horticulturae

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Comparative Evaluation of Different Extraction Techniques for Separation of Artemisinin from Sweet Wormwood (*Artemisia annua* L.)

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Abstract: Sweet wormwood (*Artemisia annua* L.) valorization is gaining importance due to the presence of the health-promoting bioactive compound, artemisinin. Considering the wide possible application of artemisinin drug formulations, new, greener technologies in their production are welcome. In this study, artemisinin was extracted from *A. annua* leaves using green extraction technologies (ultrasound-assisted extraction, supercritical CO_2 extraction, deep eutectic solvent extraction and subcritical water extraction) in combination with green solvents. Artemisinin was present up to 3.21 $\mu g/mg_{dw}$. Among the different green extraction techniques, HPLC data revealed supercritical CO_2 (SCO_2) extracts to exhibit the highest yield of artemisinin due to the solvent non-polar properties. Additionally, the volatile compounds profile of SCO_2 extract was determined, with camphor (12.23%), arteannuin b (15.29%) and artemisia ketone (10.97%) as the most abundant compounds. Obtained results encourage the use of green extraction techniques for the separation of artemisinin and are expected to find potential in pharmaceutical, cosmetic and food applications.

Keywords: *Artemisia annua* L.; sweet wormwood; extraction; artemisinin

Citation: Banožić, M.; Wronska, A.W.; Jakovljević Kovač, M.; Aladić, K.; Jerković, I.; Jokić, S. Comparative Evaluation of Different Extraction Techniques for Separation of Artemisinin from Sweet Wormwood (*Artemisia annua* L.). *Horticulturae* **2023**, *9*, 629. https://doi.org/10.3390/horticulturae9060629

Academic Editor: Charalampos Proestos

Received: 6 May 2023
Revised: 19 May 2023
Accepted: 25 May 2023
Published: 26 May 2023

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1. Introduction

Recently, there are more and more scientific investigations on the health impact of plant bioactive compounds. Increasing research interest also concerns *Artemisia annua* L. (commonly known as sweet wormwood, sweet annie or qinghao), which is an annual plant belonging to the Asteraceae family and grows in spring across the world [1–4]. Sweet wormwood plant has been used in traditional Chinese medicine for over 2000 years as a remedy for many disorders such as fever or inflammation [5,6]. It became even more significant after the discovery of the bioactive, antimalarial compound artemisinin by Chinese scientists in 1970s [1]. Since 2001, the World Health Organization (WHO) has recommended sweet wormwood for therapy against malaria [5,7]. Artemisinin and its derivatives were also reported to be effective in the treatment of other health issues. They are active against cancer cells, viruses, parasites and they exhibit immunoregulatory or liver-protective effects [1,2,6]. Moreover, sweet wormwood yields an essential oil, which is commonly used in perfumery and cosmetics. The essential oil's antimicrobial and antifungal properties are also significant in treatment of skin diseases [8,9].

The chemical compounds present in sweet wormwood can be divided into volatiles and non-volatiles. In the second group primarily terpenoids, coumarins and flavonoids are present. Artemisinin is a terpenoid, which can be found in both the leaves and flowers (between 0.01% and 1.4%). The amount of essential oil in sweet wormwood ranged from 0.04% to 1.9%$_{dw}$ [3,10]. The chemical synthesis of artemisinin is uneconomic and complicated. Thus, field cultivation of *A. annua* is currently the only source of artemisinin production. It is cultivated at a large scale mainly in China and Vietnam, but also in several African countries [1,7]. The standard method to obtain artemisinin from *A. annua* is organic solvent extraction. Due to the risks of procedures requiring significant amounts of volatile flammable fluids, the poor ecological sustainability, and the risk to human health, it is necessary to create alternative procedures that would be competitive in terms of cost and efficiency and have fewer or no disadvantages compared to hydrocarbon solvents [11]. However, obtained extracts could contain a significant concentration of contaminants such as chlorophylls that can have a negative impact on the purification procedure [7]. Different extraction methods are present in the literature. Among the most popular is hexane extraction, which is a simple industrial-scale method and the most cost-effective. However, considering other available techniques (supercritical extraction with CO_2 (SCO_2), ultrasound-assisted extraction or microwave-assisted extraction), it has a lower efficiency of extraction. Further, its ecological and safety aspects are much worse [1,12,13]. A lot of researchers focus on improving the extraction of artemisinin, thus many innovative techniques are still being explored [7]. There are several reports in the literature comparing the extraction results obtained with different methods. For example, Soktoeva et al. [8] compared maceration, ultrasonic and SCO_2 extraction for obtaining artemisinin from *Artemisia annua* L. They observed that the usage of different solvents (hexane, ethanol) does not affect the artemisinin content in the extracts isolated by ultrasonic extraction and maceration. These methods yielded 0.038–0.040%, whereas the highest amount of artemisinin was found by SCO_2 extraction (0.054% of extraction yield). They also pointed out that the minimum compound yield of 0.022% was obtained by ultrasonic extraction with ethyl acetate. Hao et al. [14] compared microwave-assisted extraction, Soxhlet extraction and supercritical fluid extraction for the recovery of artemisinin from *Artemisia annua* L. They found microwave-assisted extraction superior in the context of better extraction yield (92.1%) and shorter treatment time (12 min). Additionally, they tested several solvents, namely ethanol, trichloromethane, *n*-hexane, cyclohexane, petroleum ether and commercial solvent oil (SH0003-90, with the boiling point 60–90 °C, and SH 0004-90 with the boiling point 80–120 °C). Commercial solvent oil gave the highest extraction yield (84%), while trichloromethane gave the highest content of artemisinin (0.487%). Vidović et al. [15], however, compared different parameters of SCO_2 extraction and their impact on the total extraction yield and compared with the Soxhlet extraction and hydrodistillation results. They carried out SCO_2 at 40 °C and different pressures of 100, 200 and 300 bar and also at 60 °C setting the same pressures and obtained an extraction yield in the range 2.23–5.18% and 2.43–3.35%, respectively. Using conventional methods (Soxhlet extraction and hydrodistillation) 10.28% and 0.10% of were obtained, respectively. The authors emphasize that traditional ways of extraction have many disadvantages even though the obtained amount of extraction yield is much higher [15]. In recent years, deep eutectic solvents (DESs), first proposed by Abbott et al. [16], are becoming more and more popular in the extraction of bioactive components due to their low toxicity and good biodegradability [17–19]. In recent years, there has been growing interest in the application of subcritical water as an extraction solvent. Water is recognized as an extraction medium that can produce clean and safe extracts without environmental concerns. However, the polarity of water at ambient conditions can decrease the extraction efficiency of less polar and non-polar compounds [20]. There are some suggestions that water under subcritical conditions becomes less polar and more similar to organic solvents, which could increase extraction efficiency of less polar compounds [21].

The aim of this study was to compare different techniques (SCO_2 extraction, subcritical water extraction, ultrasound-assisted extraction (UAE) and extraction with deep eutectic solvents (DES)) for the extraction of artemisinin. Considering that SCO_2 is a non-polar solvent, this study also intends to determine the volatile profile of obtained non-polar extracts (SCO_2). The main novelty of this paper lies in novel green extraction techniques performed for the first time for the separation of artemisinin (DES and SWE) and the comparison of their efficiency with other green techniques.

2. Results and Discussion

Artemisinin has traditionally been isolated from *A. annua* using organic solvents including hexane, petroleum ether, dichloromethane, chloroform, propylene glycol methyl ether, isopropanol, and butanol [22–27]. There are various extraction methods described in the literature used to obtain artemisinin, in particular from the fresh plant. The methods are typically labor intensive, have a negative effect on the environment, and occasionally do not maximize extractive efficiency. The emergence of an alternative extraction technique for artemisinin and other substances is therefore desired. Those techniques should be compared with existing extraction techniques in terms of costs, environmental impact, risk, toxicity, energy use, extraction efficiency, universality and scalability [28].

Therefore, this study aimed to compare several green extraction techniques for separation of artemisinin from sweet wormwood (Table 1).

Table 1. Artemisinin concentration of sweet wormwood extracts obtained by different extraction techniques and analyzed by HPLC.

No	Extraction Technique	Extraction Conditions	Artemisinin Content (µg/mg)
1.	UAE	30 °C, 45 min, 20 mL/g	BDL*
2.	UAE	50 °C, 30 min, 20 mL/g	0.554
3.	UAE	50 °C, 45 min, 30 mL/g	0.350
4.	UAE	70 °C, 45 min, 20 mL/g	0.916
5.	UAE	70 °C, 30 min, 10 mL/g	2.001
6.	UAE	70 °C, 15 min, 20 mL/g	BDL*
7.	UAE	50 °C, 15 min, 10 mL/g	0.565
8.	UAE	30 °C, 30 min, 30 mL/g	0.256
9.	UAE	70 °C, 30 min, 30 mL/g	0.204
10.	UAE	30 °C, 15 min, 20 mL/g	2.232
11.	UAE	50 °C, 45 min, 10 mL/g	0.444
12.	UAE	30 °C, 30 min, 10 mL/g	0.516
13.	UAE	50 °C, 15 min, 30 mL/g	BDL*
14.	SWE	125 °C, 15 min, 20 mL/g, 30 bar	BDL*
15.	SWE	150 °C, 15 min, 20 mL/g, 30 bar	BDL*
16.	SWE	175 °C, 15 min, 20 mL/g, 30 bar	BDL*
17.	SWE	200 °C, 15 min, 20 mL/g, 30 bar	BDL*
18.	SCO_2E	40 °C, 15 min, 1.4 kg CO_2/h 300 bar	3.210
19.	DES	Choline chloride:lactic acid (1:2) with 20% water (*v*/*v*), 30 °C, 60 min	1.026
20.	DES	Choline chloride:levulinic acid (1:2) with 20% water (*v*/*v*), 50 °C, 60 min	1.417

BDL*—below detection limit, UAE—ultrasound-assisted extraction, SWE—subcritical water extraction, SCO_2E—supercritical CO_2 extraction, and DES—deep eutectic solvent.

The effects of different types of solvents (water, subcritical water, SCO_2, and DES) and extraction techniques were investigated to determine the presence of artemisinin in obtained extracts. According to the obtained results, artemisinin content was found to be comparatively high in SCO_2, a non-polar solvent, than in polar solvents (particularly in subcritical water where was under the limit of detection). Dogan et al. [29] stated that artemisinin recovery from *A. annua* depends greatly on the extraction solvent, so the extraction in this study was carried out using a variety of solvents because no single solvent

can be ensured to be effective. In the mentioned study, the most efficient solvents for the extraction were determined to be hexane, 95% ethanol, and isopropanol, which produced the greatest artemisinin yield in the range of 0.062–0.066%.

2.1. Ultrasound-Assisted Extraction (UAE)

It is widely recognized that temperature significantly affects how much mass is transferred during a chemical reaction or separation procedure. To evaluate its impact on the extraction, the temperature of the extraction was changed from 30 to 70 °C. Raising the temperature up to 45 °C did not have a significant influence on artemisinin content, but a further increase in temperature up to 70 °C showed linear growth of artemisinin content with a temperature rising (Figure 1). With the further solvent/solid ratio increase (from 10 to 30 mL/g) and prolonged time of extraction (15–45 min), there was no noticeable increase in artemisinin content.

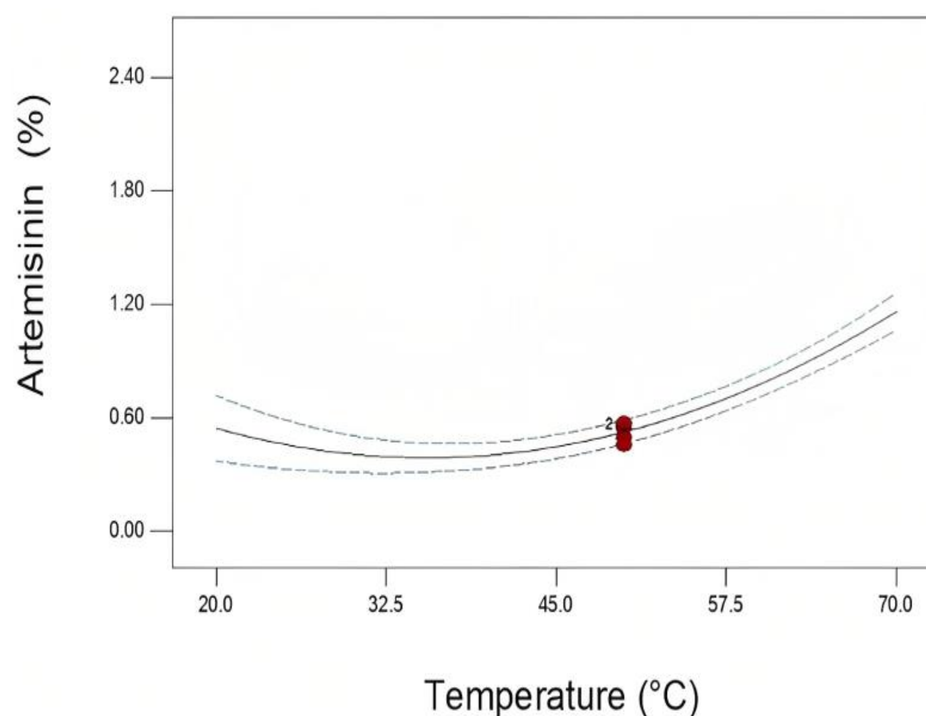

Figure 1. Influence of temperature on artemisinin content during UAE (red dot represents the central point of used parameters—temperature 50 °C).

A similar result was found in the study by Prawang et al. [30], where artemisinin was separated using UAE and polyethyleneglycol (PEG) as the solvent. They obtained high artemisinin content (up to 15.8 mg/g), probably due to the higher solubility of artemisinin in PEG than in the ethanol/water mixture with the influence of extraction condition (temperature, extraction time and solvent/solid ratio were the same as in our study). UAE is evidently very effective in the extraction of artemisinin from sweet wormwood. The result of sonication is the creation of cavitation bubbles, which have the potential to collapse asymmetrically and produce microjets of solvent that are directed to the plant material surface when created in a heterogeneous system [31]. This resulted in increased cell disruption, which is especially advantageous for artemisinin located in trichome glands at the margins of sweet wormwood leaves. Additionally, the solvent penetrates the leaf substance more deeply, improving the mass transfer and leading to an increase in artemisinin yield [32]. However, Lapkin et al. [33] stated that the ultrasound effect equally affects all other metabolites from sweet wormwood extracted into solvent. Therefore, even UAE is proven to be successful in extraction of artemisinin, the purification of these extracts could be challenging.

2.2. Subcritical Water Extraction (SWE)

In the extracts obtained with subcritical water artemisinin was not determined (its content was below the detection limit) (Table 1). Additionally, previous studies failed to extract artemisinin from sweet wormwood using water, proving its low solubility in water [29,34,35]. Even though SWE was not performed on sweet wormwood material, other techniques using water as a solvent were applied. Lapkin [34] concluded that since artemisinin is barely soluble in water, it is not practical to extract artemisinin using a hot-water. However, artemisinin tea (hot-water extraction) has long been used as a conventional folk remedy for malaria [36,37]. More reliable data on artemisinin solubility and its extraction in hot water under precisely controlled circumstances of leaf particle size, temperature, pressure, and extraction time have been previously published [38]. Although at elevated temperatures, the solubility of artemisinin should increase [32] using this extraction technique (SWE) artemisinin content was under the detection limit probably due to specific water properties under subcritical conditions [39] and possible degradation at high temperatures during SWE. Poor water solubility has already persuaded many research in the development of water-soluble artemisinin derivatives such as sodium artesunate. However, the aqueous solutions of artemisinin derivatives are usually unstable. Consequently, numerous kinds of research focus on the synthesis of stable, water-soluble artemisinin derivatives. Since artemether is more stable than artesunate, it was hypothesized that changing the ester linkage in the artesunate molecule to an ether linkage would make the derivative more stable. Deoxoartelinic acid was created and evaluated in vitro and in vivo in an effort to find stable, water-soluble dihydroartemisinin derivatives that are more effective than artesunate and artelinic acid and have a longer plasma half-life. This substance exhibited greater antimalarial action than artemisinin and has greater stability [40,41]. Considerably more work will need to be performed to determine reasons for unsuitability of SWE for artemisinin separation.

2.3. Deep Eutectic Solvent Extraction

Depending on the components used to prepare DESs, they differ in physicochemical properties and the ability to dissolve and extract certain components. Therefore, fourteen different DESs were used to test the efficiency of artemisinin extraction from sweet wormwood, of which only choline chloride: lactic acid (1:2) and choline chloride: levulinic acid (1:2) proved to be effective at temperatures of 30 and 50 °C, respectively. According to the literature, using conventional solvents for the extraction of artemisinin requires a certain degree of purification of the extract, while if DESs are used, the extraction method could be developed as a separation-free process [42]. Therefore, the efficiency of DESs in the extraction of bioactive components, as well as their cytotoxicity, is being increasingly tested, and DESs are being developed. In our case, the best results were achieved with choline chloride-based DESs with acids that show slightly higher cytotoxicity compared to choline chloride DESs with alcohol, sugars and urea. Nevertheless, according to Popović et al. [43], among acidic systems, the choline chloride: lactic acid (1:2) with 20% of water showed the lowest cytotoxicity to all four cell lines. Lactic acid itself represents an essential component of the bioresorbable and biocompatible polymers (polyesters) in order to make nanocarriers more compatible with human cells. However, it is important to note that in high concentrations it can lead to an increase in acidity in the extracellular space and increase the concentration in the cells surrounding the tumor. On the other hand, choline chloride: levulinic acid (1:2) had among the highest toxicity towards Gram-negative (*E. coli* and *S. enteritidis*) and Gram-positive (*S. aureus* and *L. monocytogenes*) bacteria among tested DESs. Nevertheless, because of their low toxicity and good biodegradability compared with traditional solvents, they can be considered green solvents [44]. Additionally, to the best of our knowledge data on the cytotoxicity of choline chloride: levulinic acid (1:2) for comparison with choline chloride: lactic acid (1:2) are not available.

Considering the growing popularity of using DESs in the extraction of bioactive components, new solvents are being developed every day, so comparison with literature results is sometimes demanding. In the available literature, it is evident that both hydrophilic and hydrophobic solvents were used for the extraction of artemisinin. According to Cao et al. [45] a hydrophobic DES named N81Cl-NBA that was tailor-made from methyl trioctyl ammonium chloride and butan-1-ol at a molar ratio of 1:4 showed the highest extraction yield in artemisinin extraction. On the other hand, l-carnitine and isosorbide (CaIs) at a molar ratio of 1:2 was also found to possess the best extraction efficiency [42]. As for the more frequently mentioned and common DESs, choline chloride: glycerol (1:2), but with 50% added ethanol, proved to be successful in the extraction of artemisinin [46]. However, artemisinin extraction with the mentioned solvent and the addition of 20% water was not successful, so there is a possibility that ethanol itself affects the extraction efficiency than this specific mixture due to favorable polarity and viscosity properties.

2.4. Supercritical CO_2 Extraction (SCO_2E)

From the results presented in Table 2, it can be seen that the most abundant volatile compounds in SCO_2 extract were arteannuin b (15.29%), camphor (12.23%), artemisia ketone (10.97%), 1,8-cineole (5.37%) and artemisic acid (4.95%). It is known that artennuin b co-occurs with artemisinin in *A. annua* and artemisinin was found by GC–MS in SC-CO_2 extract with 1.39%. The sesquiterpene lactone artemisinin derives from amorpha-4,11-diene through the pathway artemisinic alcohol → artemisinic aldehyde → dihydroartemisinic aldehyde dihydroartemisinate → dihydroartemisinic acid hydroperoxide → artemisinin), while arteannuin b is derived from artemisinic aldehyde and artemisinate [47,48]. Our research confirmed the previous findings that camphor is among the most abundant compound in *A. annua* extracts [3,4]. Other study conducted by Dobreva et al. [49] found 28 different compounds in the subcritical extract of *A. annua* detected by GC–MS analysis of the supercritical extract obtained with 1,1,1,2-tetrafluoroethane and the major ones were artemisia ketone (26.2%), camphor (10.7%), and eucalyptol (9.1%) followed by artheanin B (3.7%) and arteannuic acid (3.7%). High variability of *A. annua* volatiles obtained by hydrodistillation was reported and the existence of several *A. annua* chemotypes were found [50]: camphor and camphor/1,8-cineole types; artemisia ketone/α-pinene/1,8-cineole and artemisia ketone/camphor/1,8-cineole types; camphor/artemisia ketone/germacrene D type; germacrene D/β-caryophyllene and germacrene D/β-caryophyllene/1,8-cineole/artemisia ketone types. In vivo and/or in vitro toxicity (including hepato- and nephrotoxicity/protection), antinociceptive, antioxidant (DPPH, ABTS and superoxide radical scavenging activity assays), antimicrobial, and enzyme-inhibiting (protein kinase A and α-amylase) potential of *A. annua* oil and its constituents was evaluated [51]. The results revealed that the beneficial properties of *A. annua* are not limited only to their antimalarial properties. According to research conducted by Hu et al. [51], arteanuin b successfully inhibits the activity of the SARS-CoV-2 virus. In conclusion, SCO_2E seems to improve artemisinin content in extracts, but some volatile organic compounds are co-extracted. However, some of those compounds could contribute to artemisinin medicinal properties.

Table 2. Chemical composition (area percentages) of sweet wormwood extract obtained by SCO_2 and analyzed by GC–MS.

No	Compound	RI	%
1.	3-Methylbut-2-enoic acid	<900	0.04
2.	α-Pinene	942	0.76
3.	Camphene	958	0.48
4.	Sabinene	980	0.14
5.	β-Pinene	983	0.30
6.	β-Myrcene	993	0.30

Table 2. *Cont.*

No	Compound	RI	%
7.	2,5,5-Trimethylhepta-3,6-dien-2-ol (Yomogi alcohol)	1000	0.32
8.	*p*-Cymene	1030	0.08
9.	Limonene	1034	0.08
10.	1,8-Cineole	1037	5.37
11.	Artemisia ketone	1066	10.97
12.	*trans*-Sabinene hydrate	1072	0.36
13.	Artemisia alcohol	1086	1.45
14.	*cis*-Sabinene hydrate	1100	0.34
15.	Nonanal	1106	0.10
16.	*trans*-*p*-Mentha-2,8-dienol	1124	0.16
17.	α-Campholenal	1130	0.48
18.	*trans*-Pinocarveol	1143	1.63
19.	Camphor	1148	12.23
20.	Pinocarvone	1166	1.63
21.	Borneol	1169	0.12
22.	Terpinen-4-ol	1180	0.44
23.	α-Terpineol	1192	0.26
24.	Myrtenol	1197	0.72
25.	Verbenone	1210	0.16
26.	*trans*-Carveol	1221	0.14
27.	*trans*-Anethole	1286	0.32
28.	*p*-Cymen-7-ol	1292	0.08
29.	Eugenol	1360	0.28
30.	α-Copaene	1376	1.87
31.	β-Bourbonene	1384	0.08
32.	β-Cubebene	1389	0.28
33.	*trans*-β-Caryophyllene	1417	2.68
34.	α-Humulene	1452	0.24
35.	*trans*-β-Farnesene	1459	1.23
36.	α-Selinene	1475	0.12
37.	Germacrene D	1480	1.09
38.	β-Selinene	1484	4.39
39.	δ-Cadinene	1523	0.08
40.	Dihydroactinidiolide	1526	0.06
41.	Spathulenol	1576	0.32
42.	Caryophyllene oxide	1580	3.86
43.	Methyl jasmonate	1649	0.28
44.	Artemisic acid	1847	4.95
45.	Nonadecane	1900	0.48
46.	Hexadecanoic acid	1967	2.05
47.	Contrunculin-A	1996	4.93
48.	Arteannuin b	2054	15.29
49.	Phytol	2107	2.23
50.	(Z,Z)-Octadeca-9,12-dienoic acid (Linoleic acid)	2128	1.49
51.	Artemisinin	2187	1.39
52.	Tricosane	2300	0.91
53.	Tetracosane	2400	0.93

3. Materials and Methods

3.1. Materials

Plant material (*Artemisia annua* L.) was purchased in dry condition from herbal pharmacy store Vextra d.o.o. (Mostar, Bosnia, and Herzegovina) in January 2021. The plant material was milled to a particle size of less than 3 mm using a laboratory mill (MRC Sample mill C-SM/450-C, Holon, Israel). The grounded material was stored without light exposure on 4 °C.

3.2. Chemicals

Ethanol was obtained from Carlo Erba Reagents (Barcelona, Spain). CO_2 used for the supercritical extraction was obtained from Messer (Osijek, Croatia) with indicated purity of 99.97% (*w*/*w*). The standard compound artemisinin (98.0%) (Sigma Chemical Co., St. Louis, MO, USA) was used for the HPLC analysis. All solvents and chemicals used for DESs synthesis were of analytical grade.

3.3. Supercritical CO_2 Extraction (SCO_2E)

Supercritical CO_2 extraction (SCO_2E) was carried out in a custom-made extraction system that was explained in detail previously [52]. An amount of 100 g of the plant material was placed into the extraction vessel, and separation of the extract was conducted under 15 bar and 25 °C. Extraction was performed at a pressure of 300 bar, a temperature of 40 °C and a CO_2 flow of 1.4 kg/h according to optimal conditions determined by Vidović et al. [15]. Obtained extracts was kept at 4–6 °C until analyses.

3.4. Ultrasound-Assisted Extraction (UAE)

UAE was carried out in an ultrasound bath (Elma, Elmasonic P 70 H, Singen, Germany), at three different temperatures (30, 50 and 70 °C) during the three treatment times (15, 30 and 45 min) and the three solvent/solid ratios (10, 20 and 30 mL/g) with a nominal power of 50 W and a frequency of 37 kHz. After the extraction process, extracts were filtrated through filter paper, evaporated on SpeedVac (SPD1030, Thermo Scientific, Waltham, MA, USA) and stored in +4 °C until analysis.

3.5. Subcritical Water Extraction (SWE)

SWE was performed in a batch-type high-pressure extractor (Parr Instrument Company, Moline, IL, USA). The extraction procedure and apparatus were described previously published paper [39]. An amount of 10 g of plant material and 200 mL of distilled water are mixed and placed into the extractor. Afterwards, the obtained extracts were filtrated through filter paper under vacuum and kept at 4 °C in a dark place until analysis.

3.6. Deep Eutectic Solvent Extraction

The preparation of the choline chloride-based DESs was performed in accordance with our previously published article [53]. In this study, fourteen different choline chloride-based DESs were prepared including choline chloride: lactic acid (1:2), choline chloride: levulinic acid (1:2), choline chloride: urea (1:2), choline chloride: N-methyurea (1:2), choline chloride: thiourea (1:2), choline chloride: glucose (1:2), choline chloride: fructose (1:2), choline chloride: xylitol (1:2), choline chloride: sorbitol (1:2), choline chloride: butane-1,4-diol, choline chloride: ethane-1,4-diol, choline chloride: glycerol (1:2), choline chloride: acetamide (1:2), and choline chloride: malic acid (1:2).

Ground and dried sweet wormwood (50 mg) was mixed with 1 mL of the solvent mixture of DESs with ultrapure H_2O (Millipore Simplicity 185, Darmstadt, Germany) in an 80:20 ratio (*v*/*v*). Extraction by mixing and heating was performed with all 14 prepared mixtures of DESs. The prepared samples were mixed at 1500 rpm in an aluminum block (Stuart SHB) on a magnetic stirrer for 60 min and at different temperatures (30, 50 and 70 °C).

3.7. Gas Chromatography Coupled to Mass Spectrometry (GC–MS)

The GC–MS analysis was performed using an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph system, model 7890A equipped with a mass selective detector (MSD) model 5977E (Agilent Technologies) and HP-5MS capillary column. The GC–MS procedure was used according to the method described by Jerković et al. [54]. The SCO_2 extract was dissolved in hexane (0.002 g/mL) prior to the analysis. The compounds percentage composition was calculated by comparing the average peak area to the total areas using the normalization method (without the correction factors). The component

percentages were calculated as the mean values from duplicate GC–MS analyses of all extracts. Interpretation on the mass spectrum was conducted using the Wiley 09 MS library (Wiley, New York, NY, USA) and NIST14 (Gaithersburg, MD, USA) mass spectral database and by the comparison of GC retention data relative to C_9-C_{25}*n*-alkanes for HP-5MS column.

3.8. High-Performance Liquid Chromatography (HPLC)

The HPLC system Agilent 1260 Infinity II (Agilent Technologies, Santa Clara, CA, USA) consisted of a quaternary pump (Paris, France) coupled to a variable PDA absorbance detector operated at 216 and 254 nm. A Cosmosil 5C18–MS11, 5 μm, 250 × 4.6 mm was used at 25 °C. The solvent system consisted of water (A) and acetonitrile (B) at a ratio of 15:85%. The system remained isocratic within 10 min. The flow rate was maintained at 1 mL/min while the injection volume was 20 μL. The chromatographic data were processed on ChemStation software (Agilent Technologies). The content of artemisinin in the extracts was determined by external calibration and was calculated as mean values from triplicate analyzes of all extracts. A standard stock solution for artemisinin was prepared in methanol and calibration was obtained at six concentrations in the range of 9.8–147 mg/L. The linearity of the calibration curve was confirmed by R^2 = 0.99993

4. Conclusions

Results from the present study demonstrate that *A. annua* is an important source of artemisinin with large variations between different green extraction techniques used for its separation. SCO_2 seemed to be most effective in terms of artemisinin yield. However, SCO_2 as a non-polar solvent co-extracted the volatile organic compounds including camphor, arteannuin b and artemisia ketone. Combining all obtained results, SCO_2 extraction and UAE could have a slight advantage over DES and could be successfully used for the recovery of artemisinin in less time and consuming less solvent as well as operating at lower temperatures. However, some limitations have to be mentioned, including cost of supercritical CO_2 equipment and possible co-extracted contaminants including pigments. Future research on artemisinin extraction might extend the explanations of the interaction of some extraction parameters on extraction yield. Additionally, this research provides a good starting point for discussion and further research on the purification procedures of sweet wormwood extract.

Author Contributions: Conceptualization, S.J. and M.B.; methodology, M.B., M.J.K. and I.J.; formal analysis, A.W.W., K.A., I.J. and M.B; investigation, M.B., A.W.W., K.A., I.J. and S.J; writing—original draft preparation, M.B.; writing—review and editing, M.B., S.J. and I.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to thank the Croatian Government and the European Union (European Regional Development Fund the Competitiveness and Cohesion Operational Programme—KK.01.1.1.02.0010) for support this research through project TEHNOLOŠKO-INOVACIJSKI CENTAR VIROVITICA (KK.01.1.1.02.0010), granted to the Institution for Research and Knowledge Development of Nutrition and Health TEHNOLOŠKO-INOVACIJSKI CENTAR VIROVITICA.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. YouYouc, T. *From* Artemisia Annua *L. to Artemisinins. The Discovery and Development of Artemisinins and Antimalarial Agents*, 1st ed.; Academic Press: Cambridge, MA, USA, 2017; pp. 140, 242, 396–402.
2. El-Askary, H.I.; Mohamed, S.S.; El-Gohari, H.M.A.; Ezzat, S.M.; Meselhy, M.R. Quinic acid derivatives from *Artemisia annua* L. leaves; biological activities and seasonal variation. *S. Afr. J. Bot.* **2020**, *128*, 200–208. [CrossRef]
3. Hong, M.; Kim, M.; Jang, H.; Bo, S.; Deepa, P.; Sowndhararajan, K.; Kim, S. multivariate analysis of essential oil composition of *Artemisia annua* L. collected from different locations in Korea. *Molecules* **2023**, *28*, 1131. [CrossRef]
4. Malhotra, A.; Rawat, A.; Prakash, O.; Kumar, R.; Srivastava, R.M.; Kumar, S. Chemical composition and pesticide activity of essential oils from *Artemisia annua* L. harvested in the rainy and winter seasons. *Biochem. Syst. Ecol.* **2023**, *107*, 104601. [CrossRef]
5. WHO. *Model Monograph on Good Agricultural and Collection Practice (GACP) of* Artemisia annua *L.*; WHO: Geneva, Switzerland, 2006; p. 1.
6. D'Alessandro, S.; Scaccabarozzi, D.; Signorini, L.; Perego, F.; Ilboudo, D.P.; Pasquale, F.; Delbue, S. The Use of Antimalarial Drugs against Viral Infection. *Microorganisms* **2020**, *8*, 85. [CrossRef] [PubMed]
7. Aftab, T.; Ferreira, J.F.; Khan, M.M.A.; Naeem, M. *Artemisia Annua-Pharmacology and Biotechnology*; Springer: Berlin/Heidelberg, Germany, 2014; pp. 10, 12, 28, 29, 125, 132.
8. Soktoeva, T.E.; Ryzhova, G.L.; Dychko, K.A.; Khasanov, V.V.; Zhigzhitzhapova, S.V.; Radnaeva, L.D. Artemisinin content in *Artemisia annua* L. extracts obtained by different methods. *Russ. J. Bioorganic Chem.* **2013**, *39*, 761–764. [CrossRef]
9. Khangholil, S.; Rezaeinodehi, A. Effect of drying temperature on essential oil content and composition of sweet wormwood (*Artemisia annua*) growing wild in Iran. *Pak. J. Biol. Sci.* **2008**, *11*, 934–937. [CrossRef]
10. Rana, V.S.; Abirami, K.; Blázquez, M.A.; Maiti, S. Essential oil composition of *Artemisia annua* L. at different growth stages. *J. Spices Aromat. Crops* **2013**, *22*, 181–187.
11. Cutler, M.; Lapkin, A.; Plucinski, P.K. Comparative assessment of technologies for extraction of artemisinin. In *Summary Report Commissioned through the Malalaria Medicines Venture, Geneva, August*; 2006. Available online: https://www.mmv.org/sites/default/files/uploads/docs/artemisinin/2007_event/10_Artemisia_Extraction_Study_Lapkin-Cutler.pdf (accessed on 24 May 2023).
12. Baldino, L.; Reverchon, E. *Artemisia annua* organic solvent extract, processed by supercritical CO_2. *J. Chem. Technol. Biotechnol.* **2018**, *93*, 3171–3175. [CrossRef]
13. Lapkin, A.A.; Plucinski, P.K.; Cutler, M. Comparative assessment of technologies for extraction of artemisinin. *J. Nat. Prod.* **2006**, *69*, 1653–1664. [CrossRef]
14. Hao, J.Y.; Han, W.; Xue, B.Y.; Deng, X. Microwave-assisted extraction of artemisinin from *Artemisia annua* L. *Sep. Purif. Technol.* **2002**, *28*, 191–196. [CrossRef]
15. Vidović, S.; Simić, S.; Gavarić, A.; Aćimović, M.; Vladic, J. Extraction of sweet wormwood (*Artemisia annua* L.) by supercritical carbon dioxide. *Lek. Sirovine* **2020**, *40*, 22–26. [CrossRef]
16. Abbott, A.P.; Capper, G.; Davies, D.L.; Rasheed, R.K.; Tambyrajah, V. Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* **2003**, *9*, 70–71. [CrossRef] [PubMed]
17. Fan, C.; Shan, Y.; Wen, L.; Cao, X. Extraction of artemisinin using natural deep eutectic solvent selected by COSMO-RS. *Sustain. Chem. Pharm.* **2023**, *33*, 101096. [CrossRef]
18. Chen, J.; Ni, Y.; Zhang, P.; Liang, X.; Fang, S. Acidic natural deep eutectic solvents as dual solvents and catalysts for the solubilization and deglycosylation of soybean isoflavone extracts: Genistin as a model compound. *Food Chem.* **2023**, *406*, 134999. [CrossRef]
19. Gundupalli, M.P.; Cheenkachorn, K.; Chuetor, S.; Kirdponpattara, S.; Gundupalli, S.P.; Show, P.L.; Sriariyanun, M. Assessment of pure, mixed and diluted deep eutectic solvents on Napier grass (*Cenchrus purpureus*): Compositional and characterization studies of cellulose, hemicellulose and lignin. *Carbohydr. Polym.* **2023**, *306*, 120599. [CrossRef]
20. Jokić, S.; Gagić, T.; Knez, Ž.; Šubarić, D.; Škerget, M. Separation of Active Compounds from Food by-Product (Cocoa Shell) Using Subcritical Water Extraction. *Molecules* **2018**, *23*, 1408. [CrossRef]
21. Akiya, N.; Savage, P.E. Roles of Water for Chemical Reactions in High-Temperature Water. *Chem. Rev.* **2002**, *102*, 2725–2750. [CrossRef]
22. Kohler, M.; Haerdi, W.; Christen, P.; Veuthey, J.L. Supercritical fluid extraction and chromatography of artemisinin and artemisinic acid. An improved method for the analysis of *Artemisia annua* samples. *Phytochem. Anal.* **1997**, *8*, 223–227. [CrossRef]
23. Mannan, A.; Ahmed, I.; Arshad, W. Survey of artemisinin production by diverse Artemisia species in northern Pakistan. *Malar J.* **2010**, *9*, 310–319. [CrossRef]
24. Peng, C.A.; Ferreira, J.F.S.; Wood, A.J. Direct analysis of artemisinin from *Artemisia annua* L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector. *J. Chromatogr. A* **2006**, *1133*, 254–258. [CrossRef]
25. ElSohly, H.N.; Croom, E.M.; El-Feraly, F.S.; El-Sherei, M.M. A Large-scale extraction technique of artemisinin from *Artemisia Annu*. *J. Nat. Prod.* **1990**, *53*, 1560–1564. [CrossRef]
26. Qian, G.; Yang, Y.; Ren, Q. Determination of artemisinin in *Artemisia annua* L. by reversed phase HPLC. *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28*, 705–712. [CrossRef]

27. Woerdenbag, H.J.; Pras, N.; Chan, N.G. Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. *Planta Med.* **1994**, *60*, 272–275. [CrossRef] [PubMed]
28. Zarrelli, A.; Pollio, A.; Aceto, S.; Romanucci, V.; Carella, F.; Stefani, P.; De Vico, G. Optimisation of artemisinin and scopoletin extraction from *Artemisia annua* with a new modern pressurised cyclic solid–liquid (PCSL) extraction technique. *Phytochem. Anal.* **2019**, *30*, 564–571. [CrossRef]
29. Dogan, K.; Erol, E.; Didem Orhan, M.; Degirmenci, Z.; Kan, T.; Gungor, A.; Guzel, M. Instant determination of the artemisinin from various *Artemisia annua* L. extracts by LC-ESI-MS/MS and their in-silico modelling and in vitro antiviral activity studies against SARS-CoV-2. *Phytochem. Anal.* **2022**, *33*, 303–319. [CrossRef] [PubMed]
30. Prawang, P.; Zhang, Y.; Zhang, Y.; Wang, H. Ultrasonic assisted extraction of artemisinin from *Artemisia annua* L. using poly (ethylene glycol): Toward a greener process. *Ind. Eng. Chem. Res.* **2019**, *58*, 18320–18328. [CrossRef]
31. Banožić, M.; Banjari, I.; Jakovljević, M.; Šubarić, D.; Tomas, S.; Babić, J.; Jokić, S. Optimization of ultrasound-assisted extraction of some bioactive compounds from tobacco waste. *Molecules* **2019**, *24*, 1611. [CrossRef]
32. Briars, R.; Paniwnyk, L. Examining the extraction of artemisinin from *Artemisia annua* using ultrasound. In Proceedings of the AIP Conference Proceedings, Ft. Worth, TX, USA, 5–10 August 2012; American Institute of Physics: College Park, MD, USA, 2012; Volume 1433, pp. 581–585.
33. Lapkin, A.A. Green Extraction of Artemisinin from *Artemisia annua* L. In *Green Extraction of Natural Products Theory and Practice*, 1st ed.; Chemat, F., Strube, J., Eds.; Wiley-VCH: Weinheim, Germany, 2015; pp. 333–356.
34. Lapkin, A.A.; Walker, A.; Sullivan, N.; Khambay, B.; Mlambo, B.; Chemat, S. Development of HPLC analytical protocols for quantification of artemisinin in biomass and extracts. *J. Pharm. Biomed. Anal.* **2009**, *49*, 908–915. [CrossRef]
35. Martinez-Correa, H.A.; Bitencourt, R.G.; Kayano, A.C.A.V.; Magalhães, P.M.; Costa, F.T.M.; Cabral, F.A. Integrated extraction process to obtain bioactive extracts of *Artemisia annua* L. leaves using supercritical CO_2, ethanol and water. *Ind. Crops Prod.* **2017**, *95*, 535–542. [CrossRef]
36. Wright, C.W.; Linley, P.A.; Burn, R.; Wittlin, S.; Hsu, E. Ancient Chinese methods are remarkably effective for the preparation of artemisinin rich extracts of Qing Hao with potent antimalarial activity. *Molecules* **2010**, *15*, 804–812. [CrossRef]
37. Hsu, The history of qinghao in the Chinese materiamedica. *Trans. R. Soc. Trop. Med. Hyg.* **2006**, *100*, 505–508. [CrossRef]
38. Van der Kooy, F.; Verpoorte, R. The content of artemisinin in the *Artemisia annua* tea infusion. *Planta Med.* **2011**, *77*, 1754–1756. [CrossRef] [PubMed]
39. Jokić, S.; Aladić, K.; Šubarić, D. Subcritical water extraction laboratory plant design and application. *Annu. Croat. Acad. Eng.* **2017**, *1*, 247–258.
40. Sales, I.; Abranches, D.O.; Costa, P.; Sintra, T.E.; Ventura, S.P.; Mattedi, S.; Pinho, S.P. Enhancing artemisinin solubility in aqueous solutions: Searching for hydrotropes based on ionic liquids. *Fluid Phase Equilibria* **2021**, *534*, 112961. [CrossRef]
41. Ahirrao, P.; Batra, D.; Jain, U.K. Artemisinin, a potential antimalarial drug: Current status. *J. Chem. Pharm. Res.* **2016**, *8*, 624–636.
42. Pan, Z.; Bo, Y.; Liang, Y.; Lu, B.; Zhan, J.; Zhang, J.; Zhang, J. Intermolecular interactions in natural deep eutectic solvents and their effects on the ultrasound-assisted extraction of artemisinin from Artemisia annua. *J. Mol. Liq.* **2021**, *326*, 115283. [CrossRef]
43. Popović, B.M.; Gligorijević, N.; Aranđelović, S.; Macedo, A.C.; Jurić, T.; Uka, D.; Mocko-Blažek, K.; Serra, A.T. Cytotoxicity profiling of choline chloride-based natural deep eutectic solvents. *RSC Adv.* **2023**, *25*, 3520–3527. [CrossRef]
44. Zhao, B.-Y.; Xu, P.; Yang, F.-X.; Wu, H.; Zong, M.-H.; Lou, W.-Y. Biocompatible deep eutectic solvents based on choline chloride: Characterization and application to the extraction of rutin from sophora japonica. *ACS Sustain. Chem. Eng.* **2015**, *3*, 2746–2755. [CrossRef]
45. Cao, J.; Yang, M.; Cao, F.; Wang, J.; Su, E. Well-designed hydrophobic deep eutectic solvents as green and efficient media for the extraction of artemisinin from *Artemisia annua* leaves. *ACS Sustain. Chem. Eng.* **2017**, *5*, 3270–3278. [CrossRef]
46. Wang, J.; Zhou, Y.; Wang, M.; Bi, W.; Li, H.; Chen, D.D.Y. High-throughput analysis for artemisinins with deep eutectic solvents mechanochemical extraction and direct analysis in real time mass spectrometry. *Anal Chem.* **2018**, *90*, 3109–3117. [CrossRef]
47. Bertea, C.M.; Freije, J.R.; van der Woude, H.; Verstappen, F.W.; Perk, L.; Marquez, V.; De Kraker, J.W.; Posthumus, M.A.; Jansen, B.J.; de Groot, A.; et al. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Med.* **2005**, *71*, 40–47. [CrossRef]
48. Bouwmeester, H.J.; Wallaart, T.E.; Janssen, M.H.; van Loo, B.; Jansen, B.J.; Posthumus, M.A.; Schmidt, C.O.; De Kraker, J.W.; Konig, W.A.; Franssen, M.C. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochemistry* **1999**, *52*, 843–854. [CrossRef]
49. Dobreva, A.M.; Dincheva, I.N.; Nenov, N.S.; Trendafilova, A.B. Chemical characterization of *Artemisia annua* L. subcritical extract. *Bulg. Chem. Commun.* **2022**, *54*, 43–48.
50. Radulović, N.S.; Randjelović, P.J.; Stojanović, N.M.; Blagojević, P.D.; Stojanović-Radić, Z.Z.; Ilić, I.R.; Djordjević, V.B. Toxic essential oils. Part II: Chemical, toxicological, pharmacological and microbiological profiles of *Artemisia annua* L. volatiles. *Food Chem. Toxicol.* **2013**, *58*, 37–49. [CrossRef] [PubMed]
51. Hu, Y.; Liu, M.; Qin, H.; Lin, H.; An, X.; Shi, Z.; Song, L.; Yang, X.; Fan, H.; Tong, Y. Artemether, Artesunate, Arteannuin B, Echinatin, Licochalcone B and Andrographolide effectively inhibit SARS-CoV-2 and related viruses in vitro. *Front. Cell Infect. Microbiol.* **2021**, *11*, 680127. [CrossRef]
52. Jokić, S.; Svilović, S.; Vidović, S. Modelling the supercritical CO_2 extraction kinetics of soybean oil. *Croat. J. Food Sci. Technol.* **2015**, *7*, 52–57. [CrossRef]

53. Molnar, M.; JakovljeviĆ, M.; Jokić, S. Optimization of the process conditions for the extraction of rutin from ruta graveolens l. by choline chloride based deep eutectic solvents. *Solvent Extr. Res. Dev. Jpn.* **2018**, *25*, 109–116. [CrossRef]
54. Jerković, I.; Marijanović, Z.; Roje, M.; Kuś, P.M.; Jokić, S.; Čož-Rakovac, R. Phytochemical study of the headspace volatile organic compounds of fresh algae and seagrass from the Adriatic Sea (single point collection). *PLoS ONE* **2018**, *13*, e0196462. [CrossRef]

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