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Hydroxypropyl-β-Cyclodextrin-Glycerol-Assisted Extraction of Phenolics from *Satureja montana* L.: Optimization, Anti-Elastase and Anti-Hyaluronidase Properties of the Extracts

Marijan Marijan, Lejsa Jakupović and Marijana Zovko Končić *

Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia
* Correspondence: mzovko@pharma.hr

Abstract: A green method for hydroxypropyl-β-cyclodextrin-glycerol-assisted extraction (HCGAE) of bioactive phenolics from *S. montana*, Lamiaceae was optimized using Box-Behnken design and response surface methodology and compared conventional water/ethanol-based extraction. The procedure was aimed at obtaining extracts with the maximum content of total phenols (TP), flavonoids (TF), dihydroxycinnamic acids (TDCA), rosmarinic acid (RA), and luteolin 7-*O*-glucoside (LG). The impact of glycerol content (0–70%), 2-hydroxypropyl-β-cyclodextrin content (0–0.4 mmol), temperature (20–70 °C), herbal material weight (0.3–0.8 g), extraction duration (5–25 min), and ultrasound power (144–720 W) on the extraction efficiency was analyzed. Four extracts with maximum amounts of target phenols, OPT-TP (5.93 mg/mL), OPT-TDCA-RA (4.17 mg/mL and 1.16 mg/mL, respectively), OPT-TF (0.99 mg/mL), and OPT-LG (0.28 mg/mL) were prepared. Comparison of the content of TDCA, TF, RA, and LG with those obtained in water/ethanol-based extraction demonstrated the superiority of the HCGAE approach for the extraction of phenols from *S. montana*. The extracts displayed good anti-elastase and excellent anti-hyaluronidase activity. IC_{50} values of the anti-hyaluronidase activity (1.67 ± 0.06 µL extract/mL, 1.16 ± 0.08 µL extract/mL, 0.85 ± 0.03 µL extract/mL, and 0.79 ± 0.05 µL extract/mL for OPT-TP, OPT-TDCA-RA, OPT-TF, and OPT-LG, respectively) surpassed that of the applied positive control, tannic acid. The observed bioactivity of the optimized extracts makes them promising active ingredients for natural cosmetics. The results of this research indicate that HCGAE is an excellent alternative to conventional water/ethanol-based extraction of phenolics from *Satureja montana* L.-yielding extracts with potent anti-elastase and anti-hyaluronidase properties suitable for direct use in cosmetic products.

Keywords: cosmeceutical extracts; cyclodextrin-glycerol-assisted extraction; luteolin-7-*O*-glucoside; rosmarinic acid; *Satureja montana*; ultrasound-assisted extraction

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1. Introduction

Cosmetic products containing only natural herbal ingredients are progressively popular in the cosmetic industry, and the research on dermatologically active natural agents is growing at a similar rate. Consumers perceive them as active and non-toxic substances that promote skin health and delay or prevent skin-aging processes [1]. Numerous studies confirm that natural products and plant extracts may indeed prevent or slow down processes that harmfully affect skin health and appearance. Some of the activities include the ability to inhibit tyrosinase, hyaluronidase, elastase, collagenase, and other enzymes that act detrimentally on skin appearance. Additionally, natural products may protect skin cells from free radicals and dehydration and thus prevent the appearance of dry skin or wrinkles [2]. Among numerous plant metabolites, plant phenolics, including flavonoids and dihydrocinnamic acids, are the most valued in the cosmetic industry [3] as their antioxidant activity and various biological effects make them excellent preservatives and active ingredients in cosmetics [4]. Furthermore, their activity may be displayed in the skin,

where they protect macromolecules from exo/endogenous free radicals and harmful solar radiation [5] or inhibit the enzymes that produce wrinkles formation and premature skin aging [6].

Before being incorporated into cosmetic formulations, plant phenolics and other bioactive plant metabolites should be extracted or isolated from plant material. As contemporary consumers become increasingly aware of ecological issues, the search for green and sustainable extraction solvents and methods for bioactive natural products extraction and isolation from plant material constantly rises. Such solvents should be biodegradable and easy to handle without compromising their dissolving power. Furthermore, their toxicity should be negligible both to humans and to the environment [7,8]. Glycerol (GL) is a cheap, safe, environmentally friendly liquid of natural origin that fits this description perfectly. Regarding environmental protection, GL has the special advantage of being a by-product of biodiesel production, meaning it is produced from renewable sources [9]. In addition, GL is broadly used as an ingredient in cosmetics, especially in liquid and semi-liquid products, such as lotions and creams, where it regulates viscosity and plays an important role as a humectant and moisturizing ingredient [7].

Other liquids that may be considered "green" solvents are aqueous solutions of cyclodextrins (CDs) and cyclic, water-soluble oligosaccharides. They are built from 6–8 D-glucopyranoside subunits (α-CD, β-CD, and γ-CD) that form a ring, where individual sugars are connected by α-1,4-glycosidic bonds. The high dissolving power of CDs in aqueous solutions is due to the difference in polarity of their interior and exterior parts. Glucose hydroxyl groups are located on the outside, providing CDs with a hydrophilic exterior. On the other hand, the interior cavity of CDs is more hydrophobic due to the glycoside bond placement. Such structure allows for the formation of inclusion complexes with different small molecules and, as a result, improves their aqueous solubility and stability [10]. As CDs can encapsulate plant phenolics, such as catechin, morin, epicatechin, and quercetin [11], CD-assisted extraction of plant phenols was suggested as an eco-friendly and cheap alternative to conventional extraction with organic solvents [12]. Numerous studies have demonstrated that aqueous CD solutions have a high extraction efficiency. Furthermore, they may also shorten the extraction time and help retain the activity of the extracted compounds (e.g., antioxidant activity) [13]. Finally, they are safe for human consumption [10].

Attachment of polar side chains, for instance, 2-hydroxypropyl group, can even further improve the water solubility of CD molecules, as well as the stability of CD complexes with small molecules. For example, the stability of the rutin complex with 2-hydroxypropyl-β-CD (HP-β-CD) is greater than the stability of its complex with unmodified β-CD [14]. Furthermore, the complexation of bioactive molecules with HP-β-CD improves their stability without compromising their activity. For example, one study has shown that resveratrol retained its influence on the activity of Na^+, K^+-ATPase of streptozotocin-induced diabetic rats even after the formation of resveratrol-HP-β-CD complexes [15]. An additional advantage of CD applications in dermatological and cosmetic formulations is that CDs may improve the bioavailability [14] and migration [16] of bioactive ingredients through the epidermis. Studies on cells and animals demonstrated that HP-β-CD complexation enhances the penetration capability of flavonoids from milk thistle extracts and increases their stability during in vitro tests [17]. It is important to note that, due to their biocompatibility, both HP-β-CD and GL may be incorporated into the final products. Thus, HP-β-CD-GL-assisted extraction (HCGAE) of secondary metabolites from plant material may be considered a "green" method of extraction as no energy is dispensed for solvent removal [13]. Despite their favorable safety profile and good extraction efficiency, the combination of HP-β-CD with GL as the co-solvent has rarely been used to extract natural compounds. Among the few examples is the extraction of polyphenols from oak acorn husks [18] and olive leaves [19].

Satureja montana L. (winter savory) is a plant from the Lamiaceae family, widely distributed in the Mediterranean region. It displays numerous activities potentially beneficial

for human health. *S. montana* and its essential oil show excellent activity on various microorganisms, including dermatophytes like *Trichophyton violaceum*, *Trichophyton rubrum*, *Trichophyton tonsurans*, and *Trichophyton mentagrophytes*. Furthermore, the aqueous extract of *S. montana* exerted strong anti-human immunodeficiency virus-1 (HIV-1) activity and inhibited its reverse transcriptase. *S. montana* extracts display strong radical-scavenging properties against the 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Ethanolic extracts of *S. montana* exert diuretic activity and inhibit angiotensin-I-converting enzyme (ACE). The fertility effects of *S. montana* were investigated in male rats. It was found that potency, fertility index, litter size, testosterone level, and the number of spermatozoids in rats treated with *S. montana* were significantly improved in comparison to the control [20]. *S. montana* also shows insecticidal and insect-repellent activity [21]

S. montana is rich in various phenolic components, including phenolic acids (such as rosmarinic acid (RA) and other caffeic acid derivatives [21], as well as in derivatives of different flavonoids such as derivatives of quercetin, catechin [21] or luteolin (e.g., luteolin-7-*O*-glucoside (LG)) [22]. Additionally, *S. montana* contains triterpenic acids, such as ursolic or oleanolic acid [21]. Among numerous phytochemicals, RA and LG are among the most interesting ones, not only due to their reported abundance in *S. montana* [22] but also because they display numerous skin-related activities. For example, LG promotes wound healing and exerts anti-inflammatory, antioxidant, anti-hyaluronidase, and anti-collagenase activity [23]. In addition, LG-rich *Jasione montana* extracts displayed antioxidant and anti-inflammatory activity, promoted cell viability, and accelerated the migration of fibroblasts in an in vitro study [24]. On the other hand, RA is one of the most researched phytochemicals for skin care. It acts beneficially on many skin conditions, including skin aging [2]. It also works as an anti-microbial, immunomodulatory, anti-allergic, and anti-inflammatory agent [25].

S. montana is a plant rich in phenolic metabolites with the potential for use in cosmetics. However, a "green" method with skin-friendly solvents for their extraction has not yet been developed. HCGAE is an eco-friendly but still under-utilized method for the extraction of secondary metabolites from natural sources. Thus, the aim of this work was to develop an HCGAE method for the maximal yield of selected phenolics from flowering above-ground parts of *S. montana* and to compare it with conventional water/ethanol-based extraction. To assess the suitability of the optimized extracts for use in cosmetic products, their anti-elastase and anti-hyaluronidase activity was tested.

2. Materials and Methods

2.1. Chemicals and Apparatus

A Bandelin ultrasonic bath (SONOREX® Digital 10 P DK 156 BP, Berlin, Germany) was used for ultrasound-assisted extraction (UAE), while a FLUOstar Omega reader (BMG Labtech, Ortenberg, Germany) was used for spectrophotometric determinations. The standards used for the HPLC analysis (RA and LG) and HP-β-CD were purchased from Sigma-Aldrich (St. Louis, MO, USA). Their purity was ≥98%. Other reagents and chemicals were of analytical grade.

2.2. Plant Material

Flowering above-ground parts of *S. montana* were collected in October 2022 in the surroundings of the village Ravno (Bosnia and Herzegovina, 42°53′13″ N, 17°58′15″ E). Plant material was authenticated by Professor Antun Alegro, Ph.D. (Faculty of Sciences, University of Zagreb). A voucher specimen (SM-2022-10-1) was stored in the plant collection of the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb. Before the extraction, plant material was dried, reduced to a powder, and passed through a sieve of 850 μm mesh size.

2.3. Extraction Design and Optimization

The independent variables (factors) and their levels in Box-Behnken design (BBD) were as follows (code, −1, +1): GL content (X_1, 0% *w:w*, 70% *w:w*), HP-β-CD amount (X_2, 0 mmol, 0.4 mmol), temperature (X_3, 20 °C, 70 °C), herbal material weight (HMW) (X_4, 0.3 g, 0.8 g), extraction time (X_5, 5 min, 25 min), and ultrasound power (USP) (X_6, 144 W, 720 W). The content of total phenols (TP), dihydroxycinnamic acids (TDCA), and flavonoids (TF), as well as the content of RA and LG, were the dependent variables (responses) selected for optimization. For the CDGAE procedure, BBD protocol was followed. Plant material in the appropriate amount was placed in an Erlenmeyer flask and mixed with the appropriate amount of HP-β-CD and 10 g of water or water/GL mixture. After quick stirring, the flask was placed in an ultrasonication bath and extracted at the appropriate temperature, ultrasonication power, and for the appropriate time. The content of the selected responses in the extracts prepared according to the BBD was analyzed. Response-surface methodology (RSM) was used to calculate the conditions needed for the preparation of the extracts with the maximum amounts of the target phenolics (optimized extracts).

2.4. Comparison of Cyclodextrin-Assisted Extraction with Conventional Solvents Extraction

In order to evaluate the overall efficiency of the CDAE, additional extracts were prepared using water, ethanol, or a mixture thereof as conventional solvents. Extractions were prepared using the protocols for the optimized extracts but without the addition of GL or HP-β-CD. Contents of the target compounds were determined and compared to those determined in the optimal extracts. Five conventional solvents were used: water, 25% (*w:w*) ethanol, 50% (*w:w*) ethanol, 75% (*w:w*) ethanol, and absolute ethanol.

2.5. Spectrophotometric Determination of Total Phenolic Content

TP was determined using the Folin-Ciocalteu method modified for use in microplate readers [26]. The extract solution (80 µL) was mixed with 80 µL of Folin–Ciocalteu reagent and 80 µL of 10% (*w:w*) Na_2CO_3 solution. The absorbance at 700 nm was recorded after 1 h. TP concentration was determined from the calibration curve of caffeic acid (CA) and expressed as mg CA equivalents (CAE) per mL of the extract (mg CAE/mL extract).

2.6. Spectrophotometric Determination of Total Dihydroxycinnamic Acids Content

TDCA was determined by applying the method described by Nicolle et al. [27], modified for microplate reader. The nitrite-molybdate reagent was previously prepared using 10 g of sodium nitrite and 10 g of sodium molybdate made up to 100 mL with distilled water. The reagent (50 µL) was mixed with HCl (0.5 M, 50 µL). Following that, the extract solution (100 µL) and NaOH (50 µL, 8.5% *w:w*) were added. TDCA was calculated from the calibration curve of caffeic acid (CA) and expressed as mg of CA equivalents (CAE) per mL of the extract (mg CAE/mL extract).

2.7. Spectrophotometric Determination of Total Flavonoid Content

TF was determined using a modified colorimetric method by mixing 120 µL of the extract solution with an equal amount of 0.2% $AlCl_3$ methanolic solution [28]. After incubation at room temperature for 1 h, the absorbance was recorded at 420 nm. TF was calculated from the calibration curve of quercetin (QU) and expressed as mg of QU equivalents (QUE) per mL of the extract (mg QUE/mL extract).

2.8. HPLC Analysis

HPLC-DAD analysis of selected bioactive compounds (RA and LG) was performed using Agilent 1220 series instrument with an autosampler (Agilent Technologies, Santa Clara, CA, USA) [29]. Zorbax Eclipse XDB-C18 column (12.5 mm × 4.6 mm, 5 µm) and and corresponding guard column were used for the separation (Agilent Technologies, Santa Clara, CA, USA). The extracts and the standards' solutions (0.2 mg/mL in methanol) were filtered through a PTFE filter (CHROMAFIL Xtra PTFE, Macherey-Nagel, Düren, Germany)

with pores size 0.45 µm and subjected to chromatographic separation at a temperature of 40 °C and flow of 1.2 mL/min. The solvents A and B were 2% formic acid (*v:v*) and acetonitrile applied as follows: 0–23 min, 0–20% B; 23–27 min, 20–22.5% B; 27–28 min, 22,5–24.4% B; 28–30 min, 24.4–26.2% B; 30–50 min, 26.2–30% B; 50–60 min, 30–100% B; 60–61 min, 100–0% B and 61–66 min, 0% B. The detection was performed at 365 nm (LG) and 320 nm (RA). For the calibration curve construction, varying volumes of standard solutions were injected using an autosampler. For the peak identification, the retention times and UV spectra were compared with those of the standards. Retention times and calibration curves are reported in Table 1.

Table 1. Chromatographic parameters for HPLC method for determination of phenolic compounds in *Satureja montana* extracts.

Compound	Average RT (min)	CV RT (%)	Calibration Curve	R^2	LOD µg/mL	LOQ µg/mL
Luteolin-7-*O*-glucoside	24.4.	0.78	y = 1333.62x + 9.73	0.9999	0.008	0.028
Rosmarinic acid	28.5	0.83	y = 1945.38x + 16.34	0.9999	0.007	0.025

RT = retention time; CV RT = coefficient of variation of the RT (the ratio of the standard deviation of RT to the mean of RT); R2 = coefficient of determination; LOD = level of detection; LOQ = Level of quantification; and y = area under curve (mAU × s), x = amount of the standard (µg).

Figure 1 presents an example of a chromatogram of a prepared extract.

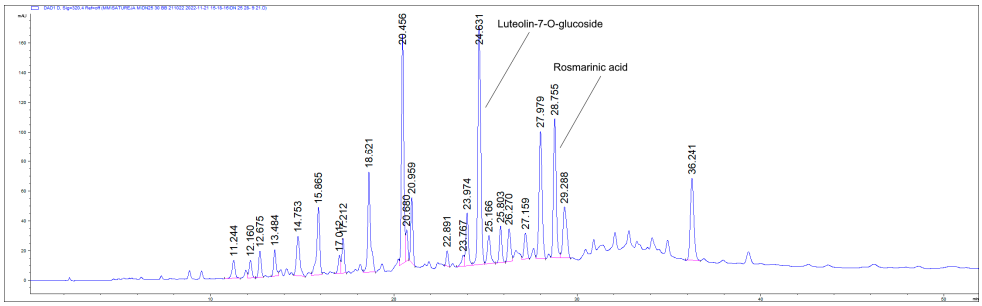


Figure 1. Chromatogram of an extract (Run 21) recorded at 320 nm.

2.9. Elastase Inhibitory Activity

For anti-elastase activity determination [30], the extract diluted in 0.1 M, pH 8.0 Tris-HCl buffer (100 µL), and 0.05 mg/mL elastase solution (25 µL) were mixed. After 5 min at room temperature, a phosphate buffer saline solution of *N*-succinyl-$(Ala)_3$-nitroanilide (70 µL of 0.41 mg/mL solution) was added. After 40 min, the absorbance was recorded at 410 nm. Elastase inhibitory activity (ELAInh) was calculated as described in Equation (1):

$$\text{ElaInh}(\%) = \frac{A_{nc} - A_{ext}}{A_{nc}} \times 100 \quad (1)$$

where A_{nc} and A_{ext} are the absorbances of the negative control and the extract solution, respectively. Ursolic acid (UA) was used as the positive control. ElaInh IC_{50}, the extract concentration that showed 50% of anti-elastase activity (µL extract/mL) was calculated.

2.10. Hyaluronidase Inhibitory Activity

For anti-hyaluronidase activity [31], the extract (25 µL) was added to 20 µL of the 4 mg/mL hyaluronidase solution and incubated for 20 min at 37 °C. To the solution, 40 µL of 12.5 mM $CaCl_2$ solution was added, and the resulting solution was further incubated at the same temperature. After an additional 20 min, 50 µL of 3.5 mg/mL sodium hyaluronate solution was added. After 40 min at the same temperature, the reaction was stopped by the addition of 20 µL of sodium hydroxide (0.9 M) and 40 µL of $Na_2B_4O_7 \times 10H_2O$

(0.2 M). After 3 min at 100 °C, 160 µL of *p*-dimethylaminobenzaldehide reagent (DMABA) (5 g DMABA dissolved in 8.8 mL of CH_3COOH and 0.6 mL of 10 M HCl) was added. After 10 min at 37 °C, the absorbance at 585 nm was recorded. Hyaluronidase inhibitory activity (HyalInh) was calculated as described in Equation (2),

$$\text{HyalInh}(\%) = \frac{A_{nc} - A_{ext}}{A_{nc}} \times 100 \quad (2)$$

where A_{nc} and A_{ext} are the absorbances of the negative control and the extract solution, respectively. Tannic acid (TA) was used as the positive control. HyalInh IC_{50}, the concentration of the extract that showed 50% of anti-elastase activity (µL extract/mL) was calculated.

2.11. Statistical Analysis

The extraction experiments were planned using Design Expert software v. 8.0.6 (Stat-Ease, Minneapolis, MN, USA). Spectroscopic analyses were performed in triplicate and calculated as the mean ± standard deviation. Analysis of variance (ANOVA), followed by Tukey's post hoc test, was used for multiple comparisons between the extracts, while Dunnett's test was used for comparison with the control (JMP, SAS, San Diego, CA, USA). Unless otherwise noted, p-values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Box-Behnken Design

The extraction of *S. montana* was performed using UAE. Short extraction time, reproducible results, and low volume of the solvent needed for the extraction make this technique widely used for the extraction of secondary metabolites from medicinal plants, including those prone to chemical degradation, such as polyphenols [32]. Due to its environmental sustainability, UAE is often referred to as a "green unique technology" [33]. Even though the combination of BBD and RSM is often used for the optimization of UAE of phenolic compounds from medicinal plants, such as *Rhodiola rosea* [34], olive leaf [35], or roselle [36], its utilization for optimization of UAE combined with CD-assisted extraction is not so common. Some of the illustrations for the use of this particular combination include the extraction of flavonoids and other phenolics from pomegranate [37], *Medicago sativa* [38], and *Lotus corniculatus* [39].

As previously pointed out, *S. montana* is rich in various phenolic metabolites, most notably hydroxycinnamic acids and flavonoids, including LG and RA, that are promising as potent ingredients in cosmetic products due to many skin-related biological activities. Therefore, the content in the prepared extracts was the focus of extraction optimization. However, these and other phenolic metabolites are characterized by different chemical and pharmacological properties, often prone to oxidation and other types of chemical degradation [20,21]. Thus, their selective extraction from plant material may be a demanding and time-consuming procedure. Extraction conditions, such as extraction technique, solvent, pH, extraction duration, temperature, and many others, can influence the yield and composition of plant extracts [40]. As the extraction conditions are so numerous, changing one condition at a time to establish how they affect extraction efficiency leads to an infinite number of experiments. Therefore, statistical analysis is necessary to recognize the variables that significantly affect the extraction process [41]. To decrease the number of experiments, experimental design (e.g., BBD) followed by RSM, a statistical technique for modeling and optimizing various processes, is often used. Such an approach is aimed at finding a relationship (model) between the independent variables that may affect process efficiency and the dependent variables of interest [42]. In this study, BBD with six independent variables was used to perform an in-depth investigation of how the extraction conditions and their interactions affect the extraction efficiency. The independent variables selected in this work were: GL content, HP-β-CD amount, temperature, herbal material weight (HMW), extraction time, and ultrasonication power. The results are presented in Table 2.

Table 2. The Box Behnken design with corresponding responses.

S	R	X_1 (%, *w:w*)	X_2 (mmol)	X_3 (°C)	X_4 (g)	X_5 (min)	X_6 (W)	TP (mg/mL)	TDCA (mg/mL)	TF (mg/mL)	RA (µg/mL)	LG (µg/mL)
41	1	0	0.2	20	0.55	15	144	3.23	0.82	0.58	<LOD	111.67
22	2	35	0.2	70	0.3	15	720	1.6	1.59	0.28	158.13	132.82
12	3	35	0.4	70	0.55	5	432	2.92	2.5	0.67	263.31	122.11
47	4	0	0.2	70	0.55	15	720	2.43	1.84	0.56	14.49	135.11
21	5	35	0.2	20	0.3	15	720	1.49	1.13	0.26	25.34	98.63
32	6	70	0.2	45	0.8	25	432	4.92	3.57	0.68	783.47	226.10
34	7	35	0.4	45	0.55	5	144	3.55	1.85	0.4	21.80	187.61
11	8	35	0	70	0.55	5	432	3.08	2.22	0.63	76.94	135.16
39	9	35	0	45	0.55	25	720	2.65	0.88	0.45	<LOD	128.67
42	10	70	0.2	20	0.55	15	144	3.22	3.2	0.39	518.51	196.25
10	11	35	0.4	20	0.55	5	432	3.04	2.14	0.38	213.93	196.21
37	12	35	0	45	0.55	5	720	2.62	0.58	0.43	<LOD	83.02
49	13	35	0.2	45	0.55	15	432	3.48	1.11	0.42	7.84	118.25
13	14	35	0	20	0.55	25	432	3.33	1.28	0.39	37.81	131.23
18	15	35	0.2	70	0.3	15	144	1.81	1.2	0.29	129.73	164.84
3	16	0	0.4	45	0.3	15	432	1.89	0.44	0.25	<LOD	63.43
31	17	0	0.2	45	0.8	25	432	3.73	0.68	0.55	<LOD	123.83
36	18	35	0.4	45	0.55	25	144	3.48	1.72	0.4	100.66	184.01
20	19	35	0.2	70	0.8	15	144	4.02	2.96	0.6	224.4	168.30
46	20	70	0.2	20	0.55	15	720	4.11	2.67	0.41	484.94	185.25
9	21	35	0	20	0.55	5	432	3.48	1.86	0.41	60.13	130.39
50	22	35	0.2	45	0.55	15	432	3.85	1.19	0.34	1.63	99.58
33	23	35	0	45	0.55	5	144	3.31	1.36	0.47	39.06	87.34
40	24	35	0.4	45	0.55	25	720	2.73	1.5	0.48	3.34	118.4
29	25	0	0.2	45	0.3	25	432	1.83	0.26	0.2	<LOD	47.47
53	26	35	0.2	45	0.55	15	432	4.03	1.5	0.38	27.80	126.81
45	27	0	0.2	20	0.55	15	720	2.68	0.86	0.45	<LOD	113.49
23	28	35	0.2	20	0.8	15	720	4.13	2.51	0.56	170.47	251.59
8	29	70	0.4	45	0.8	15	432	5.52	3.51	0.65	822.70	176.58
1	30	0	0	45	0.3	15	432	1.33	0.12	0.18	<LOD	20.06
35	31	35	0	45	0.55	25	144	3.44	0.89	0.37	<LOD	48.35
6	32	70	0	45	0.8	15	432	5.7	2.9	0.61	795.58	191.53
27	33	0	0.2	45	0.8	5	432	4.15	0.45	0.43	<LOD	92.56
19	34	35	0.2	20	0.8	15	144	4.43	3.1	0.52	258.94	208.14
24	35	35	0.2	70	0.8	15	720	3.54	2.8	0.94	57.43	205.64
38	36	35	0.4	45	0.55	5	720	2.99	1.9	0.5	<LOD	43.94
17	37	35	0.2	20	0.3	15	144	2.62	1.22	0.35	53.35	106.29
28	38	70	0.2	45	0.8	5	432	4.74	3.34	0.6	802.04	174.4
25	39	0	0.2	45	0.3	5	432	1.86	0.34	0.24	<LOD	48.34
51	40	35	0.2	45	0.55	15	432	4.04	1.27	0.44	11.75	121.85
5	41	0	0	45	0.8	15	432	3.25	0.2	0.31	<LOD	30.22
30	42	70	0.2	45	0.3	25	432	2.77	1.81	0.26	312.00	70.13
14	43	35	0.4	20	0.55	25	432	3.37	2.02	0.41	36.05	201.48
16	44	35	0.4	70	0.55	25	432	2.82	2.59	0.54	356.98	224.63
54	45	35	0.2	45	0.55	15	432	4.1	1.54	0.41	22.53	122.68
44	46	70	0.2	70	0.55	15	144	3.59	3.27	0.53	620.58	164.42
43	47	0	0.2	70	0.55	15	144	2.54	2.02	0.55	43.97	164.08
52	48	35	0.2	45	0.55	15	432	3.99	1.34	0.41	11.90	120.35
48	49	70	0.2	70	0.55	15	720	4.48	3.04	0.76	656.26	143.23
7	50	0	0.4	45	0.8	15	432	4.76	0.46	0.59	<LOD	133.9
2	51	70	0	45	0.3	15	432	2.78	1.35	0.32	317.77	96.78
4	52	70	0.4	45	0.3	15	432	1.87	1.96	0.18	336.97	67.33
15	53	35	0	70	0.55	25	432	2.73	1.87	0.58	83.71	161.58
26	54	70	0.2	45	0.3	5	432	1.77	1.94	0.38	324.72	69.77

Std = standard number of the experiment; R = run number of the experiment; X_1 = glycerol content, X_2 = 2-hydroxypropyl-β-cyclodextrin amount, X_3 = temperature, X_4 = herbal material weight, X_5 = extraction time, X_6 = ultrasound power; TP = total phenol content expressed as mg caffeic acid equivalents per mL of the extract (mg CAE/mL extract), TDCA = total dihydroxycinnamic acids content expressed as mg caffeic acid equivalents per mL of the extract (mg CAE/mL extract), TF = total flavonoid content expressed as mg quercetin equivalents per mL of the extract (mg QUE/mL extract), RA = rosmarinic acid content, and LG = luteolin 7-*O*-glucoside content; <LOD = below level of detection.

As noted in Table 2, extraction conditions strongly influenced the selected responses. TP, for example, varies from 1.33 mg CAE/mL extract (Run 30) to as much as 5.70 mg CAE/mL extract (Run 32), indicating a 4.3 increase. Similarly, TF content ranged from

0.17 mg QUE/mL extract (Run 30) to 0.94 mg QUE/mL extract (Run 35), showing an approximately fivefold increase. The content of the most prevalent flavonoid, LG, was even more dependent on the extraction conditions, varying from 20.06 µg/mL extract (Run 30) to 251.59 µg/mL extract (Run 28). The influence of the HCGAE conditions on the TDCA content was even more dramatic. The lowest and the highest TDCA content, also recorded in Run 30 and Run 6, were 0.12 mg CAE/mL extract and as much as 3.57 mg CAE/mL extract, respectively. This means that the variation in the HCGAE conditions may bring an almost 30-fold change in TDCA. Similarly, the content of RA varied strongly. In 14 out of the 54 prepared extracts, RA content was below the limit of detection. Most of those extracts (10 extracts) did not contain GL, and out of the four extracts that did, three did not contain HP-β-CD. Interestingly, a large majority of those extracts were prepared at a moderate temperature of 45 °C (12 extracts). The highest RA content was recorded in Run 29 (822.7 µg/mL extract). From the highest recorded RA and TDCA content, it may be calculated that RA makes up about 23% of TDCA in *S. montana*. Similarly, LG accounts for approximately 27% of TF in the plant.

To find the models that explain the relationships between the dependent and independent variables, RSM was used. Regression analysis revealed that polynomial quadratic equations are the best suited to describe all the models. Equations (3)–(7) are presented with independent variables as coded factors. The independent variables that statistically significantly ($p < 0.05$) affect the selected dependent variables are indicated with bold letters and an asterisk (*).

$$\text{TP (mg CAE/mL)} = 3912.73 + 516.83 \times \mathbf{X_1}\,\mathbf{*} + 25.98 \times X_2 - 149.21 \times X_3 + 1245.06 \times \mathbf{X_4}\,\mathbf{*} + 11.57 \times X_5 - 158.26 \times \mathbf{X_6}\,\mathbf{*} - 468.63 \times \mathbf{X_1} \times \mathbf{X_2}\,\mathbf{*} + 210.67 \times X_1 \times X_3 + 204.89 \times \mathbf{X_1} \times \mathbf{X_4}\,\mathbf{*} + 204.08 \times X_1 \times X_5 + 304.75 \times \mathbf{X_1} \times \mathbf{X_6}\,\mathbf{*} + 40.47 \times X_2 \times X_3 + 137.52 \times X_2 \times X_4 + 15.75 \times X_2 \times X_5 + 20.12 \times X_2 \times X_6 - 36.43 \times X_3 \times X_4 - 77.97 \times X_3 \times X_5 + 74.24 \times X_3 \times X_6 - 152.44 \times X_4 \times X_5 + 70.49 \times X_4 \times X_6 - 33.65 \times X_5 \times X_6 + 4.89 \times X_1^2 - 128.53 \times X_2^2 - 316.52 \times \mathbf{X_3^2}\,\mathbf{*} - 325.86 \times \mathbf{X_4^2}\,\mathbf{*} - 370.78 \times \mathbf{X_5^2}\,\mathbf{*} - 316.70 \times \mathbf{X_6^2}\,\mathbf{*} \quad (3)$$

$$\text{TDCA (mg CAE/mL)} = 1324.59 + 1002.42 \times \mathbf{X_1}\,\mathbf{*} + 294.99 \times \mathbf{X_2}\,\mathbf{*} + 213.02 \times \mathbf{X_3}\,\mathbf{*} + 546.70 \times \mathbf{X_4}\,\mathbf{*} - 58.64 \times X_5 - 96.29 \times X_6 + 81.32 \times X_1 \times X_2 - 218.43 \times \mathbf{X_1} \times \mathbf{X_3}\,\mathbf{*} + 351.84 \times \mathbf{X_1} \times \mathbf{X_4}\,\mathbf{*} - 5.67 \times X_1 \times X_5 - 77.58 \times X_1 \times X_6 - 2.55 \times X_2 \times X_3 - 7.21 \times X_2 \times X_4 + 33.90 \times X_2 \times X_5 + 75.03 \times X_2 \times X_6 - 34.79 \times X_3 \times X_4 + 54.84 \times X_3 \times X_5 + 61.21 \times X_3 \times X_6 + 81.19 \times X_4 \times X_5 - 129.44 \times X_4 \times X_6 + 62.56 \times X_5 \times X_6 + 142.29 \times X_1^2 - 90.71 \times X_2^2 + 736.34 \times \mathbf{X_3^2}\,\mathbf{*} - 6.92 \times X_4^2 + 89.23 \times X_5^2 + 11.33 \times X_6^2 \quad (4)$$

$$\text{TF (mg QUE/mL)} = 399.24 + 37.34 \times \mathbf{X_1}\,\mathbf{*} + 12.80 \times X_2 + 75.67 \times \mathbf{X_3}\,\mathbf{*} + 159.83 \times \mathbf{X_4}\,\mathbf{*} - 9.19 \times X_5 + 26.00 \times \mathbf{X_6}\,\mathbf{*} - 56.64 \times \mathbf{X_1} \times \mathbf{X_2}\,\mathbf{*} + 50.03 \times \mathbf{X_1} \times \mathbf{X_3}\,\mathbf{*} + 24.47 \times X_1 \times X_4 - 14.32 \times X_1 \times X_5 + 46.00 \times \mathbf{X_1} \times \mathbf{X_6}\,\mathbf{*} - 0.07 \times X_2 \times X_3 + 45.49 \times \mathbf{X_2} \times \mathbf{X_4}\,\mathbf{*} + 2.72 \times X_2 \times X_5 + 17.87 \times X_2 \times X_6 + 63.35 \times \mathbf{X_3} \times \mathbf{X_4}\,\mathbf{*} - 25.47 \times X_3 \times X_5 + 46.94 \times \mathbf{X_3} \times \mathbf{X_6}\,\mathbf{*} + 44.85 \times \mathbf{X_4} \times \mathbf{X_5}\,\mathbf{*} + 60.91 \times \mathbf{X_4} \times \mathbf{X_6}\,\mathbf{*} + 13.46 \times X_5 \times X_6 + 22.84 \times X_1^2 - 6.32 \times X_2^2 + 82.94 \times \mathbf{X_3^2}\,\mathbf{*} - 29.77 \times X_4^2 + 25.07 \times X_5^2 + 21.40 \times X_6^2 \quad (5)$$

$$(\text{RA (µg/mL)} + 0.822695)^{1/2} = 3.634 + 10.8721 \times \mathbf{X_1}\,\mathbf{*} + 1.4003 \times \mathbf{X_2}\,\mathbf{*} + 1.5811 \times \mathbf{X_3}\,\mathbf{*} + 2.3598 \times \mathbf{X_4}\,\mathbf{*} - 0.2824 \times X_5 - 1.3159 \times \mathbf{X_6}\,\mathbf{*} + 0.1258 \times X_1 \times X_2 - 0.3823 \times X_1 \times X_3 + 2.5812 \times \mathbf{X_1} \times \mathbf{X_4}\,\mathbf{*} - 0.0857 \times X_1 \times X_5 + 0.3421 \times X_1 \times X_6 + 1.3065 \times X_2 \times X_3 - 0.0067 \times X_2 \times X_4 + 0.4472 \times X_2 \times X_5 - 0.8094 \times X_2 \times X_6 - 2.2655 \times \mathbf{X_3} \times \mathbf{X_4}\,\mathbf{*} + 1.6517 \times \mathbf{X_3} \times \mathbf{X_5}\,\mathbf{*} - 0.1403 \times X_3 \times X_6 + 0.0033 \times X_4 \times X_5 - 1.1676 \times X_4 \times X_6 + 0.1529 \times X_5 \times X_6 + 5.1197 \times \mathbf{X_1^2}\,\mathbf{*} + 0.6553 \times X_2^2 + 6.1810 \times \mathbf{X_3^2}\,\mathbf{*} - 2.6877 \times \mathbf{X_4^2}\,\mathbf{*} + 0.5208 \times X_5^2 - 1.4585 \times \mathbf{X_6^2}\,\mathbf{*} \quad (6)$$

$$\text{LG (µg/mL)} = 118.25 + 28.23 \times \mathbf{X_1}\,\mathbf{*} + 19.81 \times \mathbf{X_2}\,\mathbf{*} - 0.36 \times X_3 + 41.54 \times \mathbf{X_4}\,\mathbf{*} + 12.29 \times \mathbf{X_5}\,\mathbf{*} - 6.31 \times \mathbf{X_6}\,\mathbf{*} - 23.93 \times \mathbf{X_1} \times \mathbf{X_2}\,\mathbf{*} - 18.48 \times \mathbf{X_1} \times \mathbf{X_3}\,\mathbf{*} + 16.46 \times \mathbf{X_1} \times \mathbf{X_4}\,\mathbf{*} + 2.71 \times X_1 \times X_5 - 0.63 \times X_1 \times X_6 - 10.76 \times X_2 \times X_3 + 9.35 \times X_2 \times X_4 + 9.05 \times \mathbf{X_2} \times \mathbf{X_5}\,\mathbf{*} - 35.66 \times \mathbf{X_2} \times \mathbf{X_6}\,\mathbf{*} - 22.32 \times \mathbf{X_3} \times \mathbf{X_4}\,\mathbf{*} + 15.35 \times \mathbf{X_3} \times \mathbf{X_5}\,\mathbf{*} - 4.47 \times X_3 \times X_6 + 10.44 \times X_4 \times X_5 + 15.06 \times \mathbf{X_4} \times \mathbf{X_6}\,\mathbf{*} + 20.34 \times \mathbf{X_5} \times \mathbf{X_6}\,\mathbf{*} - 14.37 \times \mathbf{X_1^2}\,\mathbf{*} - 7.37 \times X_2^2 + 50.24 \times \mathbf{X_3^2}\,\mathbf{*} + 0.97 \times X_4^2 + 1.72 \times X_5^2 - 2.44 \times X_6^2 \quad (7)$$

The results presented in Equations (3)–(7) clearly show that all the independent variables selected in this study significantly influenced the extraction outcomes. However, these influences did not always go in the same direction. While all the influences and interactions are too complex for a narrative description, a few examples will be named.

For instance, the presence of HP-β-CD positively affected LG content, but it did not affect TF content indicating that, unlike LG, most flavonoids present in the extracts are not suitable for the formation of complexes with this CD. Due to the different sizes and polarities of secondary metabolites, including phenolics, it is of utmost importance to optimize the solvent systems when utilizing CDs for the extraction of natural molecules from plant material. A fraction of extracted metabolite forming inclusion complex with CD will be in equilibrium with the fraction dissolved in the surrounding medium, and the size of the fraction will depend on the polarity of both the metabolite and the medium [43]. The addition of co-solvents is, therefore, a factor that may strongly affect the extraction of small

molecules with CDs. Their addition to the extraction mixture may have favorable effects by increasing the solubility of the compound in the surrounding medium [44]. However, they may also disrupt the stability of CD complexes [45]. In general, GL content played a favorable role in the majority of the performed extractions. This may be related to the increased solubility of the selected phenolics in the glycerol-rich surrounding medium [45]. However, it is interesting to note that while both GL and LG positively linearly affected LG concentration, their interaction exerted a negative effect. This indicates that the addition of a small amount of GL disrupts the stability of HP-β-CD-LG complexes lowering the solubility of LG, while higher amounts of GL increase the solubility of this flavonoid in the surrounding medium. Similar behavior was recorded for HP-β-CD-curcumin complexes [45]. In our previous study, it was established that the addition of GL in concentrations of up to 5% (*w:w*) had no effect on the extraction of bioactive phenolics from *H. italicum* [29]. However, in this study, GL addition strongly improved extraction efficiency. As the GL content used in this study varied from 0–70% (*w:w*), it is reasonable to assume that the complexed and free fraction ratio for each of the numerous metabolites present in the *S. montana* varied significantly among the extracts.

Another independent variable used in this study was HMW. Most commonly, larger HMW in the extraction mixture increases the concentration of the molecules in the extracts, thus improving the extraction efficiency. However, very high HMWs lead to significant swelling of plant material in water/organic solvent mixtures, thus changing the solvent mixture's composition and polarity. As a consequence, the extraction efficiency is also altered [26]. In this study, HMW mostly exerted a positive linear influence indicating that the saturation of the extraction solution did not occur under the conditions studied in this work. UAE temperature plays an important role in the extraction efficiency of phenolic compounds from plant material. High temperatures may have a dual effect on the concentration of the target compounds. On the one hand, it may enhance the efficiency of the extraction because it reduces the viscosity of the solvent. On the other hand, high temperatures may cause the degradation of sensitive compounds, such as flavonoids and phenolic acids [46]. Similarly, prolonged extraction increases the chance for the diffusion of phytochemicals from plant material into the solution but also increases the chance of degradation of sensitive molecules, especially during ultrasonication. High USP levels may improve the extraction process by increasing the kinetic energy of the molecules in the solution. However, high USP can also cause the degradation of sensitive secondary metabolites, including phenolic compounds [47].

Another important factor that exerted a significant influence over the extraction efficiency of *S. montana* phenolics is the temperature. High temperatures may have a dual effect on the concentration of the target compounds. On the one hand, it may enhance the efficiency of the extraction because it reduces the viscosity of the solvent. On the other hand, high temperatures may cause the degradation of sensitive compounds, such as flavonoids and phenolic acids [46]. Previous studies on UAE of LG and RA from *Origanum majorana*, another aromatic plant from the Lamiaceae family, also demonstrated the importance of temperature for the extraction of those phenolics. However, while the temperature exerted a negative linear influence on the extraction of RA from *O. majorana*, its influence on RA content in this study was positive. The reason may be in the technique used. While in this work ultrasonication bath with a relatively weak USP was used (up to 720 W), a stronger ultrasonic processor equipped with an appropriate probe (1500 W) was used for the extraction of phenolics from *O. majorana* [48].

The previous example also emphasizes the importance of USP levels for extracting plant molecules. UAE produces acoustic cavitation force by inducing a series of compressions and rarefactions in the solvent, causing the formation of bubbles [49], and too high USP can cause the degradation of sensitive phenolic compounds [47]. In this work, USP negatively affected both LG and RA as a linear factor. Conversely, a study examining the effect of ultrasound on RA extraction has found that the increase of USP leads to a mild increase of RA yield in *R. officinalis* extracts. However, the USP range investigated

in that work was much narrower (100 W–250 W) [50] than the range investigated in this study (144 W–720 W). These negative effects may be partly due to the hydroxyl radicals, whose production is generated by the dissociation of water molecules under the influence of ultrasound irradiation under aerobic conditions. Thus, formed hydroxyl radicals subsequently react with sensitive molecules, including phenolics [51]. For example, it was found that decomposition and polymerization reactions occur when caffeic acid is subjected to ultrasound with decarboxylation products and their dimers as the main degradation products [52]. Therefore, USP-induced degradation of RA may follow a similar pattern. Due to the potential generation of reactive hydroxyl radicals, UAE is not advisable for use as a prolonged treatment in a continuous mode, as it may bring about the degradation of sensitive phenolics compounds. This effect is most pronounced when using high frequencies (358 and 850 kHz) or high powers (750 W) [49]. In this study, time was a significant factor in most of the extractions, but usually as a part of interaction with other factors such as in LG extraction, HP-β-CD content, temperature, or USP. Previous research has shown that shorter extraction time was favorable for combined extraction of TP, RA, and LG from *O. majorana*, as it was a significant negative linear factor; however, this may relate to the high USP used in that study [48].

To determine the statistical significance of the obtained models, ANOVA was performed (Table 3). The *f*-values of the models were higher than 14, while the *p*-values for the models were lower than 0.0001. In addition, the lack of fit for all the models was statistically insignificant, with *p*-values > 0.05. The determination coefficients for the responses were rather high (between 0.9390 and 0.9698), confirming that this model well described the observed values. The predicted R^2 values for all the models were in good agreement with the adjusted ones, additionally proving the value of the models. This confirms that the selected models are highly significant and suitable for the interpretation of the experimental data.

Table 3. Analysis of variance (ANOVA) and coefficients of determination (R^2) for the fitted model equations of contents of total phenols (TP), dihydroxycinnamic acids (TDCA), flavonoids (TF), rosmarinic acid (RA), and luteolin-7-*O*-glucoside (LG).

			TP		
R^2		$R^2 = 0.9390$, $R^2_P = 0.8757$, $R^2_A = 0.6990$			
Source	SS	DF	MS	*F* Value	*p*-value
Model	53,261,408.58	27	1,972,644.76	14.82	<0.0001
Lack of Fit	3,194,717.35	21	152,129.40	2.87	0.1227
Pure Error	265,202.59	5	53,040.51		
			TDCA		
R^2		$R^2 = 0.9563$, $R^2_P = 0.9110$, $R^2_A = 0.7837$			
Source	SS	DF	MS	*F* Value	*p*-value
Model	44,788,384.08	27	1,658,829.04	21.10	<0.0001
Lack of Fit	1,899,168.22	21	90,436.58	3.13	0.1046
Pure Error	144,644.88	5	28,928.98		
			TF		
R^2		$R^2 = 0.9451$, $R^2_P = 0.8881$, $R^2_A = 0.7322$			
Source	SS	DF	MS	*F* Value	*p*-value
Model	1,148,595.53	27	42,540.58	16.58	<0.0001
Lack of Fit	60,594.57	21	2885.46	2.36	0.1731
Pure Error	6112.95	5	1222.59		

Table 3. *Cont.*

			RA		
R^2		R^2 = 0.9698, R^2_P = 0.9385, R^2_A = 0.8507			
Source	SS	DF	MS	*F* Value	*p*-value
Model	4098.25	27	151.79	30.97	<0.0001
Lack of Fit	118.26	21	5.63	3.08	0.1078
Pure Error	9.15	5	1.83		
			LG		
R^2		R^2 = 0.9630, R^2_P = 0.9247, R^2_A = 0.8180			
Source	SS	DF	MS	*F* Value	*p*-value
Model	151,680.32	27	5617.79	25.10	<0.0001
Lack of Fit	5359.43	21	255.21	2.78	0.1297
Pure Error	458.82	5	91.76		

SS = sum of squares, DF = degrees of freedom, MS = mean square, R^2_A = adjusted R^2; R^2_P = predicted R^2.

3.2. Extraction Optimization

The levels of the extraction factors that are best suited for the highest yields of the target phenolics were calculated. The HCGAE conditions needed to prepare four optimized extracts, OPT-TP, OPT-TDCA-RA (the HCGAE conditions needed for the highest TDCA and RA content coincided), OPT-TF, and OPT-LG, are presented in Table 4. In accordance with GL and HMW being the positive linear factors in all the extraction, high values of both variables were best suited for all the optimal conditions. The other factors were more dispersed between their minimum and maximum values used in HCGAE. To further confirm the suitability of the models for the extraction of phenols from *S. montana*, the relationship between their predicted and obtained amounts was calculated. The response deviations were in the range of ± 5%, indicating that the quantities of the selected responses in the extracts were in excellent accordance with the predicted ones. The results thus confirmed the suitability of the calculated models to adequately describe and predict HCGAE outcomes.

Table 4. Predicted and observed response values for the extracts prepared using calculated optimal conditions.

Extract	OR	Unit	AO	X_1 (%, *w:w*)	X_2 (mmol)	X_3 (°C)	X_4 (g)	X_5 (min)	X_6 (W)	PV	OV	RD (%)
OPT 1	TP	mg/mL	Max	70	0	45	0.80	15	504	5.76	5.93	2.98
	TDCA	mg/mL								4.19	4.17	−0.64
OPT 2	and	and	Max	70	0.15	20	0.77	9	288	and	and	and
	RA	µg/mL								1223.74	1163.33	−4.88
OPT 3	TF	mg/mL	Max	70	0.20	65	0.77	25	720	1.02	0.991	−2.36
OPT 4	LG	µg/mL	Max	57	0.34	20	0.80	14	288	286.47	283.95	−0.88

X_1 = glycerol content, X_2 = (2-hydroxypropyl)-β-cyclodextrin amount, X_3 = temperature, X_4 = drug weight, X_5 = extraction time, X_6 = ultrasound power, OR = optimized response AO = aim of the optimization, PV = predicted value, OV = observed value, RD = response deviation calculated as (OV − PV)/PV × 100, TP = total phenolic content, TDCA = total dihydroxycinnamic acids content, TF = total flavonoid, RA = rosmarinic acid, and LG = luteolin 7-*O*-glucoside.

3.3. Comparison of Cyclodextrin-Assisted Extraction and Conventional Solvents Extraction

In order to evaluate the efficiency of HCGAE, it was compared with the conventional extraction using water, ethanol, and mixtures thereof. Due to their wide availability and relative non-toxicity, water/ethanol mixtures are common solvents for extracting phenolic compounds from medicinal plants. Examples include *Helichrysum italicum* [53], *Salvia officinalis* [54], and many others. The preparation of ethanolic and aqueous extracts of *S. montana* has also been described in the literature [55]. The conditions for the preparations of water/ethanol extracts in this study were the same as those used for the preparation

of optimized extracts but without the addition of GL or HP-β-CD. The results of TP, TF, TDCA, RA, and LG determination are displayed in Figure 2. The designations of these extracts have two parts: the first part of the name denotes the extraction conditions, while the second part denotes the solvent used in their preparation. For example, OPT-TF 25% EtOH denotes the extract prepared using the same conditions as those used to prepare OPT-TF but using 25% (*w:w*) ethanol instead.

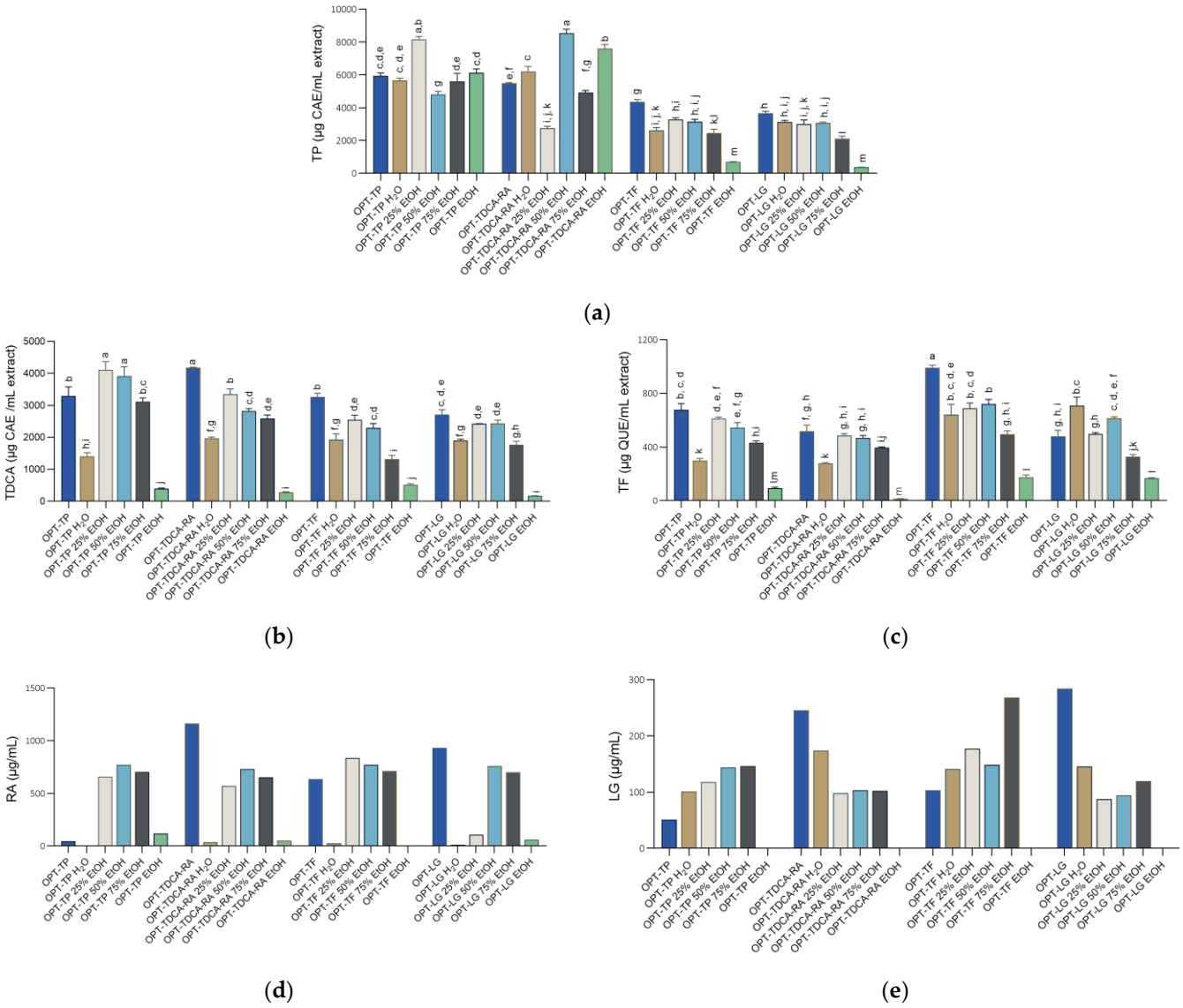


Figure 2. Total (**a**) phenolic (TP), (**b**) dihydroxycinnamic acids (TDCA), (**c**) flavonoids (TF), (**d**) rosmarinic acid (RA), and (**e**) luteolin-7-*O*-glucoside (LG) content in optimized (OPT-TP, OPT-TDCA-RA, OPT-TF, OPT-FG) *S. montana* extracts and the extracts prepared identical conditions using conventional solvents. For the extracts prepared using conventional solvents first part of the name denotes extraction conditions, while the second part denotes the solvent used for their preparation. Spectroscopic experiments were performed in triplicate, and their amounts are shown as mean ± SD. $^{a–m}$ = differences between the extracts (ANOVA followed by Tukey post-test, $p < 0.05$). Extracts not connected with the same letter are statistically different. HPLC determinations were performed as single experiments and not subjected to statistical analysis.

As observed in Figure 2, extraction conditions greatly influenced the yield of phenolic compounds. In general, the addition of HP-β-CD and GL resulted in an increase in the content of target phenolics in a large majority of extracts. However, OPT-TP, the extract

optimized for TP content, while containing a significant amount of phenolic compounds, was not the extract richest in TP. Three extracts, OPT-TP 25% EtOH, OPT-TDCA-RA 50% EtOH, and OPT-TDCA-RA EtOH, contained more phenolics (Tukey post-test, $p < 0.05$). The reason may be that TP also includes macromolecular phenols such as tannins. They are generally well extracted using diluted ethanol solutions. At the same time, depending on tannin type and concentration, they may not be fully soluble in GL solutions, for example, in the case of condensed tannins powder from wattle species that were dissolved using water/GL mixtures and sulfuric acid as a catalyst [56]. In polyol solutions, tannins may form hydrogels, as exemplified in chestnut-tannin-crosslinked organohydrogel prepared with polyvinyl alcohol and water/GL as the solvent system [57]. In general, the extraction conditions used for preparing OPT-TP and OPT-TDCA-RA were better suited for preparing the extracts rich in TP than the other two sets of conditions.

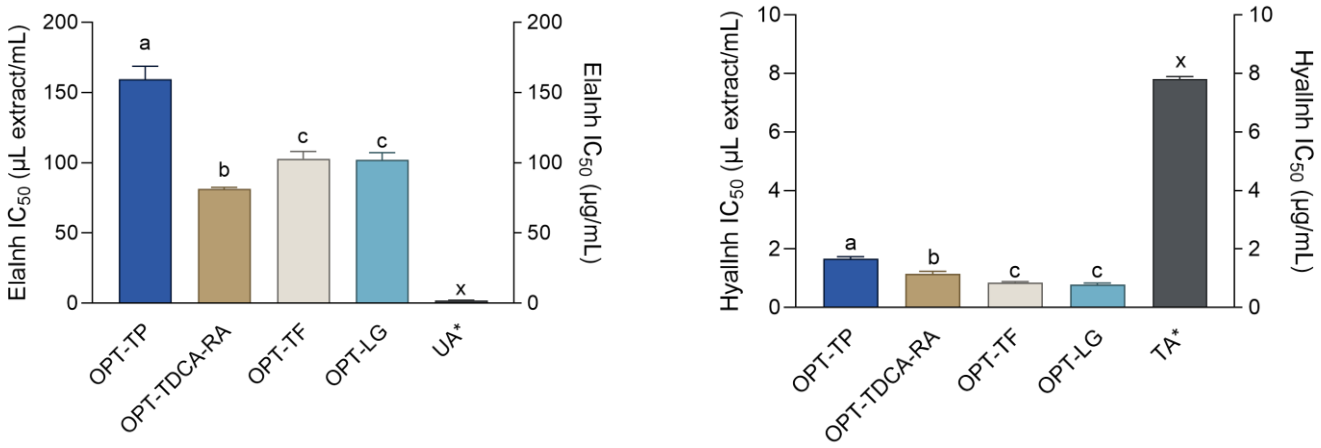
The HCGAE extract optimized for the best TDCA extraction (OPT-TDCA-RA) contained the highest TDCA content. Statistically equivalent TDCA content was also found in OPT-TP 25% EtOH and OPT-TP 100% EtOH (Tukey post-test, $p < 0.05$). In general, for all four sets of extraction conditions, pure ethanol was the least suitable solvent for TDCA extraction, followed by water. The HCGAE extract optimized for the highest TF content, OPT-TF, was superior to all the other prepared extracts (Tukey post-test, $p < 0.05$). In general, the conditions used for preparing TF-OPT were the best suited for preparing the extracts with the highest TF. In the case of TDCA, ethanol was the least effective solvent for TF extraction, regardless of the extraction conditions.

Due to a very small margin of error, HPLC determinations were performed as single experiments. As such, they were not subjected to statistical analysis. However, from Figure 2d,e it is clearly visible that the extracts prepared using optimized HCGAE methods for RA and LG described herein (OPT-TDCA-RA and OPT-LG) contained the highest amounts of those phytochemicals among the prepared extracts deeming the HCGAE highly efficient for RA and LG extraction. RA complexes with HP-β-CD [58] have been described before, as well as RA [59] extraction from plant material using HP-β-CD. However, results presented herein are, to the best of our knowledge, the first report of the use of these CDs for extraction of LG from plant material, as well as the first report of using UAE combined with CDs (either with or without added GL) for extraction of RA from plant material.

3.4. Anti-Elastase and Anti-Hyaluronidase Activity of the Optimized Extracts

Human skin aging is a complex biological process that is not yet fully understood. As the body's organ that is most exposed to the outer environment, the skin suffers from intrinsic and extrinsic aging factors. Intrinsic aging is a process that cannot be prevented, and it affects the skin similarly to all internal organs. Extrinsic aging, on the other hand, can be slowed down as it is the result of exposure to external factors. Aged skin is wrinkled, lacks elasticity, and has a rough-textured appearance. This is accompanied by changes in the skin cells, as well as structural and functional changes in the components of the extracellular matrix, like proteins and polysaccharides [60]. In this work, the anti-aging potential of the prepared *S. montana* extracts was investigated through their ability to impede the enzymes that detrimentally affect the skin macromolecules, such as elastin and hyaluronic acid. The activity of the positive controls (UA and TA) was also evaluated. However, the extracts contain solvents that cannot be evaporated because the boiling points of lactic acid and glycerol are 122 °C and 290 °C, respectively. Heating the extracts at such high temperatures would destroy the phytochemicals present in them. Thus, it is impossible to express the concentration as dry matter in mL (e.g., μg/mL). Therefore, it is important to remark that the activity of the extracts and the activity of the standards are expressed in different measurement units. Namely, the activity of the extracts is presented as μL extract/mL, while the activity of the standards is presented as μg/mL. Meanwhile, their activities may not be directly compared; they are presented for general comparison purposes, as it is possible to regard the activity of the standards as volume equivalents of 1 mg/mL solutions.

Elastin is one of the structural proteins that is found in the extracellular matrix. It is responsible for the mechanical properties of the skin [61]. The amount of elastin in the skin is regulated by the enzyme elastase, a protease whose increased activity is related to numerous dermatological problems, such as psoriasis and prolonged wound healing. In addition, elastase-mediated elastin degradation, caused, for example, by extensive UV radiation, leads to skin elasticity loss, the formation of wrinkles, and premature skin aging. Consequently, the inhibition of elastase prevents pathological changes in the extracellular matrix, as well as photoaging and related processes [6]. The elastase inhibitory activity of the prepared extracts and positive control, ursolic acid, is presented in Figure 3. While all the extracts inhibited the enzyme, their activity was lower than the applied ursolic acid solution. OPT-TDCA-RA was a stronger elastase inhibitor than the other three enzymes. In a study of *Jasione montana* extracts and their constituents, LG did not display an anti-elastase activity even though the same study has shown that luteolin, which LG is a derivative of, showed a significant anti-elastase potential [24]. This may have been related to the concentration range that LG was tested in because in silico docking analyses showed that LG can interact with elastase. LG-elastase interaction, however, was somewhat weaker than the interaction of epigallocatechin gallate, a phenolic compound that was used as the standard inhibitor of the enzyme in the study. In the same study, RA was also found to be able to interact with the enzyme, albeit to a lower extent [62]. RA may inhibit the enzyme sufficiently well even after the penetration through the skin, as demonstrated in a study aimed to evaluate the effectiveness of RA solution, RA-loaded ethosomes, and liposomes when subjected to the transdermal application. All three tested samples were able to transport RA through mouse abdominal skin in sufficient quantities to inhibit the activity of elastase [63].



(**a**) (**b**)

Figure 3. Inhibitory activity of the extracts and corresponding positive controls: ursolic acid (UA) and tannic acid (TA) on (**a**) elastase and (**b**) hyaluronidase. The activities are shown as IC_{50} values ± SD. $^{a–c}$ = differences between the extracts within a column (ANOVA followed by Tukey's post-test, $p < 0.05$). ^{x} = differences with the positive control (ANOVA followed by Dunnett's post-test, $p < 0.05$). Columns not connected with the same letter are statistically different. Asterisk indicates that the unit is placed at the right ordinate.

Skin aging is closely associated with loss of skin moisture. Reduced skin hydration is visible as a reduced turgor, flexibility, elasticity, and overall loss of youthful skin appearance. Hyaluronic acid is a glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked via alternating β-(1→4) and β-(1→3) glycosidic bonds. This polysaccharide is the most important macromolecule for skin hydration as it possesses extreme water-retaining capacity [60]. The turnover of hyaluronic acid in human skin is controlled by hyaluronidase, the enzyme whose activity

is increased in skin aging and various pathological processes. This leads to the depletion of hyaluronic acid skin content, reduced skin moisture, tone, and the appearance of skin folds and wrinkles [64]. Thus, inhibition of hyaluronidase is among the most promising approaches for maintaining a youthful skin appearance. The results in Figure 3 demonstrate that the extracts displayed excellent anti-hyaluronidase properties, with the activity greater than the activity of the positive control, tannic acid (Dunnett's post-test, $p < 0.05$). OPT-TF and OPT-LGs were more active than OPT-TP and OPT-TDCA-RA (Tukey's post-test, $p < 0.05$). The good activity of *S. montana* extracts, especially of OPT-LG in this assay, was not surprising as the bioassay-guided isolation of *Daphne oleoides* subsp. *kurdica*, a plant traditionally used for wound healing, revealed that LG was the constituent responsible for anti-hyaluronidase activity [23]. In addition, RA is a well-known inhibitor of this enzyme [44]. Similar behavior has been recorded for other caffeic acid derivatives [65], for example, chicoric and caftaric acid [66]. In silico docking analyses also showed that both LG and RA interact with the enzyme, but the interaction with LG was somewhat stronger [63]. Consequently, it may be assumed that the activity observed in this study is, at least in part, caused by RA and LG that *S. montana* extracts abound in.

4. Conclusions

The influence of UAE conditions of HCGAE on the content of phenolic compounds (TDCA, TP, TF, RA, and LG) in *S. montana* extracts was investigated. HCGAE was found to be a more efficient approach for extracting target phenolics than the extraction using conventional water/ethanol mixtures. The optimized extracts displayed notable anti-elastase and anti-hyaluronidase properties. Due to the high biocompatibility of HP-β-CD, the prepared extracts may be used as cosmetic ingredients even without solvent removal, thus saving the time and energy of production.

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