *Artemisia annua L, hydrotropes, ionic liquids/salts, malaria, natural extract, Plasmodium falciparum*

1. INTRODUCTION

According to the World Health Organization's 2021 report, estimated 247 million cases of malaria resulted in 619,000 deaths worldwide.[1] Despite the emergence of drug-resistant strains of *Plasmodium falciparum* (*P. falciparum*), artemisinin remains an effective antimalarial drug.[1] This sesquiterpene lactone is one of the most potent agents against chloroquine-resistant strains of *P. falciparum*, one of the most lethal forms of malaria.[2] Additionally, artemisinin has potential against hepatitis B, schistosomiasis, Sars-CoV-2 virus, and some cancer cell lines, such as breast cancer, human leukemia, colon, and lung carcinomas.[3−6] Consequently, the worldwide demand for artemisinin is increasing. However, the chemical synthesis of this drug is not yet economically viable because of its complexity and low yield.[7] On the other hand, artemisinin is present in the aerial parts, such as leaves, stems, buds, and flowers, of the plant *Artemisia annua* L., a herbaceous plant native from Asia, leading to an increasing interest in the extraction of this natural bioactive compound.[3,7] Conventional artemisinin extraction from *Artemisia annua* L. relies on the use of nonpolar organic solvents, such as chloroform, hexane, toluene, dichloromethane, and petroleum ether.[5,7,8] Recently, nontraditional extraction methods have emerged as alternatives, such as ultrasound-[8−10] and microwave-assisted[11] and low-pressure solid−liquid extractions using ethanol or poly(ethylene glycols)[12] and supercritical carbon dioxide.[7] These methods lead to higher extraction efficiencies compared with traditional Soxhlet extraction procedures involving volatile organic solvents. In addition, some of the volatile organic solvents, such as hexane, are not selective and may lead to the degradation of artemisinin.[7] Thus, it is essential to develop sustainable extraction methods for artemisinin from biomass while being able to keep its bioactivity.

Water would be an ideal solvent for the extraction of bioactive compounds, such as artemisinin. However, artemisi-

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Table 1. List of Compounds Used in This Work

Compound	Abbreviation	CAS Reg. No.	Supplier	Purity (wt %)
Acetonitrile	---	75-05-8	Fisher Scientific	99.0
AlbuMAXII	---	---	Gibco	100.0
Artemisinin	---	63968-64-9	Kang Biothec	99.0
Chloroquine	---	50-63-5	Sigma	98.5−101.0
Cholinium bicarbonate	---	78-73-9	Sigma	80% in water
Dichloromethane	---	75-09-2	Fisher Scientific	99.9
Dimethyl sulfoxide	---	67-68-5	Sigma	≥99.7
Ethyl acetate	---	141-78-6	VWR Chemicals	99.0
Formic acid	---	64-18-6	Honeywell Fluka	98.0
Giemsa's Azur eosin methylene solution	---	---	Merck	MQ400
Petroleum ether	---	8032-32-4	Panreac	PA-ACS-ISO
RPMI with L-glutamine	---	---	Biowest	ISO 13485
Salicylic acid	---	69-72-7	Acofarma	99.0
Sodium salicylate	Na[Sal]	54-21-7	Sigma	99.5
SYBR green I solution in dimethyl sulfoxide	---	---	Invitrogen	---
Triton-X 100	---	9036-19-5	Sigma	MQ200
ILs				
1-Butyl-3-methylimidazolium dicyanamide	$[C_4C_1im][N(CN)_2]$	448245-52-1	Iolitec	99.0
1-Butyl-3-methylimidazolium thiocyanate	$[C_4C_1im][SCN]$	344790-87-0	Iolitec	99.0
Tetrabutylphosphonium chloride	$[P_{4444}]Cl$	2304-30-5	Sigma	99.0
Cholinium salicylate	[Chol][Sal]	Synthesized by us according to the protocol described in the literature.[19] See more details in the Supporting Information.		

nin, like many other bioactive compounds, presents a hydrophobic character and low solubility in water (61 mg·L^{-1} at 30 °C, log(K_{ow}) = 2.94),[13] restricting the use of water in its extraction. Additives such as surfactants or hydrotropes, can be used to increase the solubility of bioactive compounds in aqueous solutions. Ionic liquids (ILs) have been identified as a promising class of hydrotropes,[13,14] in addition to traditional hydrotropes such as sodium salts of short alkylbenzenesulfonates, short-chain alcohols, and small organic molecules like urea. Aqueous solutions of ILs with hydrotropic character have been shown to be effective solvents for extracting a wide range of hydrophobic bioactive compounds from various types of biomass, including, among others, phytochemicals,[15] and phenolic compounds.[14,16] Studies by Bioniqs Ltd. (UK)[17] and Lapkin et al.[12] demonstrated the potential of pure ILs (*N*,*N*-dimethylethanolammonium octanoate and bis(2-methoxyethyl)ammonium bis(trifluoromethylsulfonyl)imide, respectively) in the extraction of artemisinin. These works have focused predominantly on the extraction of artemisinin alone, but with increasing malaria resistance to older drugs, there is a pressing need for extracts containing both artemisinin and synergistic compounds to boost overall efficacy against malaria. In addition to pure ILs, works on using aqueous solutions of ILs have shown that their use not only increases the solubility of target compounds in aqueous media and enables high extraction efficiency of bioactive compounds from biomass but also plays a crucial role in the stabilizing of the extracted biocompounds.[3,7] Therefore, the use of ILs in aqueous media, particularly those behaving as hydrotropes, offers several advantages and represents a promising approach for the extraction of valuable compounds from natural sources.

Considering the bioactivity of artemisinin and the advantages of using hydrotropes, we propose here the use of aqueous solutions of ILs and salts with hydrotropic characteristics as alternative solvents to obtain an extract enriched not only in artemisinin but also in synergistic compounds from the dried leaves of *Artemisia annua* L. as a promising approach to improve antimalarial drug development. An initial screening of aqueous solutions of four ILs (two imidazolium-, one phosphonium-, and one cholinium-based IL) and one sodium salt with hydrotropic character was performed. For optimization, a response surface methodology (RSM) was carried out to identify the optimal conditions for temperature, concentration, and extraction time using the most promising aqueous solutions. Then, the antimalarial activity of the extracts against the *P. falciparum* 3D7 strain was evaluated to compare the advantages of using salicylate-based hydrotropes to those of the conventional solvent. Finally, the main compounds of the obtained extracts were identified by ultrahigh-performance liquid chromatography with diode array detection in conjunction with tandem mass spectrometry (UHPLC-UV-MS^n).

2. MATERIALS AND METHODS

2.1. Materials. Dry leaves of *Artemisia annua* L. were supplied by MEDIPLANT - Centre De Recherche Sur Les Plantes Medicinales and immediately frozen at −80 °C for storage. Before extraction, *Artemisia annua* L. leaves were frozen with liquid nitrogen and ground by employing a coffee grinder until a green powder was obtained. The maximum yield of artemisinin (mg·g^{-1}) in the dry leaves determined in this work was 7 ± 1 mg·g^{-1}. Our results fall within the range of total artemisinin reported for different varieties of *Artemisia annua* L., which is between 0.10 and 14.0 mg·g^{-1} based on dry leaves weight.[12,18] The chemicals used in this work are described in Table 1. The chemical structures of the ILs and salt investigated in the artemisinin extraction are depicted in Figure 1. Ultrapure water was used, which was doubly distilled, passed through a reverse osmosis system, and treated in a Milli-Q plus 185 water purification system.

2.2. Extraction of Artemisinin. The solid−liquid extraction (SLE) of artemisinin from *Artemisia annua* L. was performed using $[P_{4444}]Cl$, $[C_4C_1im][SCN]$, $[C_4C_1im][N(CN)_2]$, [Chol][Sal], and Na[Sal] aqueous solutions, i.e., hydrotropes aqueous solutions with a concentration of 2 and 3 M. The choice of these salts and concentration was based on our previous works,[13,19] in which these compounds at these concentrations showed to be the most promising hydrotropes to increase the artemisinin solubility. The extractions were carried out in commercial Carousel Radleys Tech equipment,

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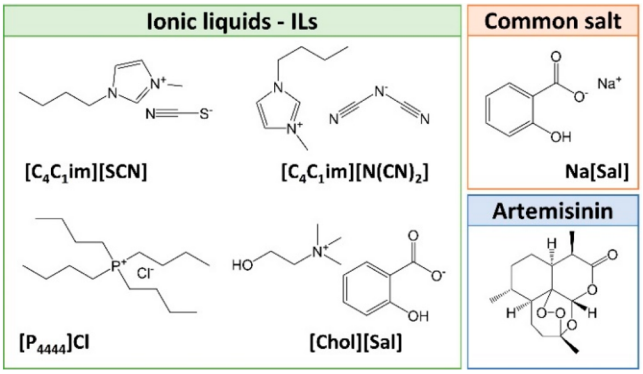

Figure 1. Chemical structures of artemisinin, ILs, and the sodium salt investigated.

which can maintain both the stir and the temperature constant. In all experiments, the stirring was kept at 250 rpm, the temperature was kept at 30 ± 1 °C, for 90 min, and a solid−liquid ratio (biomass−solvent) of 1:10 was used. Mixtures of *Artemisia annua* L. ground leaves with extraction solvents were prepared by weight taking into account the solid−liquid ratio. All aqueous solutions were prepared gravimetrically (using a Mettler Toledo XS205 Dual Range balance). At least three independent extractions with each solvent were prepared and quantified (two samples for each), from which statistical parameters such as the mean and standard deviations for the extraction yield were calculated. Several concentrations of hydrotrope, extraction temperature, and extraction time were optimized, as described below.

After extraction, the aqueous solutions were separated from biomass by centrifugation using a Thermo Scientific Heraeus Megafuge 16 R centrifuge (at 5200 rpm for 30 min) and diluted for HPLC-DAD analysis.

Conventional Soxhlet extraction of artemisinin from dry leaves was also performed with an organic solvent for comparison purposes. More specifically, ground leaves of *Artemisia annua* L. (3 g) were packed into a cellulose thimble and placed in the Soxhlet extractor. Extractions were carried out with petroleum ether and dichloromethane (350 mL) for 5 h. The extracts were then diluted in acetonitrile for the HPLC-DAD analysis.

2.3. Quantification of Artemisinin. The quantification of artemisinin in each solution was carried out by HPLC-DAD (Shimadzu, model PROMINENCE). HPLC analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 μm C18 100 A, from Phenomenex.

The mobile phase consisted of 40% acetonitrile and 60% ultrapure water. The separation was conducted in isocratic mode at a flow rate of 1.0 mL·min^{-1} and using an injection volume of 10 μL. DAD was set at 210 nm. The column oven was operated at a controlled temperature of 30 °C. Under these conditions, artemisinin displays a retention time of 6 min. Calibration curves were prepared by using pure (commercial) artemisinin.

The yield of artemisinin (mg·g^{-1}) in the dry leaves was calculated according to the milligrams of pure artemisinin present in the extract divided by the gram of dry biomass used.

2.4. Optimization of the Artemisinin Extraction Using RSM. Aiming to maximize the artemisinin yield from *Artemisia annua* L. dry leaves, an experimental design was applied to identify the most significant factors (operational conditions). Temperature (T), time of extraction (t), and concentration of hydrotrope (C) were submitted to 2^3 factorial planning to optimize the artemisinin yield, using a solid−liquid ratio of 1:10 and Na[Sal] and [Chol][Sal] aqueous solutions. A central composite design, with six replications of the central point, was used. The conditions were defined for zero level (central point) and one level (+1 and −1, the factorial points). Twenty experiments were carried out, with the conditions applied being provided in the Supporting Information (Table S2). The obtained results were statistically analyzed with a confidence level of 95%, subjected to analysis of variance (ANOVA) and regression analysis using Statistica 10.0. Response surface plots of the artemisinin yield were generated using Matlab R2019b, The MathWorks. Further details on the RSM are provided in the Supporting Information.

2.5. In Vitro Activity Characterization. *2.5.1. Sample Preparation.* Each extract was first dissolved in RPMI-1640 with L-glutamine supplemented with AlbuMAXII, hereinafter termed RPMIc, to obtain intermediate solutions containing 100 μg·mL^{-1} of artemisinin. Afterward, each solution was sterilized by filtration with a 0.20 μm filter, diluted in RPMIc to prepare a 1 μg·mL^{-1} stock solution, and maintained by the same method. The IL/salt solutions used for the extraction were prepared and diluted to 1 μg·mL^{-1} stock solution and sterilized by the same method as the extracts.

2.5.2. P. falciparum in Vitro Culture. Laboratory-adapted *P. falciparum* line 3D7, a chloroquine and mefloquine susceptible strain, was continuously cultured by using a modified method of Trager and Jensen. Parasites were cultivated in 5% hematocrit, 37 °C, and an atmosphere with 5% of CO_2. AlbuMAXII at 0.5% was used as a substitute for human serum in the culture medium.

2.5.3. Antimalarial Activity Determined Using the Whole-Cell SYBR Green I Assay. Staging and parasitemia were determined by light microscopy of Giemsa-stained thin blood smears. The antimalarial activity was determined using the SYBR Green I assay, as previously described[20] with modifications. Briefly, asynchronous parasites were cultivated for 72 h in the presence of a 1:3 serial dilution of each compound, ranging from 0.1 to 0.001 μg·mL^{-1}. Fluorescence intensity was measured with a multimode microplate reader (Triad, Dynex Technologies), with excitation and emission wavelengths of 485 and 535 nm, respectively, and analyzed by nonlinear regression using GraphPad Prism to determine the half of the maximum inhibition effect (IC_{50}) values.

2.6. In Vitro Haemolysis Assay. Haemolysis (% lysis) of the artemisinin-rich extracts, the correspondent IL/salt, artemisinin, and chloroquine, was assessed by incubating normal erythrocytes with the compounds in RPMIc, as previously described with adjustments.[21] Briefly, a 3% HTC suspension of previously purified erythrocytes at 50% HTC was prepared and cultivated for 72 h, at 37 °C and 5% CO_2, in the presence of a 1:5 serial dilution of each compound, ranging from 10 to 0.001 μg·mL^{-1}. The suspension with RPMIc (0% hemolysis) and with a 20% Triton-X 100 in RPMIc (100% hemolysis) were applied as negative and positive controls, respectively.

After the incubation period, the samples were centrifuged and the supernatant was used to evaluate the hemolytic activity by the quantification of hemoglobin release, via spectrophotometric measurement of the absorbance at 450 nm with a multimode microplate reader (Triad, Dynex Technologies). The percentage of hemolysis was calculated using the following equation:

$$\% = \frac{(\text{Sample absorbance} - \text{Negative Control absorbance})}{(\text{Positive Control absorbance} - \text{Negative Control absorbance})} \times 100 \quad (1)$$

In general, any compound below 10% of haemolysis is nonhaemolytic, while values above 25% of haemolysis are classified as haemolytic.[22]

2.7. Characterization of the Artemisinin-Rich Extracts. Artemisia extracts were first filtered using PTFE filters with 0.2 μm pore diameter for ultrahigh-performance liquid chromatography with diode array detection coupled to tandem mass spectrometry (UHPLC-UV-MS^n) analysis. Extracts (10 μL) were injected into the UHPLC system consisting of an Accela 600 LC pump, an Accela autosampler (set at 16 °C), and an Accela 80 Hz photo DAD. Components were separated in a Hypersil Gold RP C18 column (100 × 2.1 mm; 1.9 μm particle size), preceded by a C18 pre-column (2.1 mm i.d.), both supplied by Thermo Fisher, and kept at 40 °C. A binary mobile phase composed of (A) water:acetonitrile (99:1, v/v) and (B) acetonitrile, both containing 0.1% (v/v) formic acid was used. A gradient elution program was applied at a flow rate of 0.30 mL·min^{-1} for 16 min, as follows: 1−22% B from 0 to 6 min; kept at 22% B from 6 to 8 min; 22−42% B from 8 to 10 min; 42−100% B from 10 to 14 min and kept at 100% B from 14 to 16 min. Before the

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next run, the B percentage was reduced from 100 to 1% for 4 min and then kept at 1% for 4 min. The chromatograms were recorded at 225 nm, and the molecular absorption spectra were recorded between 210 and 600 nm.

The UHPLC system was coupled to an LCQ Fleet ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. The ESI-MS was operated under the positive ionization mode with a spray voltage of 5 kV and capillary temperature of 320 °C. The flow rates of nitrogen sheath and auxiliary gas were 40 and 5 (arbitrary units), respectively. The capillary and tube lens voltages were set at 44 and 225 V, respectively. Collision-induced dissociation-MS^n experiments were executed on mass-selected precursor ions in the range of m/z 100−2000. The isolation width of the precursor ions was 1.0 mass units. The scan time was 100 ms, and the collision energy was 35 arbitrary units, using helium as collision gas. The data acquisition was carried out using the Xcalibur data system.

The linearity of the method was established by creating a calibration curve of artemisinin standard over a concentration range from 101.6 to 1016.0 $\mu g \cdot mL^{-1}$. The regression equation found was: $y = 392.45x + 44740$ ($R^2 = 0.995$), where y is the peak area and x is the concentration in $\mu g\ mL^{-1}$. LOD (90.6 $\mu g \cdot mL^{-1}$) and LOQ (274.6 $\mu g \cdot mL^{-1}$) were determined as three and ten times the noise level (S/N), respectively.

3. RESULTS AND DISCUSSION

3.1. Extraction of Artemisinin. In the assays of identifying the hydrotrope aqueous solution to extract artemisinin from *Artemisia annua* L. dry leaves, aqueous solutions of $[C_4C_1im][SCN]$, $[C_4C_1im][N(CN)_2]$, $[P_{4444}]Cl$, [Chol][Sal], and Na[Sal] at 2 and 3 M were evaluated. The results obtained are depicted in Figure 2 (Table S3 in the Supporting Information). These hydrotropes were selected based on the results of previous solubility works of artemisinin.[13,19]

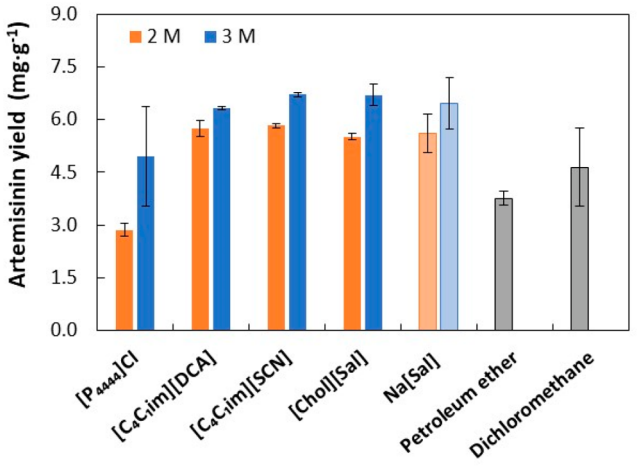

Figure 2. Artemisinin yield using aqueous solutions of hydrotropes at 2 M (orange bars) or 3 M (blue bars) by SLE or using petroleum ether and dichloromethane by Soxhlet method (grey bars). Fixed extraction conditions at SLE: S/L ratio of 1:10, 90 min, and 30 °C.

Aqueous solutions of hydrotropes are highly effective solvents for extracting artemisinin from biomass, as shown in Figure 2. Notably, the hydrotrope-based extraction solvents yielded significantly higher amounts of artemisinin (up to 6.71 ± 0.06 $mg \cdot g^{-1}$) compared to the conventional Soxhlet extraction method using petroleum ether (3.80 ± 0.21 $mg \cdot g^{-1}$) and dichloromethane (4.65 ± 1.12 $mg \cdot g^{-1}$). These unoptimized results are even better than those previously reported by Chemat et al.,[23] who used a mixture of hexane with ethyl acetate assisted by ultrasound at 40 °C, achieving an extraction efficiency of 5.98 $mg \cdot g^{-1}$. Thus, hydrotrope aqueous solutions are promising solvents for enhancing the solubility of hydrophobic compounds like artemisinin ($\log(K_{ow}) \approx 2.90$), thereby increasing the extraction yield. The remarkable results obtained for the extraction of artemisinin suggest that the high performance demonstrated by aqueous solutions of hydrotropes is related to the improvement of the solubility caused by these compounds,[13,19] and not only by the rupture of the biomass, as usually discussed in the literature.[24] It is worth noting that the amount of artemisinin in the leaves of *Artemisia annua* L. can vary based on several factors such as plant variety, climate, soil, and planting methods.[18,25]

All hydrotrope aqueous solutions showed excellent potential for the extraction of artemisinin, which is consistent with previous hydrotropic solubilization studies on artemisinin.[13,19] Hydrotrope experiments showed that all five ILs/salts exhibit a remarkable ability to solubilize artemisinin, with the salicylates exhibiting the highest solubility and $[P_{4444}]Cl$ exhibiting the lowest efficiency in the extraction of artemisinin, which are therefore excluded from further studies. The extraction yield of artemisinin is affected by the chemical structures of the ILs/salts used. ILs/salts that favor hydrogen bonding with artemisinin, such as those containing aromatic benzoyl and imidazolium rings, lead to higher extraction efficiencies. In contrast, aqueous solutions of $[P_{4444}]Cl$, involving an IL with no aromatic rings, exhibit a lower efficiency in extracting artemisinin. This trend also reveals that a strong hydrogen bond acceptor as chloride is not imperative to improve the extraction efficiency; on the other hand, IL ions with high ability to donate protons are the most prominent to extract artemisinin from biomass. This trend is in agreement with previous studies showing that deep eutectic solvents (DES) composed of benzoic acid and fenchyl alcohol perform better in artemisinin extraction.[26]

When a hydrotrope is selected, its potential toxicological effects and environmental impact must be considered. Studies with marine bacteria have shown that imidazolium- and phosphonium-based ILs are "moderately" or "slightly toxic".[27] On the other hand, cholinium-based ILs are considered stable in water, are biodegradable, and have lower toxicity compared to other ILs.[27−29] Therefore, in order to optimize the operating conditions for artemisinin extraction from biomass and to investigate the effects of the cation on extraction performance, further assays were performed only with aqueous solutions of [Chol][Sal] and Na[Sal].

3.2. Optimization of the Extraction Conditions Using RSM. With the goal of identifying the most significant process parameters and optimizing the operating conditions to maximize the extraction yield of artemisinin ($mg \cdot g^{-1}$), the RSM was used. In this work, two 2^3 (3 factors and 2 levels) factorial plannings were executed, where the concentration of aqueous solutions of hydrotropes, the extraction time, and the extraction temperature were varied. The influence of the three variables on the extraction yields of artemisinin is illustrated in Figure 3. The yields of artemisinin obtained experimentally, as well as all the statistical analyses, are provided in the Supporting Information (Tables S4−S9 and Figures S2−S7). Variance analysis (ANOVA) was used to estimate the statistical significance of variables and the interactions between them. The obtained results were statistically analyzed with a confidence level of 90%. The $R^2_{adjusted}$ value of the polynomial

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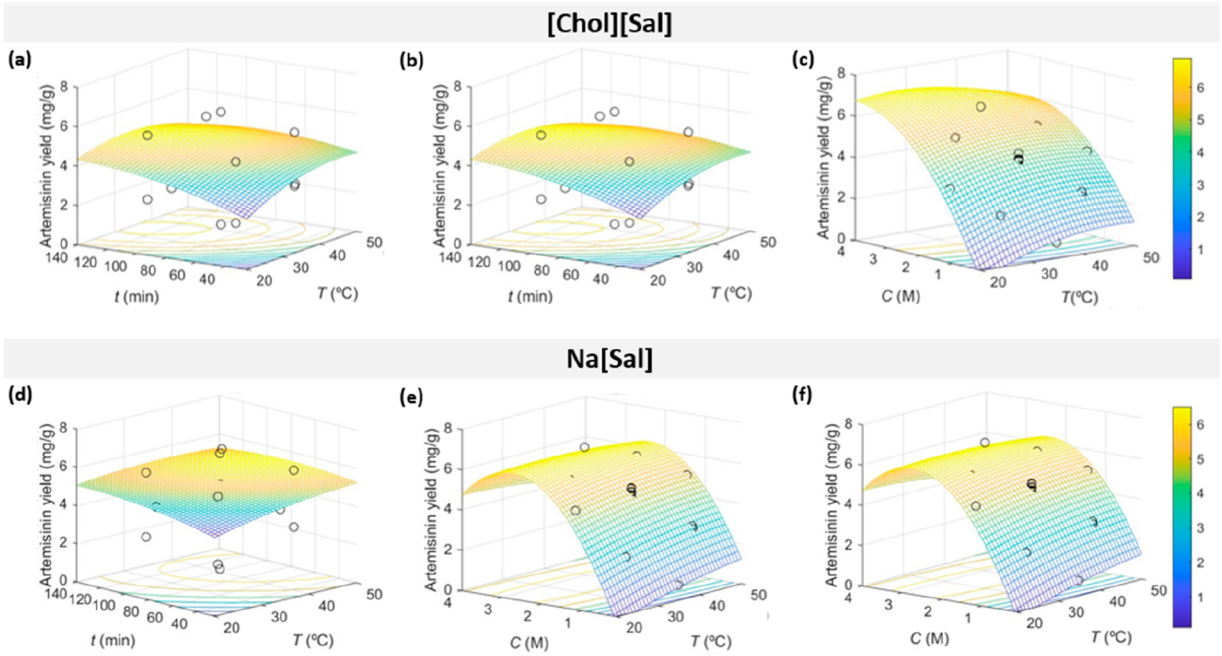

Figure 3. Response surface plots on artemisinin extraction yield ($mg \cdot g^{-1}$) using aqueous solutions of (above) [Chol][Sal] or of (below) Na[Sal] with the combined effects of (a and d) time (t) in minutes and temperature (T) in °C; (b and e) concentration (C) in M and T (°C); and (c and f) C (M) and t (min).

equation for the yield of artemisinin ($mg \cdot g^{-1}$) was higher than 0.97 for both hydrotropes, showing that no significant differences were observed between the experimental and calculated responses. This finding supports the good description of the experimental results by the statistical models developed (Figures S2−S7 in the Supporting Information).

To obtain an extract rich in artemisinin, the following operating conditions should be applied: (i) solid−liquid ratio of 1:10, at 32 °C, during 130 min, and a [Chol][Sal] concentration of 2.5 M; (ii) solid−liquid ratio of 1:10, at 32 °C, during 110 min and a Na[Sal] concentration of 2.5 M. At these points, the predicted yield of artemisinin was 6.45 and 6.49 $mg \cdot g^{-1}$, and experimentally it was found to be 6.50 ± 0.10 and 6.44 ± 0.09 $mg \cdot g^{-1}$ for aqueous solutions of Na[Sal] and [Chol][Sal], respectively. The experimental and predicted results are very similar for both aqueous solutions, demonstrating the good predictive ability of the identified models.

3.3. Antimalarial Activity Determined Using the Whole-Cell SYBR Green I Assay. We evaluated the efficacy of extracts *of Artemisia Annua* L. obtained under optimized conditions using aqueous solutions of hydrotropes and the best conventional solvent (dichloromethane) in inhibiting *P. falciparum* in vitro. The extract obtained with the conventional solvent (dichloromethane) was previously dried and resuspended in water to allow its application in the in vitro studies. The presence of dichloromethane could compromise the in vitro results. In addition to these extracts, we also included artemisinin and chloroquine (synthetic antimalarials widely used in the past to treat malaria) for comparison. All the extracts/solutions were diluted/prepared in order to have the same concentration of artemisinin, i.e., 1 $\mu g \cdot mL^{-1}$. Figure 4 (detailed data in Table S10 in the Supporting Information) shows the IC_{50} values for each solution/extract, while the dose−response curves are shown in Figure S8 in the Supporting Information. The IC_{50} values presented result from at least three independent experiments, each with five

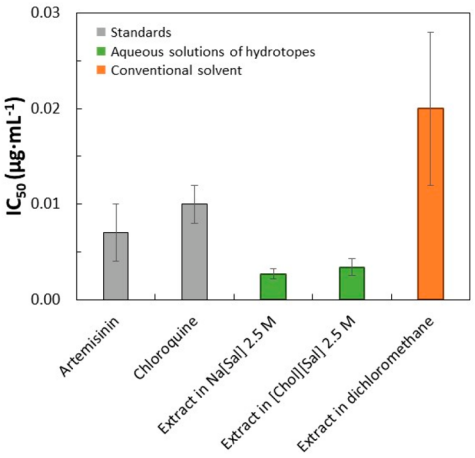

Figure 4. Average of IC_{50} of the extracts, as well as, artemisinin and chloroquine, after 72 h of exposure to *P. falciparum*.

replicates/dose. Parasite survival (%) was determined relatively to an untreated control (no drug).

Overall, the extracts obtained with aqueous solutions of hydrotropes are remarkably more effective against the malaria pathogen than pure artemisinin and chloroquine. The extract obtained from an aqueous solution of Na[Sal] at 2.5 M exhibits the highest inhibitory activity (IC_{50} = 0.0027 ± 0.0005 $\mu g \cdot mL^{-1}$). Extracts obtained with aqueous solutions of [Chol][Sal] also show promising results (IC_{50} = 0.0034 ± 0.0009 $\mu g \cdot mL^{-1}$). The biological activity is exclusively derived from the compounds extracted from biomass, since aqueous solutions of hydrotropes do not exhibit any antimalarial bioactivity. The remarkable antimalarial activity of the obtained extracts is due to a synergistic effect of other compounds that are being extracted together with artemisinin; see discussion below on the extract's characterization.

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3.4. In Vitro Haemolysis Assays. The hemotoxicity of the extracts of *Artemisia annua* L. using aqueous solutions of hydrotropes and the conventional solvent was also assessed. Artemisinin and chloroquine were used for comparison as antimalarial drugs, a Triton-X solution was used as the positive control for haemolysis, and untreated erythrocytes were used to simulate culture conditions without the presence of drugs. The hemolysis percentage was determined from at least two independent experiments, each in triplicate (Table S11 in the Supporting Information). The formula for the haemolysis percentage determination was designed to withdraw the Triton-X solution shift in absorbance by binding to the AlbuMAXII proteins and due to the pH indicator dye phenol red in RPMIc that displays absorbance at 450 nm.[30,31]

The extracts obtained with aqueous solutions of hydrotropes were assessed for haemolysis since artemisinin has a mechanism of action that involves the increase of production of reactive oxygen species (ROS) by the cleavage of the endoperoxide bridge within the parasite, and even can enhance the ROS production in uninfected erythrocytes.[32,33] Additionally, both hydrotropes (sodium salicylate and cholinium salicylate) have the same precursor—salicylic acid—which is associated with haemolytic anaemia in patients with severe glucose-6-phosphate dehydrogenase deficiency, heightened by fever, acute infections, and/or high dosages of salicylic acid.[29]

The results obtained demonstrate that sodium salicylate and cholinium salicylate extracts do not exhibit hemotoxicity at the closest dose of the IC_{50} values (Table S11 in the Supporting Information). This observation advocates in favor of the safety of the extracts obtained with aqueous solutions of hydrotropes, as these extracts are more active than pure artemisinin and the extract in the conventional solvent and are not hemotoxic at biologically active doses against *P. falciparum*.

3.5. Characterization of the Artemisinin-Rich Extracts. To better understand the reasons behind the remarkable biological activity of the obtained extracts, UHPLC-DAD-MS^n analyses were carried out, namely, on the extracts obtained with the aqueous solutions of [Chol][Sal] at 2.5 M and Na[Sal] at 2.5 M, and on the extract obtained with the conventional solvent. We were able to identify the primary compounds present in each extract, such as sesquiterpenes and flavonoids, including arteannuin B, arteannuin J, artemisinin, artemetin, casticin, and chrysosplenol D (see Supporting Information for more information; Table S12). The compounds identified in the extracts are consistent with previous studies on the extraction of artemisinin from *Artemisia annua* L.[34−37] In addition to artemisinin, the other compounds found in the extracts obtained with the salicylate salt and the IL have been reported to be effective against malaria, either alone (some of them) or in synergistic action with artemisinin to enhance antimalarial activity.[38−42] For example, arteannuin B, which is typically present in wild strains of the plant at two to four times the concentration of artemisinin, has been reported to enhance the activity of artemisinin against *P. falciparum*.[38] Our results show that arteannuin B is present at a higher concentration than just two to four times the concentration of artemisinin, but a direct comparison is not possible because we quantified arteannuin B and casticin together (see Table S13 and Figure S9 in Supporting Information). Although arteannuin J has similar activity to artemisinin, it is usually present in the plant at lower concentrations.[41] However, our study shows that this compound is in the same range of concentration or even in a slightly higher concentration than artemisinin (+ artemitin). Among the flavones, chrysosplenol-D is usually the most abundant flavonoid in the plant, which is in line with our results, and it also enhances the activity of artemisinin when used in combination.[41] The other flavonoids identified in the extracts, artemitin and casticin, have been described as having anti-inflammatory, antioxidant, and anticancer properties, while selectively enhancing the in vitro activity of artemisinin against *P. falciparum*.[39] Flavonoids may enhance the activity of artemisinin against *P. falciparum* by catalyzing the reaction of artemisinin with hemin and promoting the solubilization of artemisinin, resulting in a synergistic effect.[40,41]

The total yield of the identified compounds follows the following order: Na[Sal] 2.5 M (131.24 $mg{\cdot}g^{-1}$) > [Chol][Sal] 2.5 M (116.02 $mg{\cdot}g^{-1}$) > conventional solvent (2.40 $mg{\cdot}g^{-1}$). The yield of artemisinin follows a pattern similar to the total yield of the identified compounds. Furthermore, the IC_{50} values follow a similar trend to the total yield of the identified compounds. This suggests that these compounds may act synergistically with artemisinin. For example, the extract obtained from the aqueous solution of Na[Sal] at 2.5 M, which had the highest yield of identified compounds, showed a stronger inhibitory effect than artemisinin alone. Since both solutions were applied to the pathogen with the same concentration of artemisinin, the observed inhibition is not due to the presence of artemisinin alone. Therefore, aqueous solutions of hydrotropes extracted from *Artemisia Annua* L. contain additional compounds with antimalarial activity, being consistent with previous studies that also reported synergistic effects of the identified compounds with the target drug.[38−42]

The World Malaria Report 2022[1] indicates the emergence of artemisinin resistance in some African regions; therefore, it is highly important to explore new antimalarials. In this sense, this work shows the potential of using aqueous solutions of hydrotropes, especially salicylate salts and ILs, as alternative solvents for the extraction of artemisinin, thus paving the way for the development of new, sustainable, and biobased antimalarial drugs.

4. CONCLUSIONS

We investigated the efficacy of aqueous solutions of hydrotropes for the extraction of artemisinin from *Artemisia annua* L. Aqueous solutions of cholinium salicylate ([Chol][Sal]) and its sodium salt (Na[Sal]) were identified as the best options for artemisinin extraction. To further improve the efficiency of artemisinin extraction, the extraction conditions, including extraction time, hydrotrope concentration, and temperature, were optimized by using response surface methodology. The optimized conditions for artemisinin extraction were a solid−liquid ratio of 1:10 at 32 °C for 110 min with a Na[Sal] concentration of 2.5 M, and a solid−liquid ratio of 1:10 at 32 °C for 130 min with a [Chol][Sal] concentration of 2.5 M. Under these conditions, yields of artemisinin of 6.50 ± 0.10 and 6.44 ± 0.09 $mg{\cdot}g^{-1}$ were obtained with the aqueous solutions of Na[Sal] and [Chol][Sal], respectively. Remarkably, the extracts obtained with the salicylate salt/IL showed excellent IC_{50} values against *Plasmodium falciparum* (IC_{50} up to 0.0027 ± 0.0005 $\mu g{\cdot}mL^{-1}$) compared with pure artemisinin (IC_{50} = 0.0066 ± 0.0025 $\mu g{\cdot}mL^{-1}$), thus indicating the potential of these extracts for the treatment of malaria. The extracts obtained with aqueous solutions of hydrotropes were found to be rich not only in artemisinin but also in other antimalarial compounds such as artemitin, chrysosplenol D,

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arteannuin B, and arteannuin J, culminating in synergistic effects. Furthermore, these extracts do not exhibit haemotoxicity at the closest dose of the IC_{50} values, reinforcing their safety.

Overall, the results presented here demonstrate the potential of aqueous solutions of hydrotropes as alternative and effective solvents for the extraction of artemisinin from biomass, representing a step forward in the discovery of potential antimalarial drugs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/cbe.3c00005.

Synthesis and optimization. ^{1}H NMR and ^{13}C NMR spectra (Figure S1). Extraction of artemisinin data (Table S3). Experimental details about the optimization of the extraction conditions by RSM (Tables S1 and S2) using aqueous solutions of [Chol][Sal] (Figures S2 and S3 and Tables S4−S6) and using aqueous solutions of Na[Sal] (Figures S4−S7 and Tables S7−S9). Antimalarial activity data determined using the Whole-Cell SYBR Green I assay (Figure S8, Table S10). In vitro haemolysis assays data (Table S11). Data of the characterization of the artemisinin-rich extracts (Figure S9, Tables S12 and S13). Chemical characterization of the optimized extracts details (PDF)

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Notes

The authors declare no competing financial interest.

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