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Successive Solvent Extraction of Polyphenols and Flavonoids from *Cistus creticus* L. Leaves

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Abstract: The aim of this study was to evaluate the efficiency of successive extraction (using solvents of increasing polarity, namely hexane, ethyl acetate, acetone, ethanol, and water) of polyphenols from *Cistus creticus* L. The results were compared with the ones obtained from a single-solvent extraction (ethanol, water, and 50% ethanol: water). According to the results, each solvent used for extraction had a significant effect on the yield of extracted polyphenols and the antioxidant activity of the extracts. The highest extraction yield for successive extraction was achieved with ethanol (95.33 mg GAE/g), whereas a comparable amount could also be extracted with 50% ethanol: water mixture single-solvent extraction (96.51 mg GAE/g). The ethanolic and aqueous extracts had the highest antioxidant activity as indicated by their lowest IC_{50} values in the DPPH assay, specifically 350.99 μg/mL for ethanolic extract of successive extraction and 341.18 μg/mL for 50% ethanol: water mixture of single-solvent extraction, followed by the extract produced using acetone. However, the acetone extract contained more flavonoids than the other two extracts up to 28.03 mg QE/g. The results obtained were in line with those for the single-solvent extraction. It is concluded that using a range of solvents in succession is a more efficient way of extracting higher amounts of antioxidant compounds with varying antioxidant activity.

Keywords: polyphenols; flavonoids; successive extraction; *Cistus creticus* L.; antioxidants; HPLC-DAD

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1. Introduction

Oxidative stress is a condition that occurs in a living organism when there is an imbalance between the free radicals and the antioxidant molecules [1]. Reactive oxygen species (ROS) are highly reactive molecules that can be produced either via cellular metabolism or as a response to stimuli or exposure to compounds [2]. ROS can cause multiple damages to essential cellular components, such as membrane lipids, proteins, and DNA/RNA [3]. As such, oxidative stress is an important factor that can lead to the development of various chronic diseases, including cancer, cardiovascular disease, and neurodegenerative disorders [4]. On the other hand, antioxidants are compounds that can neutralize ROS to prevent damage to cellular components. Although organisms produce antioxidant compounds naturally, sometimes the production is not sufficient to neutralize ROS, leading to oxidative stress [5]. Therefore, it is of paramount importance to increase the consumption of antioxidant compounds. These compounds can be found in various foods, with the main sources being fruits, vegetables, and nuts [6]. Polyphenols are naturally derived compounds with inherent antioxidant properties. They have gained considerable attention due to their potent antioxidant properties, from which they exhibit health benefits [7]. They can be classified into many categories, with flavonoids being the most abundant. Due to their good antioxidant properties and the high abundance of renewable sources (i.e., fruits and vegetables), there is a growing interest in the isolation of polyphenols and flavonoids to be used as additives in foods, cosmetic products, and pharmaceuticals [7].

The extraction of polyphenols from plants is a complex process since many factors can affect the extraction yield [8,9]. To this end, various techniques have been developed, including maceration [8,9], Soxhlet extraction [8,9], ultrasound-assisted extraction [10], pulsed electric field [11], pressurized liquid extraction [12], etc. The most efficient techniques are the conventional ones, but they are characterized by high cost, complexity, and thermal degradation of thermosensitive compounds. On the other hand, techniques based on ultrasound, pulsed electric field, pressurized liquid extraction, microwave, etc., although overcoming some of these disadvantages, seem to also have other disadvantages such as the high cost for equipment and the need for specialized personnel, thus hindering their larger scale applicability [13,14]. Likewise, various solvents have been used for the extraction of polyphenols, in order to maximize the extraction efficiency. Organic solvents [10], deep eutectic solvents [15], ionic liquids [16], etc., are some of the various solvents that have been studied so far. Among the various solvents, ethanol, methanol, and water are among the most commonly used [17]. However, not all compounds are isolated using a single-solvent extraction. As such, successive extraction with solvents of increasing polarity can overcome the above hindrance [18]. This is due to the fact that different polyphenols have different solubility and varying degrees of polarity. Using a sequence of solvents with different polarities allows for the extraction of a broader range of polyphenols than a single solvent, which may not be able to extract certain types of polyphenols [19]. The successive solvent extraction method offers several distinct merits in the isolation of polyphenols from natural sources. By employing a sequence of solvents with increasing polarity, this approach enables the selective dissolution of a wider range of polyphenolic compounds compared to a single-solvent extraction [18]. This method enhances the overall extraction yield and provides a more comprehensive representation of the bioactive compounds present in the sample. Moreover, successive solvent extraction offers flexibility in tailoring the extraction process to optimize the recovery of specific polyphenols of interest. By harnessing the varying solvent polarities, this method allows for a more thorough exploration of the polyphenolic profile, offering valuable insights into the potential health benefits and applications of these compounds. Finally, the identification of the compounds is made easier, as the number of compounds extracted in each solvent is lower, compared to a single-solvent extraction, rendering the use of analytical techniques more efficient [18].

Cistus plants are a group of shrubs that belong to the *Cistaceae* family. They are widely distributed in the Mediterranean region and have been used for centuries for their medicinal properties [20]. *Cistus* plants have been traditionally used to treat various diseases, such as respiratory infections, skin disorders, and digestive problems [20]. Specifically, aerial parts of the plant such as leaves, flowers, and seeds have been used for medicinal purposes, as sitz bath, infusion, decoction, even as powder mixed with foods such as honey [21]. The genus *Cistus* has many species, including *Cistus creticus*, *C. monspeliensis*, *C. incanus*, and *C. ladanifer* [22]. *C. creticus* L. is one of the *Cistus* species that has a high content in polyphenolic compounds and therefore exhibits high antioxidant activity [23]. Its aerial parts have been used industrially, as a dietary supplement additive, and as a fixative substance in the perfume industry.

The aim of this study was to evaluate the efficiency of successive extraction with solvents of increasing polarity over the efficiency of single-solvent extraction of polyphenols from *Cistus creticus* L. To this end, successive extraction using five solvents was carried out and compared with the results from single-solvent extraction, both in terms of overall extraction yield and in individual polyphenol concentrations. This study addresses a specific research gap, since the comparative evaluation of successive extraction with solvents of increasing polarity versus single-solvent extraction specifically for *Cistus creticus* L. has not been clarified. There is a need to determine the most effective extraction method for polyphenols from *Cistus creticus* L., considering the potential advantages of successive extraction with solvents of increasing polarity. By conducting this study, we provide valuable insights into the extraction efficiency and polyphenol concentrations achieved using

these different extraction approaches, thus enlightening the effectiveness of successive extraction with solvents of increasing polarity over single-solvent extraction for obtaining polyphenols from *Cistus creticus* L. The rationale behind successive solvent extraction using solvents of increasing polarity is to selectively dissolve, separate, and identify compounds from a complex mixture based on their solubility preferences. By exploiting the solubility differences between compounds in solvents with varying polarities, this technique enables the isolation and analysis of individual components. Although the usage of multiple organic solvents in successive solvent extraction may incur additional costs in terms of post-processing, it is important to consider the overall value and potential applications. Therefore, the additional cost incurred during the extraction process can be justified by the value and potential commercialization of the extracted polyphenols.

2. Materials and Methods

2.1. Chemicals and Reagents

Hexane (≥95% purity), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), luteolin-7-*O*-glucoside, rutin hydrate, myricetin, quercetin 3-β-D glycoside, and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate, L-ascorbic acid, and formic acid (99%) were obtained from Carlo Erba Co (Milano, Italy). 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) was from Fluka (Steinheim, Germany), acetone HPLC grade was from Scharlab (Barcelona, Spain) and iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent, ethanol 99.8%, acetonitrile HPLC grade, hydrochloric acid (37%), and gallic acid monohydrate were purchased from Panreac (Barcelona, Spain). Sodium carbonate anhydrous (>99%) was from Penta (Prague, Czech Republic). All solvents used for liquid chromatography were of appropriate (HPLC) grade and the others were at least of analytical grade.

2.2. Plant Material and Handling

For this study, *Cistus creticus* L. fresh leaves were gathered from plants 2–3 years old in October 2022 in the Phthiotis region (at 38°58′22″ N and 22°19′09″ E and altitude of 510 m, based on Google Earth version 9.185.0.0), Greece. Plant material was transferred to the laboratory; the leaves were washed with water and then dried with paper towels. Subsequently, plant material was air-dried and ground in a blender. The resulting powder had an average particle diameter of 303 μm and was stored in the freezer at −40 °C, until further usage.

2.3. Preparation of Extracts

For the preparation of the extracts, a successive extraction was carried out using organic solvents and water in an increasing polarity sequence. The order of the solvents used was hexane, followed by ethyl acetate, acetone, ethanol, and, finally, water. For the preparation of extracts, 1 g of powder and 50 mL of the first solvent (i.e., hexane) were placed in a 50 mL amber-colored glass vial and extraction was carried out at 40 °C for 2 h with stirring at 500 rpm. Then, the mixture was centrifuged at 2264× *g* for 5 min and the supernatant was retracted. The solid residue was subjected to a second extraction step with the same solvent (i.e., hexane) at 40 °C for 30 min with stirring at 500 rpm, so as to ensure complete extraction. After the centrifugation, the supernatant was retracted and combined with that from the first extraction step. Finally, the residue (after drying to remove the solvent) was transferred to a new amber-colored glass vial and subjected to extraction with the next solvent (after the extraction with hexane was completed, the solid residue was extracted with ethyl acetate, then with acetone, followed by ethanol and, finally, water). The solvent from the extracts obtained in all cases was removed by vacuum evaporation. Finally, dried extracts were obtained. Solutions were made by diluting the dried extract powder in methanol at a concentration of 10 mg/mL, to be used for further analysis.

For the single-solvent extraction, ethanol, water, and a 50% *v/v* ethanol: water mixture were used separately. Extraction was carried out as mentioned above. In an amber-colored glass vial, 1 g of powder and 50 mL of the solvent were transferred and extraction was carried out at 40 °C for 2 h at 500 rpm. Then, the mixture was centrifuged at 2264× *g* for 5 min and the supernatant was retracted. The solid residue was subjected to a second extraction step for 30 min at the conditions specified above. After the centrifugation, the supernatant was retracted and combined with that from the first extraction step. Finally, the solvent was removed by vacuum evaporation.

2.4. Determination of the Total Polyphenol Yield (Y_{TP})

The measurement of total polyphenol content was performed by Folin–Ciocalteu assay [11]. In an Eppendorf tube, 0.1 mL of the sample extract was placed together with 0.1 mL Folin–Ciocalteu reagent. After 2 min, 0.8 mL of sodium carbonate (5% *w/v*) was added and the solution was further incubated for 40 min at 40 °C. Finally, the absorbance of the solution was measured at 740 nm. The determination of the total polyphenol yield was calculated as mg of gallic acid equivalents (GAE) per gram of dry weight, based on a calibration curve constructed with gallic acid. Y_{TP} was calculated by using Equation (1):

$$Y_{TP}\ (\text{mg GAE/g dw}) = \frac{C_{TP} \times V}{w} \quad (1)$$

where C_{TP} is the total polyphenol concentration of the extract (mg/L), V is the volume of the extraction medium (L), and w is the dry weight (g) of the plant material. While gallic acid itself may not be present in the extracts obtained, the total polyphenol content determination using gallic acid equivalents allows for a comparative assessment of the polyphenol content across different samples. It provides a standardized measure that facilitates comparison and interpretation of results among studies.

2.5. Determination of the Total Flavonoid Yield (Y_{TFn})

For the measurement of total flavonoid content, a previously published method was used [24]: 100 µL of the sample extract was combined with 860 µL 35% (*v/v*) aqueous ethanol and 40 µL of 5% (*w/v*) $AlCl_3$ solution in 0.5 M CH_3COONa. The absorbance was measured at 415 nm after the mixture was incubated for 30 min. Total flavonoid content was expressed as mg quercetin equivalent (QE) per gram of dry weight using a calibration curve constructed with quercetin. Y_{TFn} was determined by using Equation (2):

$$Y_{TFn}\ (\text{mg QE/g dw}) = \frac{C_{TFc} \times V}{w} \quad (2)$$

where C_{TFn} is the extract's total flavonoid concentration (mg/mL), V is the volume of the extraction medium (mL), and w is the dry weight (g) of the plant material.

2.6. Determination of the IC_{50} Value (Half Maximal Inhibitory Concentration) for DPPH Free Radical Scavenging

For the DPPH radical scavenging activity estimation, a previously published method was used [11]. In brief, 25 µL of the reconstituted extract was mixed with 975 µL DPPH (100 mM), and after the initial absorbance measurement at 515 nm (A_i) t = 0 min, it was incubated for 30 min in the absence of light and the absorbance was measured again at 515 nm (A_f) t = 30 min. The percent inhibition (I %) was calculated by using Equation (3):

$$I\% = 100\times \frac{A_f - A_i}{A_f} \quad (3)$$

For the calculation of the IC_{50} values (expressed as μg/mL), the *I*% of extracts at various concentrations (at least five concentrations) were plotted against the concentration of the extract, and the IC_{50} values were calculated from linear regression analyses. Likewise, the IC_{50} of BHT was calculated for comparison.

2.7. Determination of the Ferric Reducing Antioxidant Power (P_R)

For the determination of ferric reducing power, a previously reported method was employed [24]: 50 μL of the extract was mixed with 50 μL $FeCl_3$ (4 mM in 0.05 M HCl) and incubated for 30 min at 37 °C. Next, 900 μL of TPTZ solution (1 mM in 0.05 M HCl) was added to the solution. Finally, after 5 min of incubation at room temperature, the absorbance was recorded at 620 nm. The P_R was estimated as μmol ascorbic acid per gram of dry mass, based on an ascorbic acid calibration curve (50–500 μmol/L). P_R was calculated by using Equation (4):

$$P_R\ (\mu\text{mol AAE/g dw}) = \frac{C_{AA} \times V}{w} \quad (4)$$

where C_{AA} is the extract's concentration of μmol ascorbic acid equivalents (AAE) per liter, *V* is the volume of the extraction medium (L), and *w* is the dry weight (g) of the plant material.

2.8. High-Performance Liquid Chromatography (HPLC-DAD)

Identification and quantitation of the polyphenols contained in the extract were carried out using a Shimadzu liquid chromatograph (CBM-20A) and a Shimadzu diode array detector (SPD-M20A) by applying a previously adopted method [15]. Chromatographic separation was carried out with a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 μm) (Phenomenex Inc., Torrance, CA, USA) at 40 °C. The mobile phase was (A) 0.5% (*v*/*v*) formic acid and (B) CH_3CN with 0.5% (*v*/*v*) formic acid, a flow rate of 1 mL/min, and 20 μL of the sample was injected. The gradient profile was applied as follows: 0–40 min, 5–40% B; 40–50 min, 40–50% B; 50–60 min, 50–70% B; 60–70 min, 70%B. Quantification was performed with calibration curves (0–50 mg/L) constructed with luteolin-7-*O*-glucoside ($y = 0.00002x + 1.0794$, $R^2 = 0.9980$), myricetin ($y = 0.00002x + 0.7496$, $R^2 = 0.9990$), quercetin 3-β-D glycoside ($y = 0.00003x + 0.0625$, $R^2 = 0.9994$), and rutin ($y = 0.00003x + 0.2353$, $R^2 = 0.9990$).

2.9. Statistical Analysis

All the extracts were prepared in triplicate and each extract was analyzed three times, resulting in a total of nine (3 × 3) measurements. The results are presented as mean values ± standard deviation. Statistically significant differences ($p < 0.05$) were evaluated with ANOVA, using the SPSS (version 26) (SPSS Inc., Chicago, IL, USA) software.

3. Results and Discussion

3.1. Extraction Yields and Antioxidant Activity of the Extracts

Extraction of bioactive compounds from plant material is usually carried out using a single solvent or a mixture of two solvents. The most commonly employed solvents are water, ethanol, and methanol, as well as binary mixtures of water with the alcohols, at variable ratios. This has been proved to be an efficient way to extract polyphenols from plant species, especially highly hydroxylated aglycone forms of phenolic compounds, which are highly soluble in the abovementioned solvents [25]. However, polyphenols are a group of compounds with high diversity in means of functional groups and, as such, phenolic terpenes, flavonoid aglycones and phenolic acids, and other highly methoxylated aglycone forms need less-polar solvents, such as hexane and ethyl acetate, to be extracted [25,26]. Among the polar and nonpolar polyphenols, polar polyphenols, tend to exhibit higher antioxidant efficiency; however, nonpolar polyphenols and other phytochemicals should not be overlooked [25]. Therefore, carrying out extraction with a single solvent, although it can be rendered efficient via optimization procedures, will remain incomplete. As such,

successive extraction with solvents of increasing polarity is usually carried out to gain a better overview of the polyphenols contained in a sample, as well as their partition in the different extraction solvents [27].

In our case, a total of five solvents were used to extract the polyphenols contained in *Cistus creticus* L. The solvents were selected based on the polarity index (PI) and used in succession with increasing polarity. Hexane (PI = 0.009) was followed by ethyl acetate (PI = 0.228), followed by acetone (PI = 0.355), followed by ethanol (PI = 0.654) and finally water (PI = 1). Using these solvents, five extracts were obtained at various yields. When hexane was used, the extraction efficiency was 0.0426%. In the case of ethyl acetate, the extraction efficiency was 0.0329%. Likewise, when acetone was employed, the extraction efficiency was 0.0491%. Finally, in the case of ethanol and water, the extraction efficiencies were 0.1263 and 0.1184%, respectively. The obtained extracts were evaluated for their polyphenol and flavonoid content. Results are shown in Table 1. As expected, the content of polyphenols in the extracts was found to increase as the polarity of the solvent increased, with the ethanolic extract exhibiting the highest polyphenol content, whereas the aqueous extract contained the lower amount of polyphenols, compared to the ethanolic one. This may be attributed to the fact that most polyphenols were extracted up to ethanol, and therefore, there were not many left to extract using water as solvent. The total content of polyphenols in the five extracts was found to be 278.3 mg GAE/g. This value is comparable to the values reported in previous studies. More specifically, it was reported that the ethanolic extract from Moroccan *Cistus creticus* L. was 195.11 ± 4.14 mg GAE/g, after 24 h extraction with ethanol [28]. Likewise, the polyphenol content of methanolic and hydromethanolic extracts from *C. incanus* and *C. ladanifer* prepared by maceration or Soxhlet extraction was ranging between 269.28 mg GAE/g and 347.27 mg GAE/g [29]. Regarding the total flavonoid content, an enhanced content of flavonoids was observed as the polarity increased to medium polarity (i.e., acetone). It is noteworthy that the use of acetone resulted in nearly three times higher flavonoid content, compared to ethyl acetate. Further increase in the polarity of the solvent (i.e., ethanol and water) resulted in a decrease in the flavonoid content of the respective extracts. This can be attributed both to the lower polarity of flavonoids, suggesting better extraction by solvents of medium polarity, as well as due to the increased extraction by acetone, resulting in an overall lower content of flavonoids available to be extracted by the following solvents. The total content of flavonoids extracted using the five solvents was found to be 51.36 mg QE/g, which is comparable with a previous study focusing on *C. incanus* and *C. ladanifer* (i.e., 44.76–53.76 mg QE/g) [29].

Table 1. Total polyphenol and flavonoid content and antioxidant properties of the *Cistus creticus* L. extracts prepared by successive extraction with solvents of increasing polarity.

Solvent	Y_{TP} (mg GAE/g) [1]	Y_{TFn} (mg QE/g) [2]	A_{AR} IC_{50} (μg/mL)	P_R (μmol AAE/g) [3]	% Extraction Yield
Hexane	14.88 ± 0.88 ^{e,*}	2.53 ± 0.07 ^{d}	14,913.15 ± 879.88 ^{a}	63.75 ± 3.06 ^{e}	4.35
Ethyl acetate	24.26 ± 0.85 ^{d}	9.5 ± 0.43 ^{b}	2505.97 ± 102.74 ^{b}	177.65 ± 6.04 ^{d}	2.87
Acetone	64.37 ± 2.45 ^{c}	28.03 ± 1.74 ^{a}	486.04 ± 23.33 ^{c}	792.65 ± 19.82 ^{c}	4.40
Ethanol	95.33 ± 5.91 ^{a}	7.58 ± 0.54 ^{c}	350.99 ± 23.87 ^{d}	1103.11 ± 38.61 ^{a}	12.63
Water	79.46 ± 2.22 ^{b}	3.72 ± 0.24 ^{d}	417.69 ± 27.57 ^{c}	929.5 ± 18.59 ^{b}	11.98

* Values are expressed as the mean values of triplicates (±standard deviation). Within each column, statistically significant differences ($p < 0.05$) are denoted with different superscript letters (e.g., a–e). [1] GAE (gallic acid equivalents); [2] QE (quercetin equivalents); [3] AAE (ascorbic acid equivalents).

The next step was to evaluate the antioxidant properties of the as-prepared extracts. The extracts were evaluated using the FRAP and the DPPH assays. Regarding the DPPH assay, the IC_{50} of the extracts was calculated (Table 1). It can be seen that the IC_{50} values of the extracts prepared by hexane and ethyl acetate were increased, whereas, for the other three extracts the IC_{50} values were lower by one or two orders of magnitude. The lowest IC_{50} value was achieved by the ethanolic extract, whereas the extracts prepared with acetone and water were found to have an IC_{50} value that did not differ statistically ($p > 0.05$). It

is noteworthy that the acetone extract exhibited nearly the same IC_{50} values as the ethanol and aqueous extracts, even though it contained a lower amount of polyphenols. However, this may account for the higher content in flavonoids, compared to the two other extracts. For means of comparison, the IC_{50} value of BHT, a commonly used antioxidant compound, was calculated and found to be 363 μg/mL. The ethanolic extract was found to have a comparable IC_{50} value, compared to the BHT. Regarding the IC_{50} values reported in previous studies, lower values have been reported, ranging between 143.60 and 201.63 μg/mL [30]. These values were recorded for the leaves of *C. incanus* collected in summer and winter, respectively. Specifically, for *Cistus creticus* L., a previous study found a quite higher IC_{50} value (520 μg/mL) achieved by an aqueous single-solvent extraction [31]. Regarding the FRAP values, the same trend was observed for the prepared extracts. The highest value was recorded for the ethanolic extract (1103.11 μmol/g), followed by the aqueous and acetone extracts, and finally the ethyl acetate and hexane extracts. The variation in the antioxidant activity among the different employed solvents can be attributed to the different polarity of the solvents and the solubility of different classes of polyphenols in each solvent, which are parameters that affect the polyphenol content, as discussed above. Solvents with higher polarity, such as ethanol and water, have a greater affinity for polar polyphenols, leading to higher extraction yields. On the other hand, less-polar solvents such as hexane may selectively extract nonpolar or low-polarity polyphenols, resulting in lower extraction yields. In addition, solubility of different classes of polyphenols can vary depending on the polarity of the solvent. For example, flavonoids are generally more soluble in polar solvents, while nonpolar solvents may favor the extraction of other polyphenolic compounds. This differential solubility can contribute to the observed variations in extraction yields and subsequently influence the antioxidant activity of the extracts.

The differences observed between the values in our study and the results from previous studies can be due to the different *Cistus* species examined. Moreover, another important factor that can explain the variability in the results is the climatic conditions during the plant growth. More specifically, it has been shown that certain factors such as the month of the harvest and the solar irradiance [32], and the altitude, temperature, evapotranspiration, and soil water deficit [33], are parameters that can affect the polyphenol content of the plant. Moreover, it has been shown that *Cistus* plants harvested in October exhibit the lowest yields and as a result lower antioxidant activity [32]. Despite that, the extracts prepared in our case exhibit a total yield that is comparable to that of previous studies. Particularly for *Cistus creticus* L., the FRAP value in our study by the aqueous extract reached 929.5 μmol/g, similar to a previous study where aqueous extraction was employed (780 μmol/g) [31].

For means of comparison, single-solvent extractions were carried out using either ethanol or water, two of the most commonly employed solvents for polyphenol extraction, along with a 50% *v/v* ethanol: water mixture, also commonly employed in similar procedures [11,17,34]. Results are presented in Table 2. Regarding the polyphenol content of the extracts, the highest extraction yield was achieved with the 50% *v/v* ethanol: water mixture (96.51 mg GAE/g), while ethanol and aqueous extracts gave slightly lower extraction yields of 89.01 and 84.80 mg GAE/g, respectively. The fact that the ethanol: water mixture was found to be more efficient can probably be attributed to the fact that it exhibits an intermediate polarity, compared to the two other solvents, together with the fact that more hydrogen bonds can be formed [17]. Compared to the extracts from the successive extraction, it can be seen that the ethanolic and the aqueous extracts in both cases contained nearly the same amounts of polyphenols. However, this was not the case with the total flavonoid content of the extracts. As can be seen in Table 2, the ethanolic and the aqueous extracts from the single-solvent extractions contained nearly three times more flavonoids compared to the respective extracts from the successive extraction. Moreover, it can be observed that the ethanolic extract contained twice more flavonoids than the aqueous extract, whereas the hydroethanolic mixture contained the same amount of flavonoids as the aqueous extract. These results can be justified by the polarity of the solvents. Ethanol has lower polarity than water and the hydroethanolic mixture, and is therefore more suitable for the extraction

of the flavonoids. This can also be supported by the results presented in Table 1, since ethyl acetate could extract more polyphenols than ethanol. Regarding the IC_{50} values, all three extracts exhibited similar results, with the hydroethanolic mixture and ethanol exhibiting the strongest antiradical activity. These values are also comparable with the ethanolic and the aqueous extracts deriving from successive extraction. It is noteworthy that despite the fact that the ethanolic extract from the single-solvent extraction contained nearly three times more flavonoids than the ethanolic extract from the successive extractions, the IC_{50} and FRAP values were comparable. This can be due to the fact that not all flavonoids exhibit the same antioxidant activity [35]. Therefore, it occurs that with successive extraction, three extracts of similar antioxidant activity can be produced, maximizing the valorization of the plant leaves and retrieving all bioactive compounds, compared to the case of the single-solvent extraction. The successive extraction method, involving a range of solvents with increasing polarity, aims to extract a broader spectrum of polyphenols with varying polarities, leading to a more diverse antioxidant profile. This approach provides a greater chance in capturing a wider range of bioactive compounds compared to a single-solvent system. Moreover, the choice of solvent system may have a significant impact on the extraction efficiency for specific groups of compounds, such as flavonoids or other classes of polyphenols. By comparing the successive extraction method with the single-solvent system, we can assess the advantages and limitations of each approach and gain insights into the optimal solvent system for extracting specific compounds of interest.

Table 2. Total polyphenol and flavonoid content and antioxidant properties of the *Cistus creticus* L. extracts prepared by single-solvent extraction.

Solvent	Y_{TP} (mg GAE/g) [1]	Y_{TFn} (mg QE/g) [2]	A_{AR} IC_{50} (μg/mL)	P_R (μmol AAE/g) [3]	% Extraction Yield
Ethanol	89.01 ± 6.23 ^{a,b,*}	20.03 ± 1.5 ^{a}	358.14 ± 25.43 ^{b}	1116.18 ± 74.78 ^{a}	25.89
50% *v*/*v* Ethanol: water	96.51 ± 5.79 ^{a}	10.24 ± 0.71 ^{b}	341.18 ± 15.01 ^{b}	1237.15 ± 30.93 ^{a}	45.17
Water	84.80 ± 5.43 ^{b}	9.92 ± 0.25 ^{b}	394.18 ± 8.67 ^{a}	1094.96 ± 78.84 ^{b}	38.37

* Values are expressed as the mean values of triplicates (±standard deviation). Within each column, statistically significant differences ($p < 0.05$) are denoted with different superscript letters (e.g., a, b). [1] GAE (gallic acid equivalents); [2] QE (quercetin equivalents); [3] AAE (ascorbic acid equivalents).

3.2. Polyphenolic Composition by HPLC

In order to gain further insight into the composition of the extracts, they were analyzed using HPLC-DAD. Representative chromatograms are given in Figure 1. Chromatograms in other wavelengths (250, 270, 280, 290, 300, and 320 nm) are also given in Supplementary Materials in Figures S1–S6. Nine polyphenolic compounds were identified and quantified in the samples (Tables 3 and 4). As can be seen, the higher content in each identified compound was recorded in the acetone extract, except luteolin glucoside. The lowest content was found in the hexane extract, as expected from the measurements of extraction yields and antioxidant activities mentioned above. This could be due to the polarity of hexane and its ability to extract low-polarity flavonoids such as flavanones, methylated flavones, aglycones of isoflavones, and low-polarity flavonols [36]. Given our research focus on polyphenols and their antioxidant activity, we did not study these highly conjugated nonpolar compounds, as they were beyond the scope of our study. Future investigations specifically targeting these compounds could provide further insights into their identification and potential bioactivities. The identified compounds range in concentrations varying from 0.349 mg/g (rutin) to 19.626 mg/g (myricetin rhamnoside). The most abundant compound identified in all cases was myricetin rhamnoside. However, its concentration in the various extracts differed significantly. This can be attributed to the low solubility of all forms of myricetin in water. As such, less-polar solvents are needed to extract the compounds. Therefore, this can justify to some extent the abovementioned differences in the antioxidant activity of the obtained extracts. In terms of solubility, acetone was found to be the most efficient solvent for the identified compounds, obtaining a total yield of

30.975 mg/g for the identified compounds. Compared to a previous study that prepared extracts from *Cistus creticus* L. using deep eutectic solvents and pulsed electric field [15], the acetone extract was found to contain higher amounts of the identified compounds. More specifically, the total extraction yield for the identified polyphenols in the acetone extract was increased 535% compared to the extract obtained using a deep eutectic solvent (consisted of lactic acid: glycine) and pulsed electric field. Regarding the main identified compound (i.e., myricetin rhamnoside), an increase of 869% was recorded [15]. Additionally, in comparison with another report that studied Mediterranean northern shore *Cistus creticus* L. from different countries, an increased extraction yield of about 175% and 155% was recorded for all myricetin derivatives and quercetin derivatives, respectively [37].

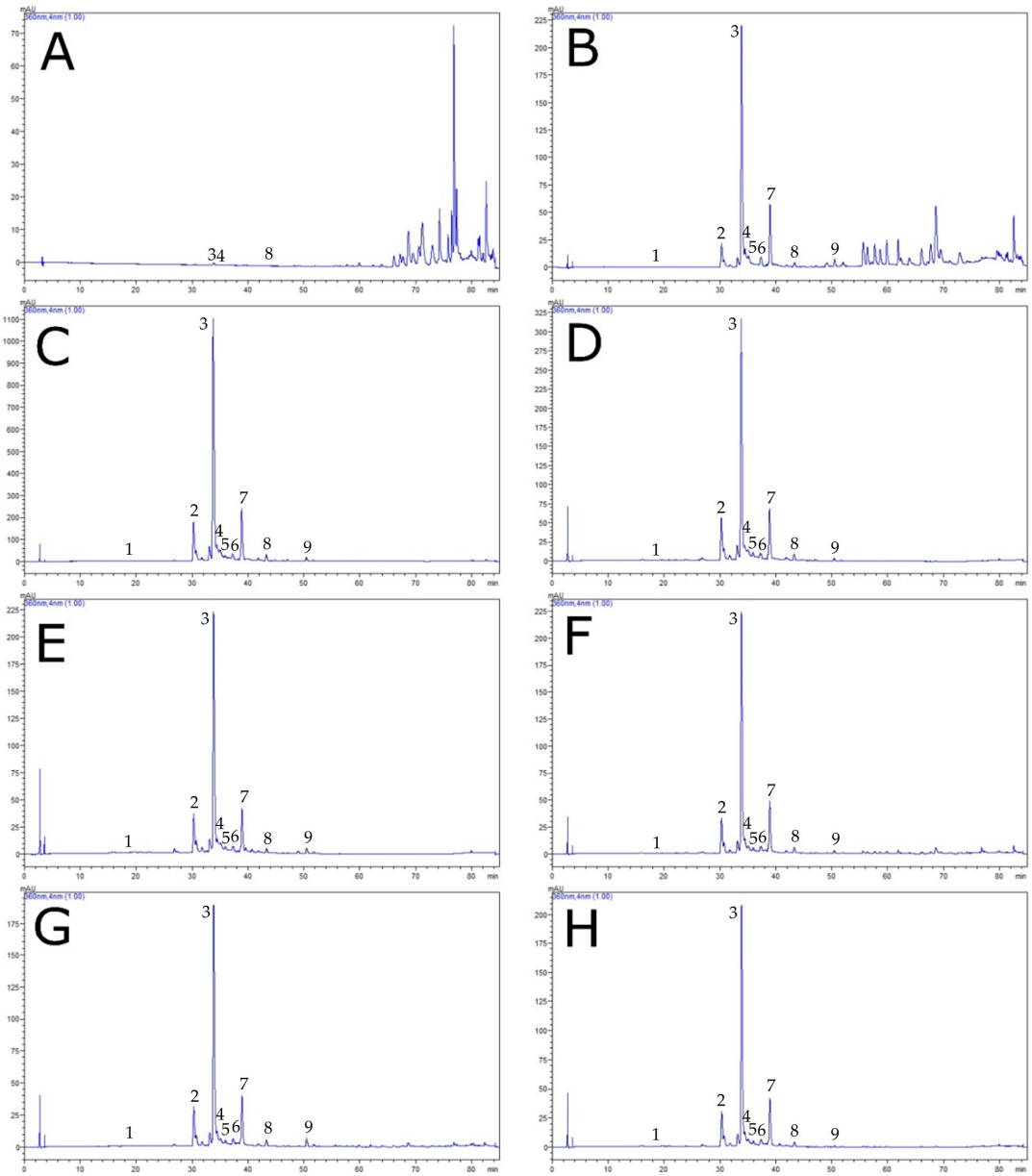

Figure 1. Representative chromatograms at 360 nm for the extract obtained using (**A**) hexane, (**B**) ethyl acetate, (**C**) acetone, (**D**) ethanol, and (**E**) water, using the successive extraction procedure and (**F**) ethanol, (**G**) 50% *v/v* ethanol: water and (**H**) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative, 5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside).

Table 3. Identified polyphenols in the extracts of *Cistus creticus* L. produced by successive extraction with solvents of increasing polarity.

Identified Polyphenol (mg/g)	Solvent				
	Hexane	**Ethyl Acetate**	**Acetone**	**Ethanol**	**Water**
Luteolin glucoside derivative	nd **	0.059 ± 0.002 ^{c,*}	0.075 ± 0.003 ^{b}	0.08 ± 0.005 ^{b}	0.112 ± 0.006 ^{a}
Luteolin 7-(2″-*p*-coumaroylglucoside)	nd	0.202 ± 0.008 ^{b}	0.393 ± 0.027 ^{a}	0.132 ± 0.004 ^{d}	0.173 ± 0.012 ^{c}
1_Myricetin glucoside	0.038 ± 0.002 ^{e}	0.347 ± 0.019 ^{d}	2.635 ± 0.184 ^{a}	0.841 ± 0.057 ^{b}	0.543 ± 0.04 ^{c}
Myricetin rhamnoside	0.046 ± 0.003 ^{d}	3.806 ± 0.217 ^{c}	19.626 ± 0.942 ^{a}	5.511 ± 0.165 ^{b}	3.715 ± 0.256 ^{c}
1_Quercetin glucoside derivative	nd	0.123 ± 0.004 ^{b}	0.428 ± 0.016 ^{a}	0.065 ± 0.002 ^{c}	0.046 ± 0.003 ^{d}
Rutin	nd	0.097 ± 0.006 ^{b}	0.349 ± 0.012 ^{a}	0.1 ± 0.006 ^{b}	0.067 ± 0.004 ^{c}
2_Quercetin glucoside derivative	nd	0.232 ± 0.008 ^{b}	0.724 ± 0.049 ^{a}	0.204 ± 0.008 ^{b}	0.136 ± 0.009 ^{c}
Quercetin rhamnoside derivative	0.006 ± 0 ^{e}	1.438 ± 0.098 ^{c}	6.227 ± 0.137 ^{a}	1.771 ± 0.113 ^{b}	1.05 ± 0.007 ^{d}
2_Myricetin glucoside	nd	0.108 ± 0.004 ^{c}	0.518 ± 0.038 ^{a}	0.18 ± 0.005 ^{b}	0.104 ± 0 ^{c}
Total extraction yield	0.09 ± 0.005 ^{d}	6.411 ± 0.365 ^{c}	30.975 ± 1.408 ^{a}	8.886 ± 0.365 ^{b}	5.946 ± 0.338 ^{c}

* Values are expressed as the mean values of triplicates (±standard deviation). Within each row, statistically significant differences ($p < 0.05$) are denoted with different superscript letters (e.g., a–e). ** nd: not detected.

Table 4. Identified polyphenols in the extracts of *Cistus creticus* L. produced by single-solvent extraction with solvents of increasing polarity.

Identified Polyphenol (mg/g)	Solvent		
	Ethanol	**50% *v*/*v* Ethanol: Water**	**Water**
Luteolin glucoside derivative	0.075 ± 0.002 ^{b,*}	0.089 ± 0.004 ^{a}	0.084 ± 0.002 ^{a}
Luteolin 7-(2″-*p*-coumaroylglucoside)	0.108 ± 0.006 ^{c}	0.353 ± 0.013 ^{a}	0.187 ± 0.011 ^{b}
1_Myricetin glucoside	1.236 ± 0.065 ^{a}	1.168 ± 0.084 ^{a}	1.16 ± 0.079 ^{a}
Myricetin rhamnoside	7.52 ± 0.376 ^{a}	7.032 ± 0.527 ^{a,b}	6.39 ± 0.147 ^{b}
1_Quercetin glucoside derivative	0.081 ± 0.006 ^{b}	0.1 ± 0.006 ^{a}	0.068 ± 0.005 ^{c}
Rutin	0.123 ± 0.007 ^{a}	0.126 ± 0.009 ^{a}	0.105 ± 0.006 ^{b}
2_Quercetin glucoside derivative	0.267 ± 0.015 ^{a,b}	0.29 ± 0.006 ^{a}	0.264 ± 0.015 ^{b}
Quercetin rhamnoside derivative	2.165 ± 0.076 ^{b}	2.473 ± 0.121 ^{a}	2.041 ± 0.114 ^{b}
2_Myricetin glucoside	0.219 ± 0.006 ^{a}	0.198 ± 0.005 ^{b}	0.157 ± 0.009 ^{c}
Total extraction yield	11.793 ± 0.561 ^{a}	11.829 ± 0.776 ^{a}	10.457 ± 0.388 ^{b}

* Values are expressed as the mean values of triplicates (±standard deviation). Within each row, statistically significant differences ($p < 0.05$) are denoted with different superscript letters (e.g., a–c).

Next, we also evaluated the content of the three abovementioned extracts from the single-solvent extraction, in the same polyphenols (Table 4). It can be seen that ethanol and the hydroethanolic mixture achieved the same ($p > 0.05$) extraction yield for the identified compounds, strengthening the notion that the solubility of the compounds plays a major role in the extraction process. The amount of luteolin glucoside derived increases with polarity. The highest amounts of luteolin 7-(2″-*p*-coumaroylglucoside) were achieved by the hydroethanolic mixture. For the other compounds examined, ethanol achieved the highest yields. The similarity in HPLC profiles observed between the extracts obtained from successive extraction and single-solvent extracts can be attributed to the presence of common polyphenolic compounds that are efficiently extracted by both methods. Despite using different solvents, the extraction efficiency for certain classes of polyphenols might be similar, resulting in comparable HPLC profiles. It is important to note that the choice of solvents in both the successive extraction and single-solvent extraction methods was based on their varying polarities. While this range of solvents aims to extract a broad spectrum of polyphenols with different polarities, there may be some overlap in the compounds extracted due to the shared extraction capabilities of certain solvents. Furthermore, polyphenolic compounds often exhibit complex chemical structures, including various

derivatives and isomers, which can result in similar retention times during HPLC analysis. This similarity in retention times might contribute to the resemblance of the HPLC profiles observed in our study.

4. Conclusions

In conclusion, this study explored the extraction of polyphenols from *Cistus creticus* L. using solvents of varying polarities. The successive extraction method with solvents of increasing polarity provided a comprehensive overview of the bioactive compounds present in the samples. The results demonstrated that the solvent polarity and solubility significantly influenced the yield of extracted compounds and the antioxidant activity of the extracts. Specifically, the ethanolic extract exhibited the highest polyphenol content, while the acetone extract showed the highest flavonoid content. Both the ethanolic and aqueous extracts demonstrated the highest antioxidant activity, as evidenced by their low IC_{50} values in the DPPH assay. The findings highlight the efficiency of employing a successive extraction approach using solvents of increasing polarity for extracting a wider range of antioxidant compounds from *Cistus creticus* L. Overall, the successive solvent extraction method offers a powerful and versatile approach to isolating polyphenols from natural sources. It provides a more comprehensive extraction of polyphenolic compounds, enhances the overall extraction yield, and allows for the targeted extraction of specific classes of polyphenols. This method contributes to a deeper understanding of the bioactive compounds present in natural products and their potential applications in various fields, including pharmaceuticals, nutraceuticals, and functional foods. Future studies should aim to further elucidate the relationship between extraction efficiency and solvent polarity. Additionally, it would be valuable to explore alternative solvents and extraction techniques to optimize the extraction process and enhance the yield of specific bioactive compounds. The results highlight the importance of solvent choice and can act as a springboard to further improve the extraction of antioxidant compounds from *Cistus* species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/oxygen3030018/s1, Figure S1. Chromatograms at 250 nm of the extract obtained using (A) hexane, (B) ethyl acetate, (C) acetone, (D) ethanol, and (E) water, using the successive extraction procedure and (F) ethanol, (G) 50% *v/v* ethanol: water and (H) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative, 5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside). Figure S2. Chromatograms at 270 nm for the extract obtained using (A) hexane, (B) ethyl acetate, (C) acetone, (D) ethanol, and (E) water, using the successive extraction procedure and (F) ethanol, (G) 50% *v/v* ethanol: water and (H) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative, 5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside). Figure S3. Chromatograms at 280 nm for the extract obtained using (A) hexane, (B) ethyl acetate, (C) acetone, (D) ethanol, and (E) water, using the successive extraction procedure and (F) ethanol, (G) 50% *v/v* ethanol: water and (H) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative, 5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside). Figure S4. Chromatograms at 290 nm for the extract obtained using (A) hexane, (B) ethyl acetate, (C) acetone, (D) ethanol, and (E) water, using the successive extraction procedure and (F) ethanol, (G) 50% *v/v* ethanol: water and (H) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative, 5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside). Figure S5. Chromatograms at 300 nm for the extract obtained using (A) hexane, (B) ethyl acetate, (C) acetone, (D) ethanol, and (E) water, using the successive extraction procedure and (F) ethanol, (G) 50% *v/v* ethanol: water and (H) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative,

5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside). Figure S6. Chromatograms at 320 nm for the extract obtained using (A) hexane, (B) ethyl acetate, (C) acetone, (D) ethanol, and (E) water, using the successive extraction procedure and (F) ethanol, (G) 50% *v/v* ethanol: water and (H) water, using the single-solvent extraction. 1: Luteolin glucoside derivative, 2: 1_Myricetin glucoside, 3: Myricetin rhamnoside, 4: 1_Quercetin glucoside derivative, 5: Rutin, 6: 2_Quercetin glucoside derivative, 7: Quercetin rhamnoside derivative, 8: 2_Myricetin glucoside, 9: Luteolin 7-(2″-*p*-coumaroylglucoside).

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