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Enhanced Recovery of Artemisinin from *Artemisia annua* L. by Pressurized Extraction Using Biobased Solvents

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Abstract

Artemisinin is a key antimalarial compound extracted from *Artemisia annua* L. and widely used to treat drug–resistant malaria. In this work, an integrated strategy combining predictive solvent selection with pressurized extraction using water/biobased solvent mixtures was developed for the recovery of artemisinin and its biosynthetic precursor dihydroartemisinic acid (DHAA) *via* accelerated solvent extraction. COSMO-RS was used to pre-screen promising solvent candidates, which were then evaluated experimentally. Among the evaluated biobased solvents, γ–valerolactone (GVL)–water mixtures showed the highest extraction performance. Process conditions were optimized using response surface methodology, yielding 8.13 ± 0.34 mg/g of artemisinin and 3.19 ± 0.03 mg/g of DHAA at 85 °C, 13 min, and 80 wt.% GVL. Stability, antiplasmodial activity, and safety of the resulting extracts were evaluated without solvent removal. The extracts retained approximately 78% of their initial artemisinin content after 30 days at 30 °C and exhibited *in vitro* antiplasmodial activity against *Plasmodium falciparum* (IC_{50} = 2.22 ± 0.96 ng/mL, expressed as artemisinin equivalents), comparable to pure artemisinin and dihydroartemisinin. Control experiments demonstrated negligible solvent contribution at biologically relevant dilutions, while hemolysis and cytotoxicity assays indicated a high selectivity index and wide safety margin. Overall, combining predictive solvent screening with pressurized extraction allows for rapid solvent selection and efficient recovery. The use of biobased solvents such as GVL offers the possibility of avoiding the solvent removal step in the downstream processing of the extract, although further toxicological and regulatory evaluation is necessary prior to any pharmaceutical application.

Keywords: Gamma–valerolactone; Solvent screening; COSMO-RS; High–pressure extraction; Natural product extraction; Antimalarials.

1. Introduction

Artemisinin, a sesquiterpene lactone peroxide, is one of the most effective antimalarial compounds, particularly against *Plasmodium falciparum* strains resistant to chloroquine and piperaquine [1]. Artemisinin–based combination therapies (ACTs), which combine artemisinin or one of its derivatives with a partner drug from a different chemical class, are recommended by the World Health Organization as first–line treatments for uncomplicated *P. falciparum* malaria [2]. Despite major advances in malaria control, the disease remains a global health challenge, with 263 million cases and approximately 597,000 deaths reported in 2023, mostly in endemic regions [2]. Artemisinin can be obtained via total synthesis, semi–synthesis, or direct extraction from natural sources. Among these routes, extraction from *Artemisia annua* L. remains the most economically viable option for large–scale production [3], owing to the relatively high artemisinin content of the plant. In addition to artemisinin, *A. annua* contains structurally related metabolites such as artemisinic acid and arteannuin B, which may contribute to the biological activity if co–extracted [4]. Nonetheless, artemisinin remains one of the primary targets for pharmaceutical applications [5].

Conventional extraction of artemisinin relies on techniques such as Soxhlet extraction, maceration, or percolation using volatile organic solvents, including hexane, dichloromethane, or petroleum ether [6]. While effective, these approaches involve high solvent consumption, high energy demand, and extensive downstream purification, resulting in increased environmental impact and production costs [7]. Moreover, the relatively low artemisinin content in *A. annua* leaves (0.1–14 mg/g dry weight) limits process efficiency and limits access to treatment in low–income regions [8].

To address these limitations, alternative extraction techniques have been explored to improve mass transfer and reduce solvent usage. Microwave–assisted extraction (MAE), ultrasound–assisted extraction (UAE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE, also referred to as accelerated solvent extraction, ASE) have demonstrated improved performance compared to conventional methods [6,9]. MAE and UAE typically use organic solvents or ethanol–water mixtures [10,11], whereas SFE relies on carbon dioxide, often modified with ethanol to increase solubility [12]. In particular, PLE/ASE maintains solvents in the liquid phase above their boiling points, increasing solvent penetration and solute diffusion [13], although many studies rely on pressurized hot water, which may promote degradation of thermolabile compounds such as artemisinin [14].

Regardless of the extraction technique, solvent selection remains a key determinant of process efficiency, sustainability, and safety. Alternative solvents, including deep eutectic solvents and ionic liquids, have shown promising results in artemisinin extraction [15,16]. For example, a eutectic mixture composed of benzoic acid and fenchyl alcohol significantly increased artemisinin yield relative to petroleum ether [15]. Similarly, aqueous solutions of ionic–liquid–based hydrotropes exhibited superior extraction efficiency compared to conventional organic solvents [16].

Biobased solvents have attracted increasing attention due to their renewable origin, favorable toxicity profiles, and biodegradability. Compounds such as γ–valerolactone (GVL), ethyl lactate, and alkanediols are already used in food and pharmaceutical applications and have demonstrated high efficiency in the extraction of phenolics and other bioactive compounds. GVL, in particular, exhibits low toxicity [17] and is classified under EU REACH with a favorable safety and environmental profile [18]. Although the relatively high viscosity of some biobased solvents may

limit mass transfer [19], the addition of water reduces viscosity and improves solute diffusion. This can enhance extraction performance compared to neat solvents and conventional organic solvents through polarity change and hydrotropic interactions that favor solvent–solute affinity and the solubilization of hydrophobic compounds [20].

To rationalize solvent selection, computational tools such as the Conductor–like Screening Model for Real Solvents (COSMO–RS) have emerged as valuable predictive methods. COSMO–RS predicts activity coefficients and solubility trends across a wide range of solvents that allows efficient pre–screening prior to experimental validation [21], and has been shown to be effective in guiding solvent selection for artemisinin extraction [15]. Nevertheless, the selection of biobased solvents for artemisinin recovery using COSMO-RS remains limited, particularly in combination with intensified extraction techniques. In addition, most studies on artemisinin extraction focus primarily on recovery yields, with little attention paid to the direct use of the resulting extracts without solvent removal or further processing. As a result, approaches integrating predictive solvent screening, pressurized extraction, and downstream–relevant evaluation of extract stability, bioactivity, and safety remain largely unexplored.

In this work, an integrated strategy was developed for the recovery of artemisinin and DHAA from *Artemisia annua* using water/biobased solvent mixtures under pressurized extraction conditions, with the aim of assessing whether the extract can be processed without conventional solvent evaporation and redissolution steps, while preserving its biological activity and safety profile *in vitro*. Solvent selection was guided by COSMO–RS predictions of infinite–dilution activity coefficients, followed by experimental validation and optimization using response surface methodology, with performance benchmarked against dichloromethane. The extracts were evaluated in terms of storage stability, antiplasmodial activity against *Plasmodium falciparum*, and

safety profile, including cytotoxicity and hemolysis, and were compared with reference compounds. This approach aims to simplify downstream processing and reduce the use of conventional organic solvents.

2. Materials and methods

2.1. Materials

Dried leaves of *Artemisia annua* L. were provided by MEDIPLANT – Centre de Recherche sur les Plantes Médicinales (Conthey, Switzerland) and stored at –80 °C with no additional drying step. The residual moisture content is 6–10 wt.%, as reported by the manufacturer. The same batch of dried leaves was used throughout all experiments reported in this work, including solvent screening, optimization, stability assessment, and biological assays. Prior to extraction, leaves were ground using a commercial coffee grinder until a fine powder was obtained. All compounds used in this study are listed in Table S1 (see Supplementary Material). Ultrapure water (resistivity 18.05 MΩ cm at 25 °C) was obtained by double distillation and further purified using a Milli–Q Plus 185 water purification system (Merck, Darmstadt, Germany).

2.2. *In silico* prediction of the activity coefficients of artemisinin using COSMOtherm

Solvent selection for screening and optimization was guided by COSMO–RS predictions, enabling the identification of the most promising candidates for experimental validation. COSMO–RS is a quantum chemistry–based model that estimates activity coefficients from the calculation of excess Gibbs energies using σ–surfaces derived from molecular charge density distributions.

Calculations were performed using the BIOVIA COSMOtherm 21 software package (COSMOlogic GmbH, Germany) with the BP_TZVPD_FINE_21 parametrization. The absence of multiple stable conformers was verified using COSMOconf, and a single conformer was

optimized for each molecule using TURBOMOLE (version 7.4, 2019) with the COSMO–BP–TZVPD–FINE template. Density functional theory (DFT) calculations were carried out using the BP86 functional with the def2–TZVPD basis set, including diffuse functions, and a fine tessellation grid.

Solvent screening was performed by predicting the infinite dilution activity coefficient (γ^{∞}) of artemisinin at 25 °C in a set of 26 pure solvents. To assess the influence of water content, γ^{∞} values were also calculated for water–solvent mixtures containing 25 wt.% water (hydrated solvents) and 75 wt.% water (aqueous solutions).

2.3. Extraction of artemisinin using accelerated solvent extraction

The biobased solvents identified as the most promising by COSMO–RS screening were selected for experimental validation. Pure water and selected conventional organic solvents were included as benchmarks. To assess the effect of water content, each biobased solvent was evaluated in its pure form and in mixtures containing 25 wt.% and 75 wt.% water. Water–solvent mixtures were prepared gravimetrically using a Sartorius PRACTUM224–1S analytical balance (Sartorius AG, Göttingen, Germany; ± 0.1 mg precision).

All extraction experiments for solvent screening and optimization were performed using a Thermo Fisher Dionex ASE 350 system (Thermo Fisher Scientific, MA, USA). Extractions were carried out in 10 mL stainless-steel cells loaded with 1.0 g of ground *Artemisia annua* L. leaves, corresponding to a solid-to-liquid ratio of 1:10. The system was operated in fixed solvent volume mode, with the entire solvent volume (10 mL) delivered to the extraction cell. Extractions were performed with one static cycle, no flush volume, and a nitrogen purge of 60 s at the end of each

extraction. Under these conditions, the final extract volume collected per run was approximately 10 mL.

For solvent screening (Section 3.1), extractions were conducted at 100 °C for 5 min, while the conditions used for the optimization step are described in Section 2.3.1. For each solvent, at least three independent extraction runs were performed, and mean extraction yields and standard deviations were calculated. Prior to the solvent screening experiments, extractions using pure ethanol were also performed under fixed-pressure mode (1500–1700 psi) under otherwise identical extraction conditions to evaluate the influence of extraction mode on extraction performance. Because no significant differences were observed between fixed-pressure and fixed solvent volume extraction modes, the latter was selected for all subsequent experiments.

Following extraction, all liquid extracts were filtered prior to analysis. For each extraction, at least two analytical replicates were quantified according to the procedure described in Section 2.4.

2.3.1. Optimization of artemisinin extraction

Response surface methodology (RSM) based on a central composite rotatable design (CCRD) was applied to optimize artemisinin yield from dried *A. annua* leaves. The experimental design included factorial points (±1), axial points (±α), and three central replicates (level 0), to capture curvature effects. Three variables were chosen for optimization: extraction time, solvent/water composition (wt.%), and temperature, using the solvent identified as most effective during the screening step (Section 3.1). A total of 17 experiments were conducted under pressurized liquid extraction conditions, with the complete experimental matrix reported in Tables S2 and S3 (see Supplementary Material). The ranges of independent variables were defined based on the experimental solvent screening (Section 3.1), the operational limits of the ASE 350 system, and

the thermal stability of artemisinin (decomposition above 190 °C,[22]). The lower temperature limit (40 °C) corresponds to the minimum controllable temperature of equipment, while the upper limit (100 °C) corresponds to the screening conditions and ensures liquid-phase operation without significant thermal degradation of artemisinin. Extraction time was varied from 5 to 25 min to evaluate extraction kinetics beyond the initial screening conditions. The solvent composition interval (25–90 wt.% GVL) was established based on the screening results, which identified hydrated GVL mixtures within this compositional window as the most effective extraction media for artemisinin recovery.

Experimental data were analyzed using analysis of variance (ANOVA) and regression modeling at a 95% confidence level. Model adequacy was evaluated using the coefficient of determination (R^2) and lack–of–fit tests. Statistical analyses and response surface plots were generated using Design–Expert 13 and Statistica 10.0 software.

2.4. Quantification of artemisinin and derivatives (qualitative and quantitative UHPLC–DAD–MS^n analysis)

Extracts and standard compound solutions were filtered through a 0.2 μm PTFE syringe filter before injection (20 μL) in a UHPLC system equipped with a variable loop Accela autosampler (set at 16 °C), an Accela 600 LC pump, and an Accela 80 Hz photodiode array detector (DAD) (Thermo Fisher Scientific, San Jose, CA, USA). The column used for separation was a Hypersil Gold C18 (100 mm × 2.1 mm × 1.9 μm) column protected with a pre-column (10 mm × 2.1 mm × 1.9 μm), both supplied by Thermo Fisher Scientific (San Jose, CA, USA). The mobile phase was composed of acetonitrile (A) and water:acetonitrile (99:1, v/v) (B), each containing 0.1% (v/v) of formic acid. The gradient elution was carried out at a flow rate of 0.3 mL min^{-1}, at 40 °C, over 24

min, as follows: 0–6 min, 99–78% B; 6–8 min, 78% B; 8–10 min, 78–58% B; 10–14 min, 58–0% B; 14–16 min, 0% B; 16–20 min, 0–99% B; followed by re-equilibration of the column at 99% B for 4 min. UV spectra were recorded in the 190–600 nm range, and chromatograms were also obtained at 190, 210 and 235 nm.

An LCQ Fleet ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was coupled to the UHPLC system through an electrospray ionization (ESI) source operating in positive mode, following the procedure previously described by Santos *et al*. [23]. Peak identification was confirmed by MS fragmentation patterns and comparison with reference standards. Artemisinin and DHAA were dissolved in HPLC-grade ethanol at least five concentrations to obtain the standard curves. The quantification of each compound was carried out through the corresponding linear regression equation (Table S4), obtained by MS detection.

2.5. Thermostability of the extracts

To evaluate the stability of the extracts, samples obtained under optimal extraction conditions (Section 3.2) were stored in liquid form, without further processing, in sealed glass vials at 4 °C and 30 °C for up to four months, protected from light to prevent photodegradation. Artemisinin and DHAA concentrations were quantified after 3, 7, 30, 60, 90, and 120 days to assess the effect of storage conditions on compound stability. At each time point, the stability assay was performed in duplicate.

2.6. *In vitro* bioactivity and cytotoxicity assays

2.6.1. Antiplasmodial activity characterization

The extract exhibiting the highest artemisinin yield, obtained under optimal extraction conditions using biobased solvents (Section 3.2), was evaluated for *in vitro* antiplasmodial activity against the chloroquine– and mefloquine–sensitive *Plasmodium falciparum* 3D7HT–GFP strain (MRA–1029, MR4, ATCC®, Manassas, VA, USA), which constitutively expresses green fluorescent protein (GFP). The corresponding pure solvent (GVL) was tested as a vehicle control, while artemisinin, dihydroartemisinin, and chloroquine were included as reference compounds for comparison at equivalent concentrations.

Parasitemia was monitored daily by light microscopy of Giemsa–stained thin blood smears. Unsynchronized parasite cultures (0.6% hematocrit, 0.5% parasitemia) were incubated with the tested extracts in three–fold serial dilutions in 96–well flat–bottom plates for 72 h at 37 °C under a controlled atmosphere containing 5% CO_2 [24]. Parasite growth inhibition was quantified by flow cytometry (CytoFLEX, Beckman Coulter, CA, USA), acquiring 100,000 red blood cells per well, with GFP fluorescence detected in the FL–1 channel. Data were analyzed using FlowJo software (Tree Star Inc., OR, USA), and half–maximal inhibitory concentrations (IC_{50}) were determined by nonlinear regression (sigmoidal dose–response) using GraphPad Prism 10.4.1 (GraphPad/Dotmatics, MA, USA). Three independent assays were performed in duplicate.

2.6.2. In vitro hemolysis

The hemolytic activity of the extracts was evaluated using red blood cells (RBCs) as a model system. RBCs were incubated in 96–well microplates with three–fold serial dilutions of the test extracts prepared in culture medium for 72 h at 37 °C under a 5% CO_2 atmosphere. Dihydroartemisinin was included as a reference compound, while RBCs incubated with culture medium alone served as a negative control. A 20% (v/v) Triton X–100 solution was used as a

positive control to induce complete hemolysis. The corresponding solvent (GVL) was also tested as a control to assess potential solvent effects. All experimental conditions were tested in triplicate. Absorbance was measured at 450 nm using a microplate reader (Triad™ Series Multimode Detector, Dynex Technologies, VA, USA). Samples exhibiting hemolysis values above 10% were considered hemolytic, according to the literature [25]. The percentage of hemolysis was calculated according to the following equation:

$$\%\text{Hemolysis} = \frac{\text{Sample Abs} - \text{Negative Control Abs}}{\text{Positive Control Abs} - \text{Negative Control Abs}} \times 100 \quad \text{(eq. 1)}$$

2.6.3. Cytotoxicity assay

The cytotoxicity of the extracts obtained under optimal conditions (Section 3.2) was evaluated using the MTT assay (3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide, MTT). V79 cells (Chinese hamster lung fibroblasts) were seeded in 96–well plates at a density of 2×10^4 cells/mL and allowed to adhere for 24 h prior to treatment. Cells were then exposed to the extracts for 72 h using the seven highest concentrations of a three–fold serial dilution. Dihydroartemisinin and chloroquine were included as reference compounds, and the corresponding solvent (GVL) was also tested as a control to assess potential solvent effects.

After incubation, MTT solution (5 mg/mL, Sigma–Aldrich, Merck KGaA, MA, USA) was added to each well, and plates were incubated for an additional 3 h to allow for formazan crystal formation. Crystals were subsequently solubilized with dimethyl sulfoxide, and absorbance was measured at 570 nm using a Triad^{TM} Series Multimode Detector (Dynex Technologies, VA, USA). Cell viability was expressed as a percentage relative to untreated controls according to the following equation:

$$\%\text{Cell Viability} = \frac{\text{Sample Abs}}{\text{Control Abs}} \times 100 \quad \text{(eq. 2)}$$

In accordance with ISO 10993–5 and ISO 10993–12, cell viability below 70% was considered indicative of cytotoxicity. The concentration reducing cell viability to 50% (CC_{50}) was determined by nonlinear regression analysis using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicate.

2.6.4. Selectivity Index (SI)

The selectivity index (SI) was calculated for each extract as the ratio between the concentration that reduces cell viability to 50% (CC_{50}) and the half–maximal inhibitory concentration (IC_{50}). Values of SI > 10 were considered indicative of selective antiplasmodial activity with low cytotoxicity [26].

3. Results and discussion

3.1. Solvent selection

3.1.1. COSMO–RS analysis of solvent–solute interactions

COSMO–RS predictions were used as a pre-screening approach to estimate the relative affinity between artemisinin and a range of conventional and biobased solvents prior to experimental evaluation. To support this analysis, the σ–profile and σ–surface of artemisinin were generated using COSMO–RS and are shown in Figure 1.

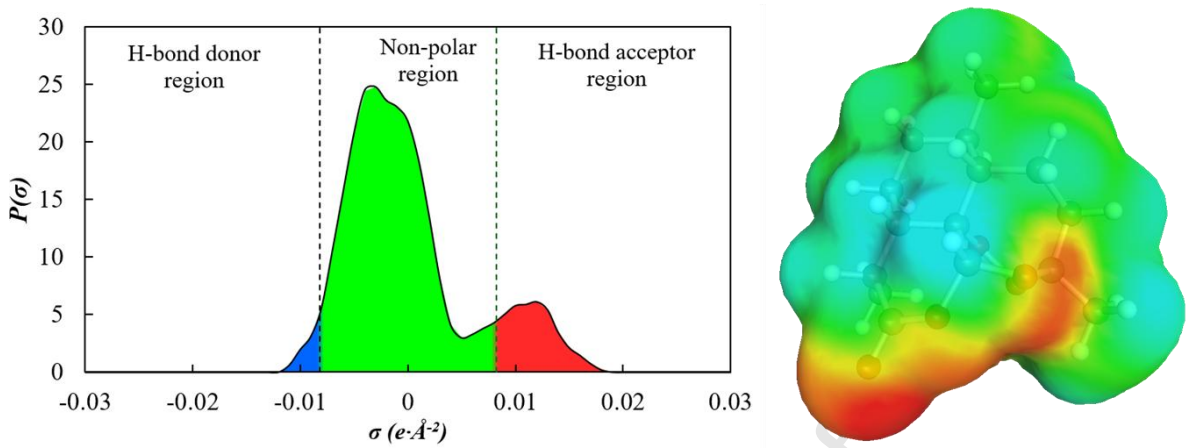

Figure 1. σ–profile (left) and σ–surface (right) of artemisinin. Different colors of the σ–surface indicate different polarities within the molecule: positively charged (blue), non–polar (green), and negatively charged (red).

Artemisinin exhibits a pronounced hydrogen bond acceptor (HBA) character, with a peak at approximately 0.010 e·Å^{-2} associated with the lone pairs of its oxygen atoms. A broad non–polar region between −0.0082 and 0.0082 e·Å^{-2} reflects the contribution of hydrocarbon groups. As the molecule lacks hydrogen bond donor functionalities, artemisinin is classified as a lone HBA compound. These features are consistent with the limited solubility of artemisinin in water and suggest a higher affinity for solvents with polar aprotic character or weak hydrogen bond donor (HBD) capacity, such as lactones, alcohols, and diols.

The infinite dilution activity coefficient (γ^{∞}) of artemisinin was predicted at 25 °C for a set of conventional and biobased solvents, as well as for solvent mixtures containing 25 wt.% and 75 wt.% water (Figure 2). The solvent set included 9 conventional organic solvents [1,3,9], and 17 alternative solvents (including water), including biobased compounds and solvents approved as pharmaceutical excipients. Several diols included in the screening, such as 1,2–propanediol and ethylene glycol, are recognized for their low toxicity and listed as pharmaceutical excipients [27].

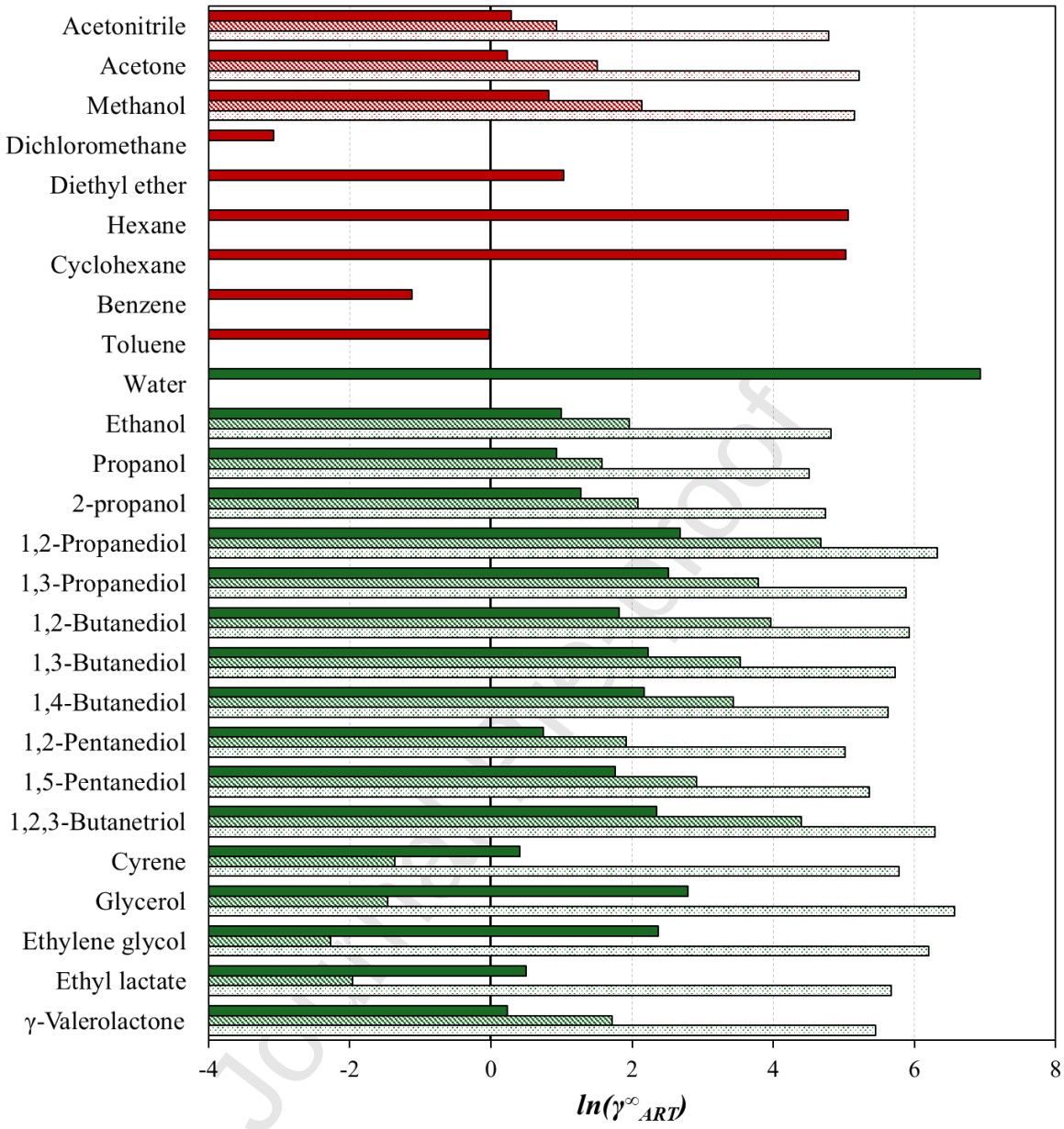

Figure 2. Infinite dilution activity coefficient (γ^{∞}) of artemisinin at 25 ºC predicted by COSMO–RS for conventional (red) and alternative (green) solvents, considering pure (full bars), and water/solvent mixtures with 25 wt.% water content (dashed bars), and 75 wt.% water content (dotted bars). The solid line at ln (γ^{∞}) = 0 corresponds to an ideal solution.

COSMO–RS predictions indicated that the activity coefficient values of artemisinin in pure solvents ranged from strong negative deviations in dichloromethane ($\ln(\gamma^{\infty})$ = –3.08) to high positive deviations in water ($\ln(\gamma^{\infty})$ = +6.93). Apolar solvents such as hexane and cyclohexane exhibited poor compatibility with artemisinin ($\ln(\gamma^{\infty})$ = +5.06 and +5.03, respectively), reflecting

their limited solvation capacity. For most other pure solvents, behavior closer to ideality was observed, with $\ln(\gamma^{\infty})$ values ranging from –1.12 (benzene) and +2.79 (glycerol).

Dichloromethane, the only tested solvent with weak HBD character, exhibited the lowest activity coefficient among all solvents evaluated. Most biobased solvents were predominantly HBA, similar to artemisinin. Among these, GVL showed the lowest $\ln(\gamma^{\infty})$, consistent with its lactone structure [28], followed by Cyrene, ethyl lactate, and 1,2–pentanediol. These results are consistent with the σ–profile analysis, which highlights the large non–polar surface area and HBA sites of artemisinin, in accordance with the "like dissolves like" principle [29].

Therefore, solvents combining a large apolar region with mildly polar functional groups (either weak HBA, such as acetonitrile, methanol, and ethanol, or weak HBD, such as dichloromethane) presented the lowest γ^{∞} values, indicating higher affinity for artemisinin. Benzene, although non–polar, has an aromatic ring that is strongly polarizable due to the delocalization of π–electrons [30], which enables interactions with solutes containing apolar or slightly polar regions. For pure alkanediols, the infinite dilution activity coefficient of artemisinin decreased systematically with increasing carbon chain length, due to improved compatibility with its largely non–polar structure. A notably high activity coefficient ($\ln(\gamma^{\infty}) = 6.93$) is predicted in water, consistent with the poor experimental solubility of artemisinin (mole fraction solubility = $(3.95 \pm 0.28)\cdot10^{-6}$ at 30 °C [31]). The addition of water generally increased the predicted activity coefficients relative to the pure solvents, particularly at 75 wt.%, indicating reduced solvation capacity. Some biobased solvents such as GVL, alkanediols, and Cyrene have been reported to exhibit hydrotropic behavior in aqueous media [32], which may contribute to the solubilization of hydrophobic compounds. However, such effects are not explicitly captured by COSMO–RS, highlighting the importance of experimental validation of the computational predictions.

3.1.2. Experimental solvent screening using accelerated solvent extraction

To validate the COSMO–RS predictions, solvent extraction experiments were performed. Dichloromethane was selected due to its low predicted activity coefficient, while several diols were included to assess the effect of alkyl chain length. Additional biobased solvents were tested based on their favorable predicted behavior. Although ethanol is a conventional solvent, it was classified here as biobased owing to its renewable origin, low toxicity, and widespread use in extraction processes [33]. Extractions were carried out using ASE. However, due to equipment operational limitations, glycerol, Cyrene, and the alkanediols could not be evaluated in their pure form or at 25 wt.% water content because of their high viscosity. These solvents were therefore evaluated at 75 wt.% water, where reduced viscosity allowed stable ASE operation. In addition, because DHAA is a biosynthetic precursor of artemisinin [34], extraction yields of both compounds were evaluated to identify the best-performing solvents. The corresponding experimental extraction yields are presented in Figure 3, with values summarized in Table S5 (see Supplementary Material).

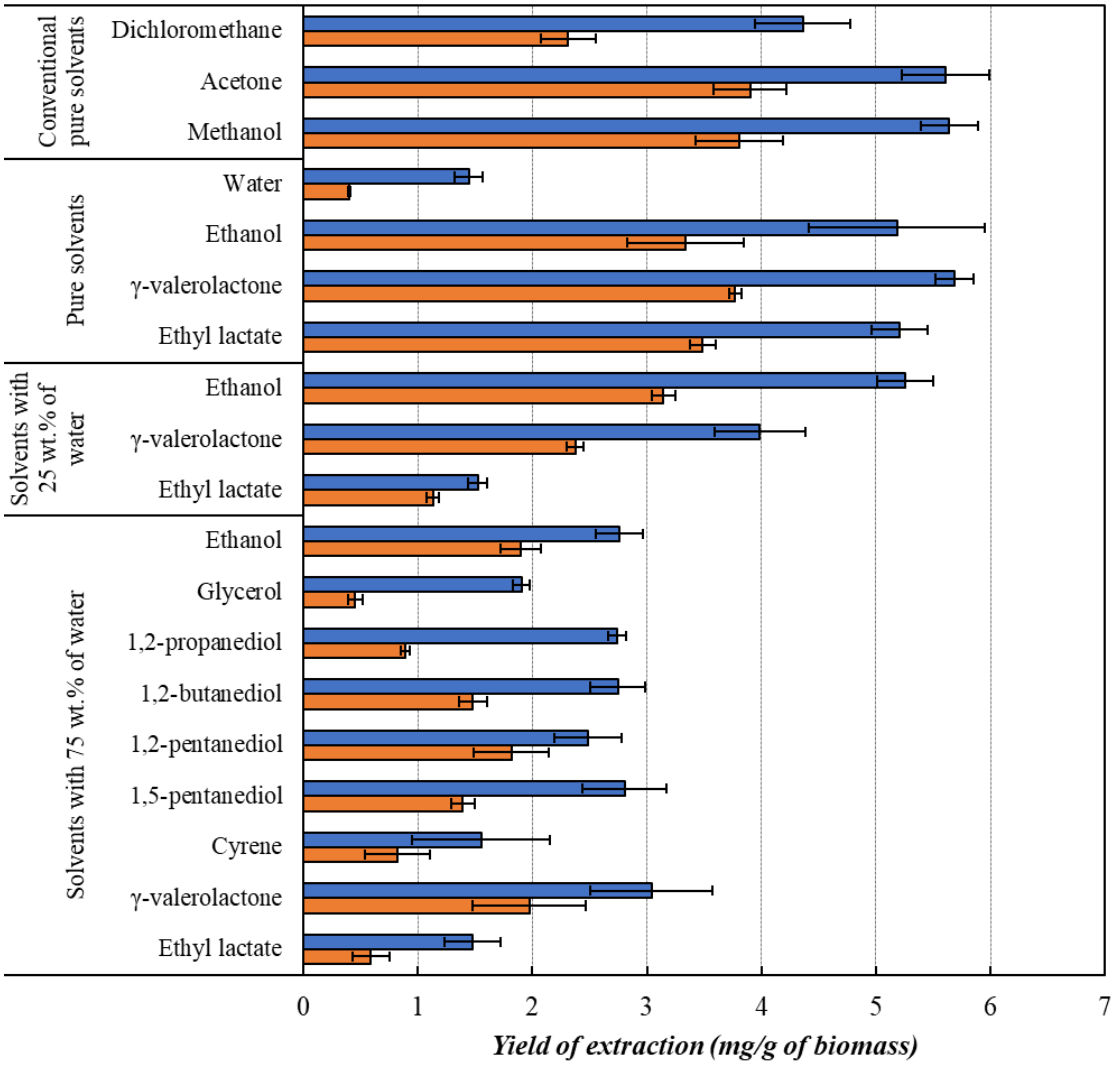

Figure 3. Extraction yields of artemisinin (blue bars) and DHAA (orange bars) from *A. annua* using conventional and alternative solvents at different solvent/water compositions. Extraction conditions: solid–liquid ratio of 1:10, temperature of 100 °C, and extraction time of 5 min.

Experimental extraction yields generally followed the trends predicted by COSMO–RS, supporting its applicability for preliminary solvent selection prior to experimental validation. Among all solvents tested, conventional solvents such as acetone, ethanol, and methanol provided the highest artemisinin yields. However, methanol is highly toxic and classified as a Class 2 solvent under ICH Q3C guidelines, making it unsuitable for pharmaceutical or food–related applications. Acetone and ethanol, although less toxic, are volatile and flammable.

Water led to a significant reduction in extraction yields for solvents containing 75 wt.% water, consistent with the increased ln (γ^{∞}) values relative to the pure solvents as predicted by COSMO–RS. The impact of water addition also varies between solvents. Pure ethanol and ethanol with 25 wt.% water presented similar extraction yields for artemisinin (5.18 ± 0.77 and 5.26 ± 0.24 mg/g, respectively) and DHAA (3.34 ± 0.51 and 3.15 ± 0.10 mg/g, respectively). GVL exhibited a comparable extraction yield to ethyl lactate in the pure solvent (5.69 ± 0.17 and 5.21 ± 0.25 mg/g of artemisinin, respectively), but a decrease in extraction capacity is observed upon water addition, particularly at 75 wt.% (3.99 ± 0.40 mg/g for GVL and 1.52 ± 0.08 mg/g for ethyl lactate).

In agreement with COSMO–RS predictions, aqueous solutions of GVL and 1,2–pentanediol were the best–performing biobased solvents, with GVL yielding slightly higher artemisinin recovery. This behavior may be associated with hydrotropic effects previously reported for GVL, Cyrene, and alkanediols [32]. In such mixtures, the poor interactions of both solute and hydrotrope with water promote the formation of hydrotrope–solute aggregates that locally concentrate hydrophobic compounds. According to the cooperative hydrotropy model, these dynamic aggregates reduce unfavorable water–solute interactions, thereby enhancing solubility. This mechanism has been confirmed for phenolic compounds such as syringic and ferulic acids [32], and may help explain the efficient extraction of artemisinin despite its low intrinsic solubility in water and the high water content (75 wt.%) used in the biobased solvent mixtures.

Despite the low infinite dilution activity coefficients predicted for artemisinin in Cyrene, this solvent performed poorly in practice, likely due to its reversible equilibrium between ketone and geminal diol forms in aqueous solutions, which alters polarity and hydrogen–bonding properties [35]. Such effects are not fully described by COSMO–RS, contributing to discrepancies between predicted and experimental performance [36].

The activity coefficient is only one of several factors governing solvent performance, reflecting the thermodynamic affinity between the solute and solvent at equilibrium. The extraction of target compounds from biomass is also governed by mass transfer kinetics, which depend on factors such as solvent viscosity, diffusivity, particle size, and contact time [37], effects that are not described by COSMO–RS. This limitation highlights the importance of experimentally validating computational predictions, particularly when hydrotropy or other non-classical solvation mechanisms may influence extraction efficiency.

GVL is a non–flammable, low–volatility solvent derived from renewable biomass and classified under the European Union REACH regulation with a favorable safety and environmental profile [18]. Although recognized as safe for use as a flavoring agent (FEMA GRAS 3103) [38], GVL is not yet approved as a direct food additive in the EU. Nevertheless, its low toxicity, biodegradability, renewable origin, and favorable physicochemical properties have led to its extensive investigation for pharmaceutical, cosmetic, and food–related applications [39]. Based on its extraction performance and reported hydrotropic behavior, hydrated GVL was selected for further optimization using RSM.

3.2. Optimization of the extraction

The extraction yields of artemisinin and its biosynthetic precursor DHAA were considered when determining optimal extraction conditions. Response surface methodology (RSM) based on a central composite rotatable design (CCRD) was applied using temperature (T, °C), extraction time (t, min), and solvent composition (GVL/water mixture, wt.%) as independent variables. Yields were expressed as milligrams of compound per gram of dry biomass. The experimental matrix and corresponding experimental and predicted yields for artemisinin and DHAA are reported in Tables

S6 and S7 (see Supplementary Material), respectively, with response surface plots shown in Figure 4. Statistical significance was evaluated by ANOVA at a 95% confidence level.

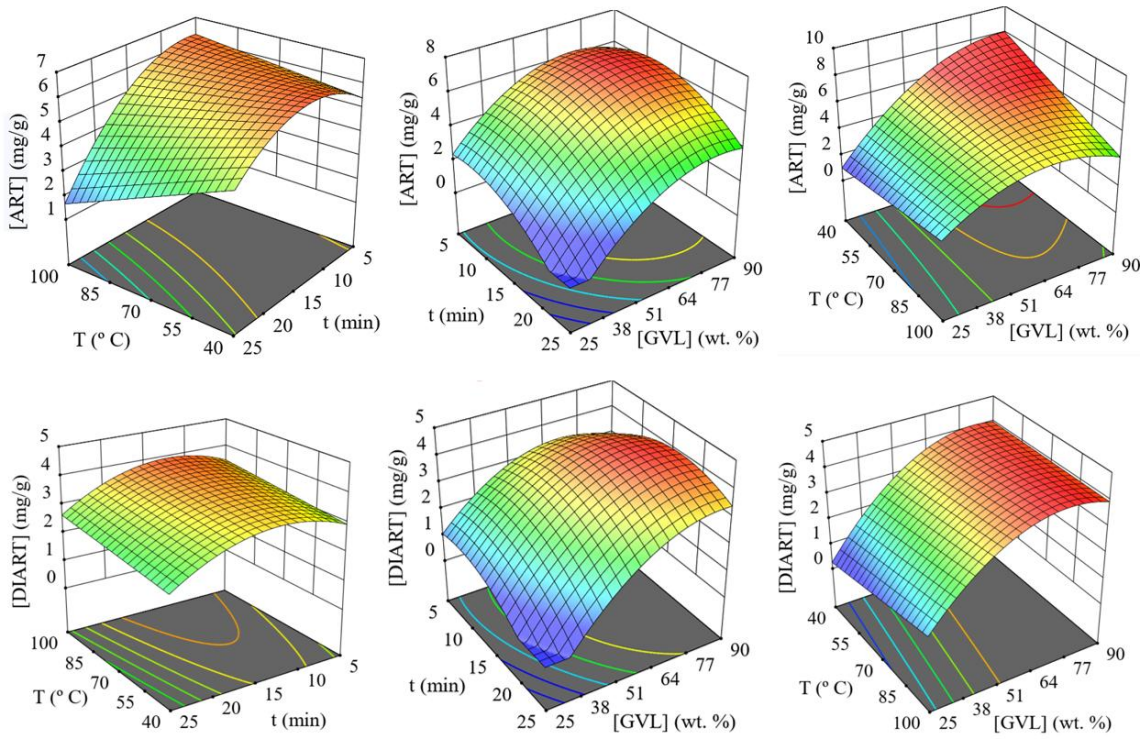

Figure 4. Response surfaces of artemisinin (ART, top) and DHAA (bottom) yields (mg/g of dry plant) using hydrated mixtures of γ–valerolactone with the combined effects of temperature (T, °C) and extraction time (t, min); extraction time (t, min) and concentration of γ–valerolactone ([GVL], wt.%); temperature (T, °C) and concentration of γ–valerolactone ([GVL], wt.%) (from left to right).

For artemisinin, the reduced quadratic model exhibited adjusted and predicted R^2 values of 0.93 and 0.82, respectively (Table S8, Supplementary Material). For DHAA, the corresponding values were 0.94 and 0.87 (Table S9, Supplementary Material). The lack-of-fit term is not significant for the artemisinin model ($p = 0.2312$) and significant for the DHAA model ($p = 0.0115$), indicating limitations of the polynomial description for DHAA. The models are therefore used here primarily as descriptive tools to identify the main effects and interactions governing extraction performance, with operating conditions confirmed by experimental validation.

Regression models obtained from the RSM analysis are expressed in terms of coded factors, which allows direct comparison of the magnitude and direction of each main effect and interaction term on the extraction yield. Full quadratic models were reduced by backward elimination ($p < 0.05$) while respecting model hierarchy. The fitted coded-factor equations for artemisinin and dihydroartemisinic acid extraction yield (Y, in mg/g) are:

$$Y_{ART} = 5.81 - 0.33A - 0.80B + 1.30C - 0.35AB - 0.49AC - 0.46B^2 - 0.66C^2 \quad \text{(eq. 3)}$$

$$Y_{DHAA} = 3.63 + 0.15A - 0.25B + 0.95C + 0.25BC - 0.30B^2 - 0.45C^2 \quad \text{(eq. 4)}$$

where A, B, and C correspond to coded values of temperature, extraction time, and GVL concentration, respectively. The complete ANOVA outputs, regression coefficients, and diagnostic plots are reported in Tables S8–S11 and Figures S1–S6 of the Supplementary Material.

For artemisinin, all three variables (temperature, extraction time, and GVL concentration) were statistically significant. The linear effects of GVL concentration and extraction time were the most influential, whereas temperature had a smaller linear effect (Table S10 in the Supplementary Material). Negative quadratic terms were observed for GVL concentration and time, and significant interaction effects were identified for temperature × time and temperature × GVL concentration. According to the Pareto chart (Figure S3 in the Supplementary Material), GVL concentration was the dominant factor, followed by extraction time. Response surface analysis (Figure 4, upper row) showed that artemisinin yield increased between 60 and 85 °C and with GVL concentrations up to approximately 80 wt.%, reaching a plateau thereafter, with maximum yields obtained at extraction times of 13–15 min. Artemisinin is thermostable, with a melting point of 156–157 °C and thermal decomposition occurring only above 190 °C [22], supporting the use of elevated temperatures when required.

A similar response pattern was observed for DHAA. According to the Pareto chart (Figure S4 in the Supplementary Material), the linear effect of GVL concentration was the dominant factor, followed by the quadratic terms for GVL concentration and extraction time. Both extraction time and GVL concentration were statistically significant in their linear and quadratic forms. DHAA yield increased with temperature and GVL concentration, reaching maximum values at the upper end of the studied range (Figure 4, bottom row). Temperature consistently enhanced extraction efficiency, while the effect of GVL concentration plateaued above approximately 80 wt.%. Extraction time had a limited influence, with yields peaking between 10 and 15 min before declining, in agreement with the negative quadratic term. These trends are consistent with those observed for artemisinin and indicate operational flexibility.

To validate the performance of the optimized biobased solvent, extraction conditions were selected to simultaneously maximize the yields of artemisinin and DHAA. The selected conditions, namely 85 °C, 13 min, and 80 wt.% GVL, correspond to a compromise between artemisinin recovery and DHAA co-extraction, since the two compounds do not present identical optima within the studied experimental range. The experimental yields obtained under these conditions followed the trends predicted by the model, supporting the suitability of the selected conditions for simultaneous recovery of artemisinin and co-extraction of DHAA. The corresponding recoveries were 87.0% for artemisinin and 81.4% for DHAA, estimated based on the total extractable contents of ART and DHAA in the biomass. These total extractable contents were determined through three consecutive extraction cycles performed under the same conditions until no further compound was detected and corresponded to 9.34 ± 0.35 mg/g for artemisinin and 3.92 ± 0.18 mg/g for DHAA.

To benchmark the performance of the GVL–water mixture, parallel extractions were conducted using dichloromethane, a conventional solvent widely employed in large-scale artemisinin

recovery, under the same optimized ASE conditions (85 °C, 13 min) used for the GVL–water mixture. Dichloromethane yielded 6.34 ± 0.08 mg/g of artemisinin and no detectable DHAA, whereas the optimized GVL–water mixture enabled higher artemisinin yield (8.13 ± 0.34 mg/g) together with the simultaneous extraction of DHAA (3.19 ± 0.03 mg/g). These results highlight the advantage of the hydrated biobased solvent for target-compound recovery and co-extraction. The ability to co-extract artemisinin and DHAA using a single biobased solvent supports the use of GVL in integrated extraction strategies. The biological evaluation of the resulting extract is reported in Section 3.4.

While a wide range of extraction methods and solvents has been explored, direct comparison across studies remains challenging due to differences in biomass characteristics, including cultivar, geographical origin, seasonality, and soil composition [40]. For example, Ferreira et al. [16] reported maximum artemisinin yields of approximately 6.5 mg/g using salicylate-based ionic liquids and salts as hydrotropic solvents at 32 °C for 110–130 min, comparable to the yield obtained in this work (8.13 ± 0.34 mg/g for artemisinin). Prawang et al. [41] obtained 15.8 mg/g using PEG–200 under ultrasound-assisted extraction (60 min at 50 °C), while Soxhlet extraction with petroleum ether yielded 14.9 mg/g after 12 h. Similarly, Zhang et al. [42] reported comparable efficiencies using ultrasound-assisted extraction with monoether-based solvents. In contrast, Rodrigues et al. [12] employed supercritical CO_2, achieving yields between 0.8 and 8 mg/g of biomass, comparable to those obtained with conventional ethanol extraction (7 mg/g).

Overall, the water/GVL mixtures proposed in this work delivered artemisinin yields comparable to or exceeding those of conventional methods, while requiring shorter extraction times and milder operating conditions. In addition, this may enable processing of the extract without the solvent evaporation step typically required for conventional organic solvents, thereby simplifying

downstream processing. The ability to co-extract artemisinin and DHAA using a single biobased solvent further supports the use of GVL in integrated extraction strategies. These findings support the use of water/GVL mixtures as effective alternatives to conventional organic solvents for the extraction of bioactive compounds.

3.3. Thermostability of the extracts

The use of natural extracts in pharmaceutical applications raises challenges related to chemical stability during storage, which may compromise efficacy and shelf life due to degradation [43]. In this context, the stability of GVL–based extracts obtained under optimized conditions was evaluated over 120 days. Samples were stored at 4 °C and 30 °C, and artemisinin and DHAA contents were monitored at defined intervals (Figure 5).

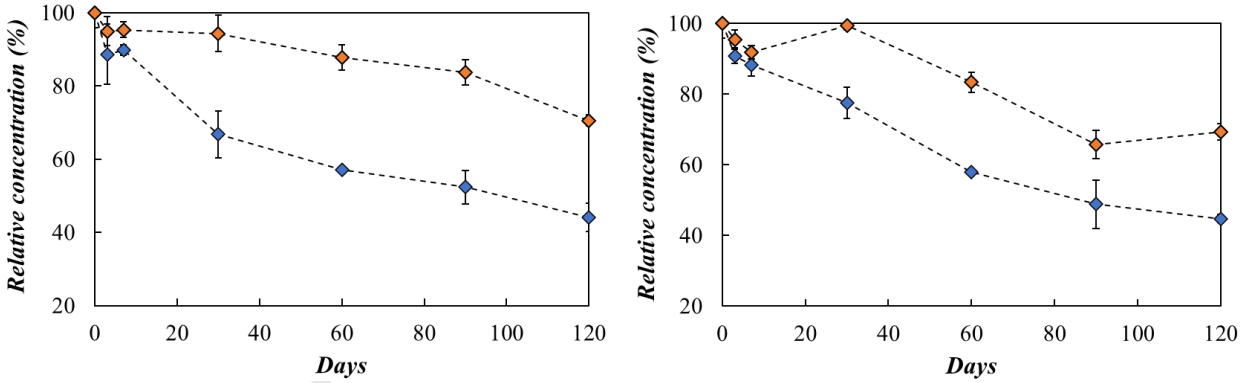

Figure 5. Stability profile of *Artemisia annua* L. extracts obtained using GVL at 4 °C (left) and 30 °C (right), showing artemisinin (blue dots) and DHAA (orange dots) content. Each point represents the mean of at least two replicate determinations ± standard deviation.

After 30 days, extracts retained approximately 78% of their initial artemisinin content at 30 °C and approximately 67% at 4 °C, while DHAA retention was above 95% at both temperatures. After 120 days, the two storage conditions converged to similar values (approximately 45% for artemisinin and 70% for DHAA), indicating that the long-term degradation profile is not strongly influenced by storage temperature within the tested range. The decline observed for both

compounds over time is consistent with the known instability of artemisinin in liquid solutions, where the reactive endoperoxide bridge is susceptible to degradation by dissolved oxygen and other matrix components. DHAA, however, lacks the endoperoxide moiety and is a comparatively more stable compound in solution.

The lower artemisinin retention observed at 4 °C at the 30-day time point does not follow the expected degradation trend. One possible explanation is the complexity of crude plant extracts, where co-extracted matrix components may influence compound stability and quantification over time. Furthermore, the stability assay was performed in duplicate at each time point, limiting the interpretation of small differences in storage temperatures. These results indicate that hydrated GVL mixtures are capable of maintaining measurable levels of artemisinin and DHAA over extended storage periods and may therefore represent suitable media for extract preservation under conditions where refrigeration is limited.

Previous studies have reported that artemisinin stability depends strongly on formulation and storage conditions. Zime–Diawara et al. [44] observed that solid artemisinin retained approximately 95% of its content after 60 days under accelerated degradation conditions (40 ± 2 °C, 75 ± 5% RH), whereas saturated aqueous solutions showed around 66% degradation, consistent with the trends observed in this work. Fleming and Freyhold [45] reported no significant loss in ethanolic *Artemisia annua* extracts stored at room temperature for 45 days, suggesting a protective effect of ethanol.

Overall, hydrated GVL mixtures exhibited higher stability than aqueous solutions but lower stability than solid–state or ethanolic formulations. Despite differences at intermediate time points, both storage conditions showed comparable long–term degradation profiles, supporting their applicability in settings without reliable cold storage. Together, the moderate thermal stability and

the favorable properties of GVL support the use of biobased solvents as alternatives to conventional organic solvents. To further assess their practical relevance, the antiplasmodial activity of the extracts was subsequently evaluated.

3.4. Antiplasmodial activity

The antiplasmodial activity of the extracts was assessed against *P. falciparum* 3D7HT–GFP, a chloroquine- and mefloquine-sensitive strain routinely used for the initial screening of natural extracts and antiplasmodial compounds prior to evaluation against resistant strains. Extracts were obtained under optimized conditions (85 °C, 13 min) using either an 80 wt.% GVL/water mixture or pure dichloromethane. Prior to biological evaluation, dichloromethane was removed due to its inherent cytotoxicity, whereas GVL was maintained in the extract because its contribution to the observed antiplasmodial activity at the tested dilutions was considered negligible ($3.00 \cdot 10^{-4}$ wt.% at the IC_{50}). Artemisinin, chloroquine, and dihydroartemisinin were used as positive controls, the latter representing the pharmacological gold standard among artemisinin derivatives. The assay was designed to confirm that the GVL-based extract preserves the antiplasmodial activity of the active fraction relative to pure artemisinin, dihydroartemisinin, and the dichloromethane extract, under identical normalization to artemisinin equivalents. IC_{50} values were determined after 72 h of parasite exposure (Figure 6), with corresponding dose–response data provided in Table S12 and Figure S7 in the Supplementary Material. Each IC_{50} value corresponds to the mean of at least three independent experiments, in which each concentration was assessed in duplicate. All samples were normalized to an initial artemisinin concentration of 11 μg/mL to ensure comparability between samples.

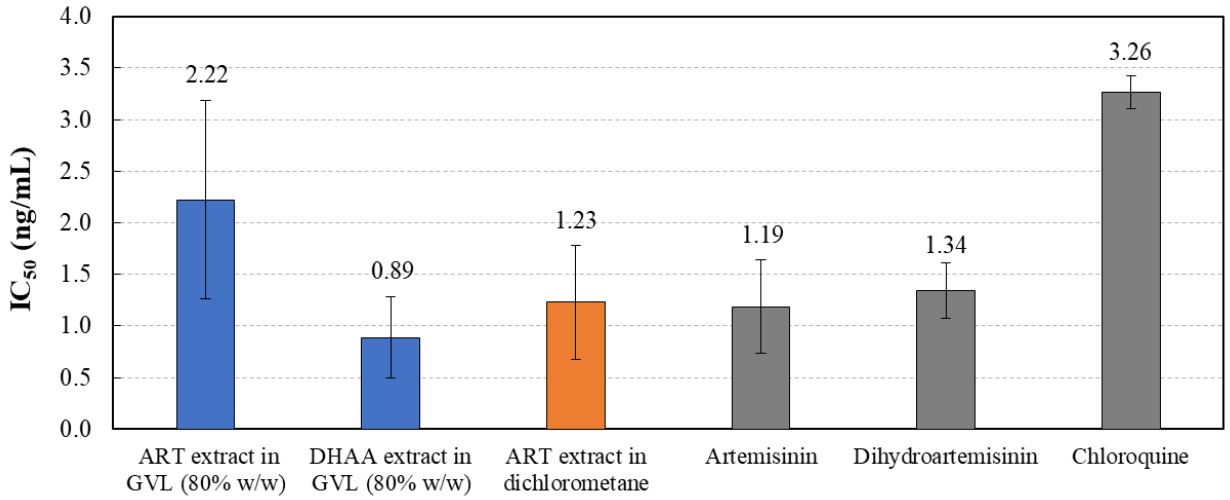

Figure 6. *In vitro* antiplasmodial activity of *Artemisia annua* L. extracts and positive controls against the 3D7HT–GFP strain of *P. falciparum* after 72 h of exposure. IC_{50} values were obtained from three independent assays. Legend: IC_{50} of artemisinin (ART) and DHAA present in γ–valerolactone 80 wt.% extract (blue bars); artemisinin (ART) extracted with dichloromethane (orange bar); artemisinin, dihydroartemisinin, and chloroquine as reference compounds (grey bars).

As shown in Figure 6, the GVL–based extract exhibited high antiplasmodial activity, with an IC_{50} expressed as artemisinin equivalents of 2.22 ± 0.96 ng/mL and a DHAA–equivalent IC_{50} of 0.89 ± 0.39 ng/mL. The IC_{50} value for DHAA reflects its relative concentration in the extract and does not indicate higher intrinsic antiplasmodial potency compared to artemisinin. As expected, artemisinin (1.19 ± 0.45 ng/mL), dihydroartemisinin (1.34 ± 0.27 ng/mL), and chloroquine (3.26 ± 0.16 ng/mL) exhibited IC_{50} values comparable to or lower than those of the extracts, consistent with their known high potency [46]. The pure solvent GVL showed negligible activity (IC_{50} = 0.138 ± 0.009 wt.%), and its estimated concentration at the artemisinin IC_{50} point was only $3 \cdot 10^{-4}$ wt.%, confirming that the observed antiplasmodial effect is not associated with the solvent. The dichloromethane extract also displayed strong activity (IC_{50} = 1.23 ± 0.55 ng/mL).

IC_{50} of the GVL extract (2.22 ± 0.96 ng/mL, expressed as artemisinin equivalents) was comparable to those of pure artemisinin (1.19 ± 0.45 ng/mL), dihydroartemisinin (1.34 ± 0.27 ng/mL), and the dichloromethane extract (1.23 ± 0.55 ng/mL), indicating that the GVL-based extraction process

preserves the intrinsic antiplasmodial activity of the active fraction under the tested conditions. The literature regarding the contribution of co-extracted compounds in *A. annua* extracts remains inconclusive. Some studies suggest that the in vitro antiplasmodial activity of *A. annua* extracts largely follows artemisinin content, with limited evidence of synergistic enhancement relative to pure artemisinin [47,48], whereas others report enhanced in vivo potency associated with pharmacokinetic modulation, including increased artemisinin absorption or altered metabolism [4,49]. The *in vitro* activity observed in the present work is therefore consistent with preservation of antiplasmodial activity rather than evidence of synergistic enhancement.

When compared with extracts obtained using hydrated hydrotropes such as sodium salicylate and cholinium salicylate (IC_{50} = 2.7–3.4 ng/mL), the GVL–based extract showed comparable or slightly higher activity [16]. This result supports the efficiency of hydrated biobased solvents for extraction and highlights the potential of GVL as a medium for producing bioactive multi–component extracts. Although GVL is classified as safe under REACH [18] and exhibits low acute toxicity towards aquatic organisms [50], it is not yet approved for pharmaceutical use, and further toxicological and regulatory evaluation will be required prior to clinical translation.

3.5. *In vitro* hemolysis and cytotoxicity of the extracts

To evaluate the safety of the GVL–based extract, hemocompatibility was assessed by in vitro hemolysis assays using non–infected erythrocytes. The tested concentration range included the IC_{50} for antiplasmodial activity (expressed as artemisinin equivalents) and was compared with pure artemisinin, chloroquine, and a dichloromethane–based extract. Pure GVL was also tested at concentrations equivalent to those present in the diluted extract to assess whether the solvent contributed to the observed toxicity. Hemolysis was quantified by hemoglobin release into the

medium, a standard marker of membrane damage [51]. This analysis is particularly relevant for artemisinin–derived compounds, whose mechanism involves endoperoxide cleavage in the presence of iron, generating reactive oxygen species (ROS) that can damage both *Plasmodium* proteins and host erythrocytes [52].

The hemolytic and cytotoxic profiles of the extracts and controls were quantified by calculating the HC_{10} (minimum concentration inducing 10% hemolysis) and the CC_{50} values (minimum concentration reducing cell viability to 50%). Detailed results of cytotoxicity and hemolysis are provided in Tables S13–S18 (see Supplementary Material).

As shown in Table S19 (see Supplementary Material), pure GVL showed hemolytic activity at concentrations above 1.53 wt.% (HC_{10}), while cytotoxicity was detected at concentrations above 0.23 wt.% (CC_{50}), as detailed in Tables S15 and S18 in the Supplementary Material, respectively. For the extract prepared with 80 wt.% GVL, the artemisinin concentrations corresponding to the toxicity thresholds were 14,000.33 ng/mL for hemolysis (HC_{10}) and 614.50 ng/mL for cytotoxicity (CC_{50}). Although this extract displayed slightly higher cytotoxicity than the dichloromethane extract or the reference antimalarial drugs, the concentrations associated with toxicity remained more than three orders of magnitude above those required for antiplasmodial efficacy. In contrast, the GVL content present in the extract at its IC_{50} for antiplasmodial activity was only $3.00 \cdot 10^{-4}$ wt.%, far below both its HC_{10} and CC_{50} values. The extract prepared with 80 wt.% GVL showed a CC_{50} of 0.08 ± 0.02 wt.% (614.50 ± 174.27 ng/mL) and a hemolytic threshold of 1.93 ± 0.51 wt.% (14,000.33 ± 3,731.16 ng/mL), the latter comparable to that of pure GVL.

To further assess selectivity, the selectivity index ($SI = CC_{50}/ IC_{50}$) was calculated (Table S19 in the Supplementary Material). Pure GVL was the only sample with an SI below 10. In contrast, the artemisinin extract in 80 wt.% GVL and the dichloromethane extract displayed SI values of 276.80

and 718.34, respectively. These values are substantially higher than the commonly accepted threshold of 10 for selective compounds with low cytotoxicity, confirming that both extracts exhibit strong selectivity for *P. falciparum* over mammalian cells.

While the GVL–based extract showed slightly higher cytotoxicity than the dichloromethane extract and the positive controls (dihydroartemisinin and chloroquine), these effects occurred only at concentrations far above therapeutic levels. GVL itself has demonstrated low systemic toxicity in vivo, with an oral LD_{50} of 8800 mg/kg in rats [39] and a NOAEL (No Observed Adverse Effect Level) of 500 mg/(kg·day) in a subchronic study using Osborne–Mendel rats [53]. Although mild hemolytic and cytotoxic effects were observed at high concentrations, the GVL content at the IC_{50} was <0.0003 wt.%, well below both its HC_{10} and CC_{50} values [25]. This wide safety margin, together with the biodegradability of GVL, supports its potential as a biobased solvent for extract preparation in antiplasmodial applications.

Taken together, these results indicate that GVL-based extracts maintain strong antiplasmodial activity while exhibiting low solvent contribution at biologically relevant concentrations. The GVL concentration at the IC_{50} remained several orders of magnitude below the levels associated with hemolysis and cytotoxicity. Nevertheless, although GVL exhibits low acute toxicity and is classified as safe under REACH, further toxicological and regulatory evaluation will be required prior to pharmaceutical or clinical application.

4. Conclusions

This study presents an integrated strategy for the recovery of artemisinin and its biosynthetic precursor dihydroartemisinic acid (DHAA) from *Artemisia annua* L., combining COSMO–RS–guided solvent selection with pressurized extraction using water/biobased solvent mixtures. γ–

Valerolactone/water mixtures showed the highest performance, yielding 8.13 ± 0.34 mg/g of artemisinin and 3.19 ± 0.03 mg/g of DHAA under mild conditions (85 °C, 13 min, 80 wt.% GVL), outperforming dichloromethane.

GVL–based extracts retained ~78% of artemisinin after 30 days at 30 °C, and converged to similar retention values at both storage temperatures after 120 days, indicating that long-term degradation is not strongly influenced by storage temperature within the tested range. The extracts also exhibited *in vitro* antiplasmodial activity (IC_{50} = 2.22 ± 0.96 ng/mL, expressed as artemisinin equivalents), comparable to those of artemisinin and dihydroartemisinin. No solvent contribution was observed at biologically relevant concentrations. The extracts showed a high selectivity index and a wide safety margin, indicating selective activity against *Plasmodium falciparum* with low cytotoxicity.

Combining predictive solvent selection with pressurized extraction increases artemisinin and DHAA recovery while preserving antiplasmodial activity and extract stability. The use of GVL may enable processing of the extract without the solvent evaporation step typically required for conventional organic solvents, thereby simplifying downstream processing. Although further toxicological and regulatory evaluation remains necessary prior to clinical translation, the present results support biobased solvents as viable alternatives to conventional organic solvents for the recovery of bioactive compounds.

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Appendix A. Supplementary data

E–supplementary data for this work can be found in the e–version of this paper online.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of generative AI and AI–assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT, an AI–assisted language model, to support language editing and improve clarity of the manuscript. After using this tool, the authors reviewed and edited the content as necessary and take full responsibility for the content of the published article.

Data Availability

Data will be made available on request.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- COSMO-RS used as pre-screening tool for biobased solvent selection
- Experimental screening identified GVL as the most effective biobased solvent
- Hydrated GVL (80 wt.%) under ASE yielded 8.13 mg/g artemisinin and 3.19 mg/g DHAA
- GVL-based extracts showed in vitro antiplasmodial activity comparable to artemisinin
- GVL extracts displayed high selectivity index and wide *in vitro* safety margin