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Article

Cistus incanus from Strandja Mountain as a Source of Bioactive Antioxidants

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Abstract: The purpose of the present study is survey of extraction conditions and exploring antioxidant potential of the non-traditional for the Bulgarian ethno-medicine wild herb *Cistus incanus* widespread in Strandja Mountain. The influence of the extraction time (0–500 min) and solvent composition (0–50% ethanol in water) on the polyphenols, flavanoids yields and on antioxidant capacity of the extracts of leaves, stalks (wood parts) and buds mixture were studied. The antioxidant capacity (AOC) was evaluated by use of scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Total phenolic and flavonoid contents were quantified using UV–vis spectrometry. Optimal yield of desired components has been obtained with 30% ethanol in water solvent at 390th min extraction time. In addition, the evaluation of the influence of the seasonality (winter and summer *Cistus incanus*), and of the different areal parts—hard-coated seeds; buds, and mixture of leaves and stalks of the wild plant through the presence of polyphenols, flavanoids and AOC were investigated. Present work revealed the high values of the polyphenols, flavanoids, the high AOC not only in the summer leaves, but also found in the winter leaves, hard-coated seeds, buds and stalks. Based on the obtained results the *Cistus incanus* from Strandja mountain could be a new excellent source of natural antioxidants in food and pharmaceutical industries.

Keywords: *Cistus incanus*; Strandja; antioxidants; polyphenols; flavonoids; seasonality; buds; hard-coated seeds

1. Introduction

Medicinal plants, especially with antioxidant activity, are the main source of drugs for the treatment of complications induced by oxidative stress. Today, about half of the available drugs are estimated to come from plants [1]. The synthesized drugs may appear different adverse effects [2]. So, it is important to look for new sources of phytomedicines in nature.

Cistus incanus L. within the habitat of temperate ericoid communities or European dry heaths and in Bulgaria covers almost the whole most intriguing region– Strandja mountain [3]. The plant is not included in the "Law of medicinal plants" and protected and declared as medicinal plant.

In the Quaternary, among unaffected by glaciations parts of Europe, only Strandja seaside rests almost untouched and keeps climatic conditions similar to tertiary "eternal spring" [4]. Many plants grown there with therapeutic action are not commonly used and popular in Bulgarian folk medicine, such as sub endemic *Cistus incanus* L. or "Pamukliyka" (local name). It is most known only as food for goats and sheep in our lands, while the history of the "Holly rose" and it ethno-medicinal usage in Mediterranean began in ancient times [5]. The wild herb has provided antibacterial, antimicrobial, anti-inflammatory and strong gastroprotective beneficial effects [6]. Many research studies have demonstrated that the main components of the leaves of the different *Cistus* species are polyphenolic compounds from flavanols, flavan-3-ols family such as (+) - catechins, gallic acid, rutin, flavonoid

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aglycones based on quercetin, kaempferol, and mycein [7, 8]. It is well established that phenolics content in plants is mainly responsible to their antioxidant activities and scavenging power.

This work contributes to establishing of the *Cistus incanus* beneficial properties of our geographical longitude, due to its tendency to polymorphism or alteration of phytochemical composition under different environmental factors, conditions and seasonality. An appropriate extraction of phenolic compounds depends on multiple factors, such as their chemical nature, row material size, storage time and conditions. Not at last place, it depends on the extraction and quantification methods, choice of standards, and presence of interferences [9, 10]. Thus, it is necessary to adjust sample preparation procedures to achieve the best possible estimation of the phenolic compounds. Results on evaluation of operational extraction conditions of *Cistus incanus* will provide a better understanding of the antioxidant potential of the wild herb and will allow to be used as high added value dietary antioxidant additive.

In this investigation we selected to follow the steps of extraction optimization of *Cistus incanus* by total polyphenols, flavanoids and antioxidant capacity. Initially, the effect of the solvent (ethanol in water mixtures) concentration was evaluated for a previously chosen extraction time. Once it has been found it was evaluated the extraction time at constant chosen previously extractive parameters - temperature, particle size, solid-to-solvent ratio. Also, it was followed the kinetics by total dry residue and by the yield of the extract in ml to establish equilibrium of the extraction process likewise for better understanding the essence of the extraction process of the herb studied. In addition, the evaluation of influence of the seasonality and evaluation of the different areal parts of *Cistus incanus* on the presence of polyphenols and flavanoids also was investigated.

The total polyphenol content (TPC) was determined spectrophotometrically through the method of Folin-Chiocalteau at the wave length of 765 nm. Total flavonoid content was measured by the aluminium chloride colorimetric assay at wave length 510 nm. The antioxidant capacity (AOC) was studied by DPPH assay at wavelength 517 nm. The total dry residue was found gravimetrically after evaporation of 10 ml of the extract for the liquid phase and through drying of pressed exhausted drug to constant weight in the oven at 105° C for the solid phase. All analyses were investigated spectrophotometrically.

2. Materials

2.1. Chemicals

Ethanol 96% was supplied by Valerus, Bulgaria, methanol, HPLC grade; sodium carbonate (> 99%); gallic acid anhydride (> 99%), sodium nitrite, aluminium chloride hexahydrate - by Merck, Germany, Folin-Ciocalteau reagent – 2M solution, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin hydrate, quercetin hydrate (≥ 95%), tannic acid (≥ 91%), pyrogallic acid (≥ 98%), (+) - catechin hydrate (≥ 96%), sodium hydroxide, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97%) were supplied by Sigma Aldrich, Germany. Ammonia-iron alum - by Sharlau, Germany. Deionized water from water deionizer - Elix70C Gulfstream-Merck.

2.2. Plant material

For this study were used wild *Cistus incanus L.* leaves, stalks (wood parts) and buds collected in the end of the May (2015) in the beginning of the flowering and hard-coated seeds of the plant collected of September (2015); leaves and stalks collected in the end of the March (2016). The drugs were gathered from the area "Parnara" around the village Varvara (Tsarevo municipality) according to the rules of conservation of biodiversity of the National Park Strandja, Bulgaria.

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3. Extraction procedure

For the experiments were used the following mixtures of *Cistus* drugs - leaves, stalks, buds (80:10:10, *w:w*); *Cistus* stalks and leaves (50:50, *w:w*); hard-coated seeds; leaves and stalks (90:10, *w:w*) gathered in summer and respectively in the winter harvest seasons. All samples were dried at room temperature and kept before to be ground into grinder and sieved. All samples were used with LOD (loss on drying) not more than 10%. For the experiments a fraction of 0,5 - 2,0 mm particle size was used. The initial solid to solvent ratio was fixed to 1:20 (2 g *Cistus* in 40 mL solvent). The temperature used for the extraction was the room temperature and was kept constant as far as possible. Extractions were done through magnetic stirring at 3000 rpm (rotation per minute) with a Magnetic stirrer (MS-H-Pro+, Dragon Lab). The influence of the solvent composition water or water-ethanolic solution (10, 20, 30, 40, 50, *v:v*) were studied for the 80th min extraction time. The extraction kinetics of *Cistus* samples were followed during 8,3 h (5, 10, 30, 50, 80, 120, 180, 390, 500 min) with the chosen constant extraction condition. Each exhausted raw material was carefully pressed, and the extract was filtered through cotton and filter paper, measured and analyzed immediately after appropriate dilutions.

4. Methods

4.1. Total polyphenolic assay by the method of Folin-Ciocalteu

Folin-Ciocalteu method (FCM) is usually used only to assess the total number of phenolic compounds in plant extracts [11]. The Folin-Ciocalteu phenol reagent is used to estimate roughly the number of phenolic compounds present in an extract.

A volume of 0.1 ml of Folin–Ciocalteu's reagent was added to a tube, containing 0.02 ml of the extract (previously diluted to 150 ml/L with 50% ethanol for all analysis) and 1.58 ml of deionized water. A minute later 0.3 ml of a 20% Na_2CO_3 solution was added to the tube. The samples were kept in dark place for two hours and then the absorbance was measured at 765 nm against the reagent blank with a UV-VIS-spectrophotometer (T60UV/VIS ver. 1.0) using 10 mm path length cuvette [12]. The results were calculated as gallic acid equivalents ($y = 0.9119x$, $R^2 = 0.9892$), pyrogallic acid equivalents ($y = 1.2114x$, $R^2 = 0.9907$) and tannic acid equivalents ($y = 0.4601.x$, $R^2 = 0{,}9912$). The standard calibration curves were obtained with the following standard solution concentration diapasons: gallic acid solution (0.1 - 1.0 mg/ml), pyrogallic acid solution (0.1 – 0.75 mg/ml), and tannic acid solution (0.5 – 2.0 mg/ml). The total phenolic contents of the *Cistus incanus* extracts was expressed as mg of Gallic acid, Pyrogallic acid, Tannic acid equivalent per gram dry weight sample (mg GAE, PGAE, TAE/g dw) and calculated by following formula:

$$TPC = C \times Ve \times F / M, \quad (1)$$

where: C - total phenolic content, mg GAE/g dw, mg PGAE/g dw, and TAE/g dw; C - concentration of used standard, mg/ml; V- volume of used solvent, ml; F - dilution coefficient of sample; M- mass of the sample, g.

4.2. Flavonoids assay

The total flavonoid content (TFC) of plant extracts was expressed as quercetin, rutin, and (+) - catechin equivalents and measured by the aluminium chloride colorimetric assay [13]. An aliquot of 1 ml extract (previously diluted to 150 ml/L with 50% ethanol for all analysis) was mixed with 4 ml of deionized water and 0.30 mL of a $NaNO_2$ solution (10%, w/v). At 6th min, 0.30 mL of $AlCl_3$ solution (10%, w / v) was added, followed by 2.0 mL of NaOH solution (1 M). Immediately, after thorough mixing the absorbance was measured at 510 nm versus the blank sample. The calibration curves of the used standards were obtained with quercetin (100 - 1000 mg/L; $y = 0.0005x$; $R^2 = 0.9977$), rutin (20 – 100 mg/L; $y = 0.001x$; $R^2 = 0.9958$) and (+) - catechin (10 – 200 mg/L; $y = 0.0034x$; $R^2 = 0.9968$), respectively. The

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results are expressed as quercetin, rutin and (+) - catechin equivalents per gram dry weight (mg QE, RE, CE/g dw) and calculated by the following formula:

$$TFC = C \times Ve \times F / M, \quad (2)$$

where: TFC - total flavonoids content, mg QE/g dw, mg RE/g dw, mg CE/g dw; C – concentration of used standard, mg/L; Ve - volume of used solvent, L; F – dilution coefficient of sample; M- mass of the sample, g.

4.3. Antioxidant activity by the method of DPPH

This is the most commonly used method for quantification of antioxidant activity. The method is described by *Brand-Williams, Cuvelier, and Berset* [14]. later changed by *Sánchez-Moreno, Larrauri, and Saura-Calixto* [15]. DPPH solutions show high absorption at 517 nm due to the deep violet color. The absorbance gradually disappears because of discoloration, which is stoichiometric to the degree of reduction of free radicals. The remaining DPPH measured after a certain time inversely corresponds to free radical scavenging ability of antioxidants.

One thousand microliters of various concentrations of the extracts in ethanol were added to 4 mL of 0.004% methanol solution of DPPH. After an hour incubation period at room temperature, the absorbance was measured against a methanol as a blank at 517 nm. Antioxidant activity defined as the extract concentration necessary to neutralize 50% of free radicals - IC_{50} is calculated by plotting the correlation between concentration of the extract (ml/L) and inhibition (%) - C/I. The graph was constructed by preparing a series of extracts with various concentrations (0.05 - 0.25 μg/ml). Free radical scavenging ability of the tested samples was calculated using the formula (Yen & Duh) [16]:

$$IC\ (\%) = (Ao - Aa / Ao) \times 100, \quad (3)$$

where: A_o - value of absorbance blank; Aa - value of absorbance AOA; IC - inhibition capacity; 100 - percent, %.

After recalculation, the results were expressed as the IC_{50} values (μg/ml).

The results derived were also recalculated using the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) which is an antioxidant vitamin E derivative. It is regularly used as an antioxidant standard. The calibration curve of the Trolox was used at a linearity range of 2,5 – 175 μmol/L and obtained equation of rights was y = 1.332x + 0.5634. The data obtained were expressed in μmol Trolox equivalent antioxidant capacity (TEAC) per gram dry weight (μmol TEAC/g dw) of the extracts. The concentration of the TEAC was calculated using the formula (4):

$$C\ teac = \%\ IC\ sample - a/b * DC, \quad (4)$$

where: C teac – Trolox equivalent antioxidant capacity, μmol TEAC/g dw; IC: inhibition capacity, %; a, b – coefficients from the regret ion equation (y = ax + b); DC: dilution coefficient.

The TEAC assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements [17].

4.4. Total dry residue of extracts

The total dry residue of extracts was determined in accordance with the method of Ph. Eur. (European pharmacopeia) with some modifications [18]. In flat-bottomed dishes were introduced rapidly exhausted drug and 10 ml of 50% ethanol in water extract to be examined. The samples were dried at 105° C in an oven (“Robotica”, Velingrad) to constant mass and after that were cooled in desiccator under anhydrous silica gel and weighted. The results were calculated as gram dry mass.

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5. Results and discussion

Detailed literature research on the phenolic compounds present in *Cistus incanus* was done. There is no data available concerning kinetic studies of the selected drug by total polyphenol and flavonoid content, antioxidant power, and total dry residue, except the extraction kinetics presented by *Dimcheva and Karsheva, 2017* [19] of Bulgarian *Cistus incanus* leaves with 50% ethanol in water solution. In addition, the influence of the seasonality by the presence of examined bioactive components, thus AOC of the wild herb has never been studied. There is no data on the literature concerning the polyphenol content and AOC of the areal parts (stalks, buds, hard-coated seeds) of *Cistus incanus*.

5.1. Effect of the solvent used

The conventional method of polyphenol recovery from plant is based on the solid-liquid solvent extraction. It is generally known that the yield of extracted polyphenols depends on the chemical composition and physical characteristics of the samples as well as on the type of solvents used, their different polarity, extraction manner, contact time and temperature. The results can vary even by one order of magnitude when one or another procedure is used for the same sample. Thus, it is necessary to adjust the extraction method for each new crude drug.

Solvents such as methanol, ethanol, acetone, ethyl acetate and combinations of them are most commonly used to extract phenolics from plants, often in different ratios with water. Choosing the right solvent is essential for the industry, it must be safe, cheap and non-toxic. The ethanol is a good solvent for the extraction of polyphenols and preferable for the extraction of *Cistus incanus* according to Patent Publication for *Cistus extracts [20]*. That is why ethanol was chosen as solvent in present investigation.

To be evaluated appropriate solvent composition was used deionized water or ethanol in water solution (10 – 50%, v / v) to establish the best yield of total polyphenols, flavanoids and antioxidant capacity. The extractions were done by magnetic stirring for the 80 minutes. The results of TPC, TFC, and AOC calculated as IC_{50} (DPPH) are shown in Figure 1.

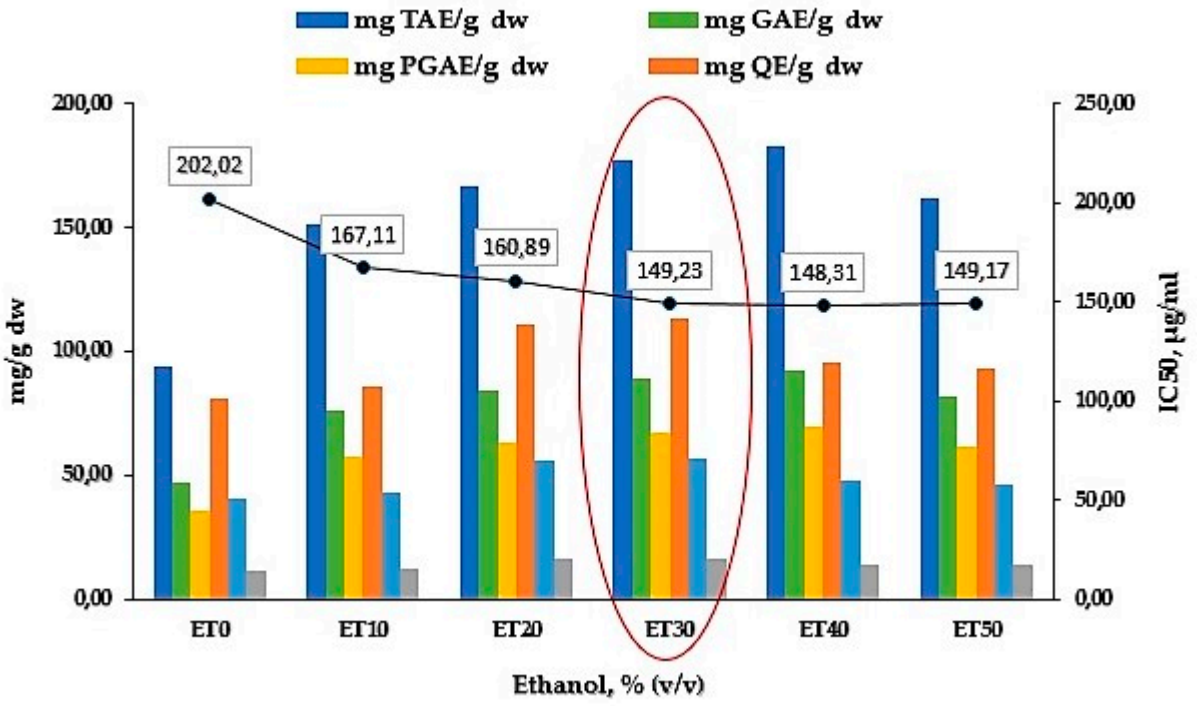

Figure 1. Effect of the solvent concentration on the extraction of *Cistus incanus* leaves, stalks and buds at 80th min through TPC, TFC, and DPPH antioxidant capacity.

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The pure deionized water (ET0) showed worst values of the desired components at the expense of medium polar mixtures, such as ET30 and ET40. It can be seen the AOC, calculated as IC_{50} rests constant with decreasing of the polarity of extracting solvent. Because of the almost constant values of the AOC, of the highest flavanoids contents, and because of the economic reasons the ET30 is chosen as the optimal solvent concentration and used in the further examinations.

5.2. Effect of the extraction time

In the present investigation was evaluated an extraction parameter – the extraction time (0 – 500 min) by total polyphenols, total flavonoids and antioxidant capacity by DPPH of *Cistus incanus* leaves, stalks and buds. The temperature and solid to solvent ratio were kept constant during the whole extraction kinetics procedures, which was carried out through magnetic stirring extraction and ET30 as a solvent. In conventional methods, sampling is manual at chosen time intervals which are not precise, as there is always a time gap between sampling and analysis, which may lead to errors during kinetic measurements. Nevertheless, in the present study, we have tried to make the interval between the various extractions relatively small. On the other hand, the measured raw material was kept how as possible with the same ratio of leaves, stalks and buds. Evaluation of the extraction time was investigated by TPC, TFC, AOC by DPPH, and total dry residue all shown below.

5.3. Polyphenols

Phenolic compounds act as essential metabolites for plant growth and reproduction, and as protecting agents against pathogens. These compounds involve a large group of about 8000 compounds with different structures and chemical properties [21]. In general, these substances containing one or more aromatic rings with one or more hydroxyl groups and can be classified in three main categories: simple phenols, which include phenolic acids; polyphenols constituted by flavonoids and tannins; and a miscellaneous group that comprises compounds such as coumarins, stilbenes and lignans.

Phenolic acids are widely distributed in the plant kingdom and are second only to flavonoids in terms of their dominance, suggesting that naturally occurring [22].

Gallic acid is phenolic acid widely distributed and found in many plants, teas, oak species, and fruits. It is found both free and as part of hydrolysable tannins. Gallic acid is commonly used in the pharmaceutical industry for determining the total phenol content by the Folin-Ciocalteau assay [23]. The phenolic acid is mostly used to express the content of phenolic compounds in most of foods [24].

Pyrogallic acid is a polyphenol compound commonly found in mango and many citrus plants. On the other hand, it is used as a standard for determination of total polyphenols according to the Eur. Ph. [25].

Tannic acid is a plant polyphenol which is found, along with other condensed tannins, in several beverages including red wine, beer, coffee, black tea, green tea, and many foodstuffs such as grapes, pears, bananas, sorghum, black-eyed peas, lentils and chocolate [26]. Similarly, to many polyphenols, tannic acid was proved to possess antioxidant [27], antimutagenic [28] and anticarcinogenic properties [29]. The antioxidant mechanism of tannic acid is still far from being fully understood; therefore, it requires further investigation.

The total phenolic content for 30% ethanol extracts was estimated by Folin Ciocalteu's method using gallic, pyrogallic and tannic acids as standards. According to the results a statistically significant effect of time of each extract is presented in Table 1, where the content of total polyphenol compounds is shown, expressed by represented above phenolic acids.

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Table 1. Total polyphenol kinetic expressed as tannic acid, gallic acid and pyrogallic acid equivalents in mg per g dry weight of *Cistus incanus* leaves, stalks and buds.

Extraction time, min	mg PGAE/g dw	mg GAE/g dw	mg TAE/g dw
5	27,30	36,26	71,88
10	35,67	47,38	93,91
30	52,07	69,17	137,09
50	68,47	90,95	180,27
80	67,26	89,35	177,08
120	75,07	99,73	197,66
180	82,89	110,11	218,24
390	86,81	115,32	228,56
500	82,55	109,66	217,34

The total amounts of polyphenols, expressed as Gallic acid in the extracts vary between 36.26 and 115.32 mg GAE/g dw as a function of time. The quantity of the polyphenols expressed as tannic acid equivalents ranged from 71.88 to 228.56 mg TAE/g dw and from 27.30 to 86.81 for the PGAE/g dw. The lower phenolics contents were detected at the 5th min and the highest at 390th min, as shown in Table 1. But it can be concluded that the equilibrium is achieved at 180th min, because the obtained values for the desired polyphenols are only 4.7% less than obtained after a 3.5 h extraction and 0.41% less than those obtained after 5.3 h stirring.

The used Folin-Chiocalteau assay is specific not only for polyphenols but to any other substance that could be oxidized by the Folin reagent: many non-phenolic compounds like ascorbic acid and saccharides can reduce the amount of reagent [15].

5.4. Flavonoids

Flavonoids are the low molecular weight polyphenolic secondary metabolic compounds, universally distributed in green plant kingdom [30]. Flavonoids represent a broad family of more than 4000 secondary plant metabolites such as 4-oxoflavonoids (flavones and flavonols), isoflavones, anthocyanins, and flavan-3-ol derivatives (tannins and catechins) [31]. For centuries, preparations that contain flavonoids are applied as the primary physiologically active components that have been used for treating human diseases [32].

Quercetin is one of the important bioflavonoids present in more than twenty plants materials and which is known for its anti-inflammatory, antihypertensive, vasodilator effects, antiobesity, antihypercholesterolemic and antiatherosclerotic activities [33].

Rutin (quercetin-3-rutinoside) is a bioflavonoid commonly found in buckwheat bran, black tea, and citrus fruits [34]. Rutin contributes to many positive health effects such as powerful antioxidant, protects against free radicals [35].

Catechins have been also reported to effectively inhibit lipid peroxidation and scavenge free radicals [36].

The total flavonoid content for ethanolic extracts was measured through the aluminum chloride colorimetric assay using quercetin, rutin and (+) - catechin as standards. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition, it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The extraction kinetics' data for different species are shown at Table 2.

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Table 2. Total flavonoid content, expressed as quercetin, rutin and (+) - catechin equivalents in mg per g dry weight of *Cistus incanus* leaves, stalks and buds.

Extraction time, min	mg QE/g dw	mg CE/g dw	mg RE/g dw
5	40,80	6,00	20,40
10	46,81	6,88	23,40
30	68,21	10,03	34,10
50	85,61	12,59	42,80
80	113,37	16,67	56,68
120	120,07	17,66	60,04
180	133,35	19,61	66,67
390	138,44	20,36	69,22
500	119,20	17,53	59,60

The total flavanoids, expressed as quercetin equivalent in the extracts show higher values varying between 40.80 and 119.20 mg QE / g dw from 5th to 500th min extraction time. The lower quantities of the flavanoids are calculated as (+) - catechin equivalent ranged from 6.0 to 17.53 mg CE/g dw and the middle ones from 20.40 to 59.60 mg RE/g dw for the flavonoids calculated as rutin equivalent. The presented kinetics in Table 2 show the same tendency as the total polyphenols - equilibrium is achieved at 180th min but the difference here is their decrease after 390 minutes, which is probably due to their unstable nature or to error due to time between experiments and other random factors.

No previous study on kinetics of total content of polyphenols and flavonoids in *Cistus incanus* exists, including Bulgarian *Cistus incanus* as it was already mentioned. Hence, the data obtained can only be compared with those found for *Cistus* species grown in different regions and extracted using different extraction procedures and different conditions to those used at present study.

For example, for the aqueous extracts of *Cistus ladanifer* and *Cistus populifolius* from Spain values of TPC at levels of 229.3 mg GAE/g dw and 318.9 mg GAE/g dw, respectively were found. The values of TFC in these plants were found to be 30.4 mg QE/g dw and 59.5 mg QE/g dw, respectively [37].

Similarly results for TPC obtained for aqueous extracts of Turkish *Cistus laurifolius* were 289.9 mg GAE/g extract [38].

Lower levels of TPC and TFC were reported for methanol and ethanol extracts of Moroccan *Cistus ladanifer*: 18.43 mg GAE/g extract, 64.33 mg RE/g extract and 11.87 mg GAE/g extract, 61.40 mg RE/g extract, respectively [39].

In another study for the extracts obtained from *Cistus incanus* grown in Turkey and Cyprus the following values for the valuable components were obtained: 258.42 mg GAE/g dw and 202.95 mg GAE/ g dw for the aqueous extracts and 105.02 and 114.18 mg GAE/g dw for hydromethanolic extracts for the total polyphenols content. The total flavanoids for the same extracts were 4.27 and 3.97 mg QE/g dw and 2.39 and 2.27 mg QE/g dw, respectively [40].

From the research made it can be concluded that the Bulgarian *Cistus incanus* contain the greatest total flavanoids content (138.44 mg QE/g dw and 69.22 mg RE/g dw) in comparison not only with *Cistus ladanifer* from Morocco and *Cistus populifolius* from Spain and Turkey but in comparison with Turkish and Cyprian *Cistus incanus* leaves' extracts. The results found in the literature for the total polyphenols of the *Cistus* species are higher than those obtained in this study for *Cistus incanus* leaves, stalks and buds hydroethanolic extracts. The quantities of extracted polyphenolic compounds in the plants depends on the differences in extractive parameters, the solvent used. The various biological and environmental factors at which the plant had grown also contribute on the plant antioxidant power [41].

5.5. Antioxidant capacity

It is well established that the flavonoids and phenolic acids have antioxidant activities due to the presence of structural hydroxyl groups significantly contributing in protection against the oxidative

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damage due to endogenous free radicals [42, 43]. Many of them are reported to have high levels of antioxidant activities [44]. Due to their redox properties, these compounds contribute to overall antioxidant activities of plants. Usually, the antioxidant activity is to neutralize lipid free radicals and to prevent decomposition of hydroperoxides into free radicals [45].

The kinetics of the inhibition capacity at 50% (IC_{50}) and TEAC is presented in Figure 2, expressed as the concentration of the extract it varies from 305.71 to 122.16 μg/ml and expressed as Trolox equivalent, it varies from 303.88 – 747.13 μmol TEAC/g dw. The best antioxidant capacity of *Cistus incanus* leaves, stalks and buds is obtained at 390th min and it is 768.44 μmol TEAC/g dw or just 119.25 μg/ml from the extract can reduce the 50% of the free radicals.

In the literature study there are data on 15 different samples of *Cistus incanus* from different countries the results showed that the values of DPPH for hydromethanolic and aqueous extracts were varied in the range 20.06 – 96.69 μmol TEAC/g dw and 1.52 – 96.85 μmol TEAC/g dw, respectively.

These results are much lower than those obtained in the present study. That means that the Bulgarian *Cistus incanus* is a rich source of antioxidants and the environmental factors of Strandja mountain are obviously suitable for their formation.

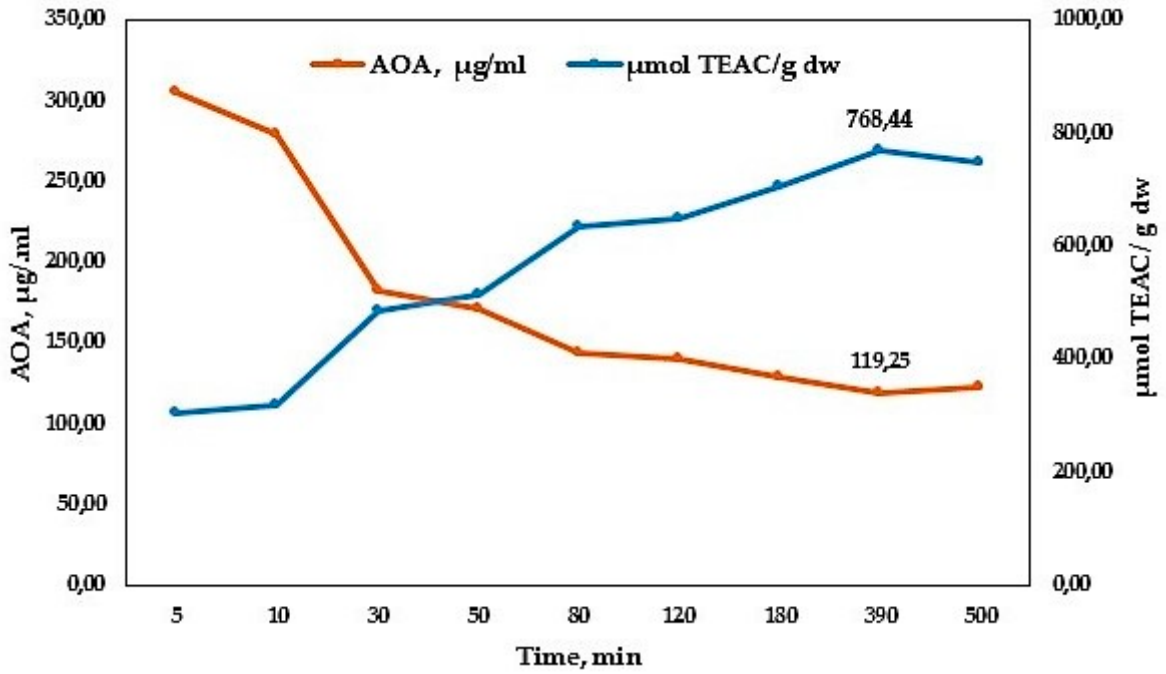

Figure 2. Kinetic curves by DPPH AOC (IC%) in μg/ml and μmol TEAC/g dw of extracts of *Cistus incanus* leaves, stalks and buds.

5.6. Total dry residue

In the evaluation of the extraction of plants it is good to know the kinetics of the process also by total dry residue, when equilibrium is achieved and not at last for the better understanding of the row material extraction. By gravimetric method described above the kinetics of the total dry residue (TDR) of *Cistus incanus* leaves, stalks and buds picked up in summer harvest season in the solid and liquid phase, respectively, was examined. The results were expressed in grams dry weight v/s extraction time, and the kinetics curves obtained are shown in Figure 3.

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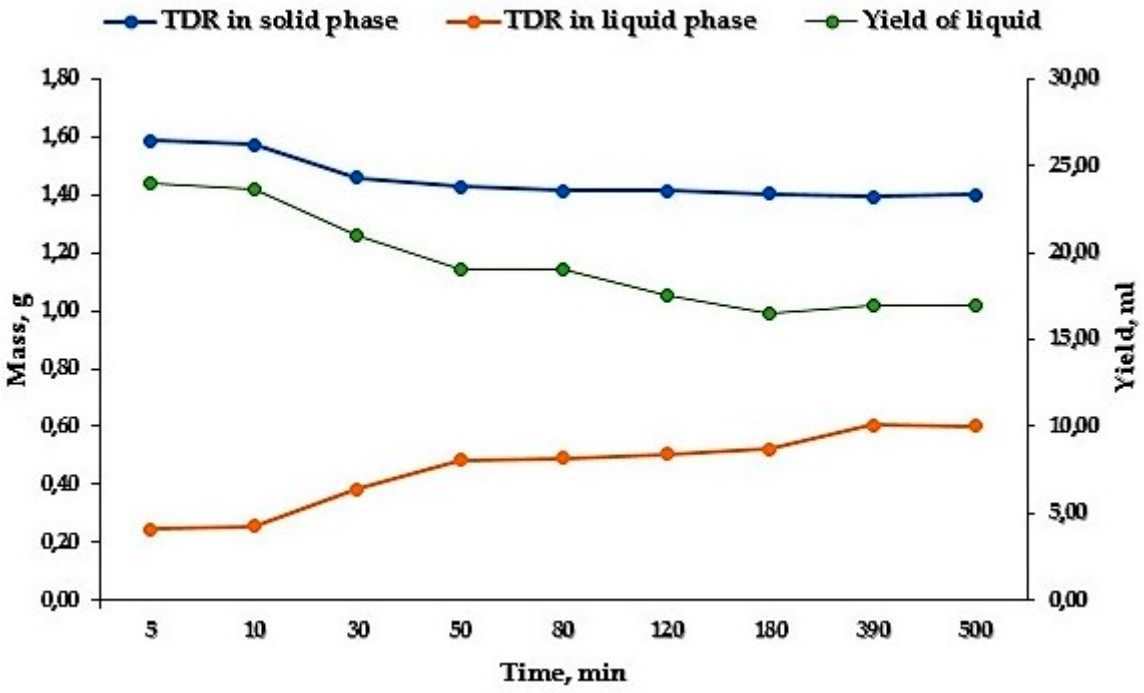

Figure 3. Kinetic curves by total dry residue (TDR) of extracts, exhausted dry material of extract and volume yield of extract (ml) of *Cistus incanus* leaves, stalks and buds.

In the kinetics presented water contents (9.70%) is not recalculated and respectively the presence of volatile substances is quite probable. As shown, the kinetic curves have three parts with different character. The increase of TDR in liquid phase (extract) corresponds to the decrease of TDR in the solid phase. The total yield of extract in ml liquid phase (extract) was also done because it is essential parameter for the industrial production of extracts. The initial steep part of the graphic corresponds to the dissolution of the readily available substances on the surface of the sample particles. The second curved part could be explained by the simultaneous dissolution of the rest from the surface and from inside the sample particle (the mixed zone control). Based on the total yield kinetic the plateau or the extraction equilibrium is achieved after 180th min. Likewise, there is an increasing after 180th min illustrated on the TDR kinetic responsible for the liquid phase and the plateau for this kinetic can be seen at 390th min. However, based on the TDR of the solid phase the plateau is reached approximately on 80th min, where quantity of total dry residue of extract is 1.4135 g and slowly decreasing with 1.0% up to 500th min. These results may be due to the uneven raw material used or measurement errors. Based on kinetics by total polyphenols, flavanoids and AOC, it can be concluded that the 390th min or 6.5 hours is the optimal extraction time also in relation to yield of extract and TDR in liquid phase. The high extraction time probably shows that the magnetic stirring is not the best way to extract the examined mixtures of drugs or there are bioactive substances in the hard buds and stalks which need more time for discharging. In both cases, further extraction optimization is required, may be with increasing of the extraction temperature or changing the applied extraction manner.

5.7. Evaluation of the *Cistus incanus* areal parts

In this study different areal parts were used, as follows: hard-coated seeds, young buds as well as mixture of stalks and leaves (50:50%, *w:w*). They were extracted for 80 min with 30% ethanol in water solution.

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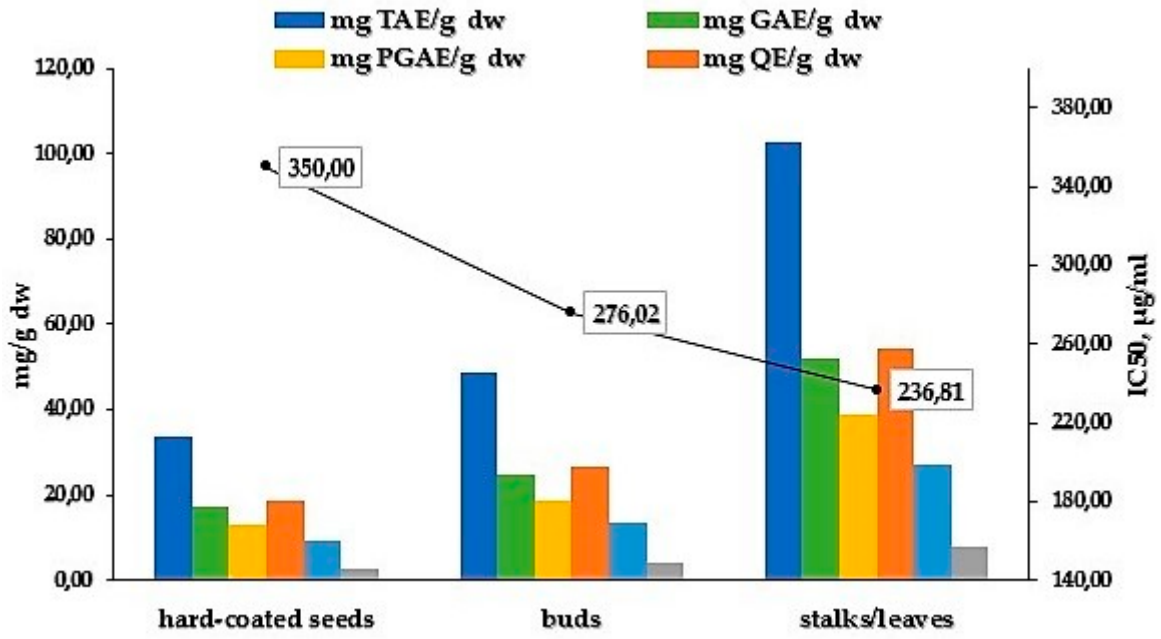

Figure 4. Evaluation of total polyphenol, flavonoid content and DPPH antioxidant capacity of *Cistus incanus* hard-coated seeds, buds and mixture of stalks and leaves.

The total polyphenol and flavanoids in the buds and in the hard-coated seeds give good results. The buds should contain much more of the desired components of the seeds because they are picked up during plant flowering, when it is in its polyphenol power. It is known that the woody parts of the aromatic herbs contain also flavanoids and polyphenols playing an important role in protecting the plant. As shown in Figure 4, the mixture of leaves and stalks, in ratio 50:50, gives the best results, which is normal because the main quantities of polyphenols are concentrated in the leaves. The obtained results show that the hard-coated seeds, buds and stalks also can be used as a row material for production of antioxidants in the nutraceutical industry or for making a tea (infusion) at home.

5.8. Evaluation of the *Cistus incanus* winter and summer leaves

In this study, were compared mixtures in mass percent concentration of 90:10 of *Cistus incanus* leaves and stalks picked up in summer and winter harvest seasons by yield of antioxidants. The samples were extracted for 80 min with the 30% ethanol in water solution. It is known that the wild plant is evergreen shrub which blooms from May to September, and then it is assumed that the flavanoids and polyphenols reach their highest value. The data of other authors about polyphenolic content and AOA of *Cistus incanus* gathered through the winter is missed. This can be confirmed by the results obtained and summarized in Figure 5.

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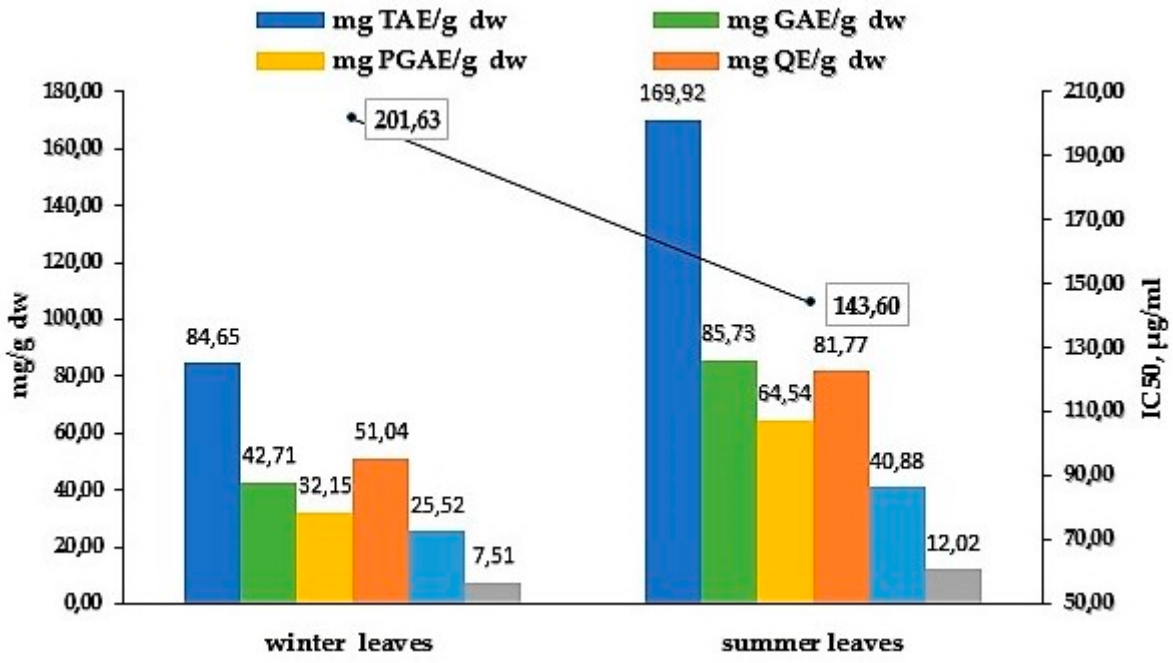

Figure 5. Evaluation of total polyphenol, flavonoid content and DPPH antioxidant capacity of *Cistus incanus* summer and winter leaves

Summer *Cistus incanus* leaves and stalks extract give better antioxidant capacity - 143.60 µg/ml (IC50) or 579.70 µmol TEAC/g dw. The extract of winter sample gives as good results for antioxidant capacity 201.63 µg/ml (IC50) or 377.93 µmol TEAC/g dw. This means that *Cistus incanus* from Strandja should be collected and used even during the winter season.

6. Conclusions

It can be concluded that the sub endemic plant - *Cistus incanus* growing in all Strandja Mountain content high values bioactive components, not only in picked up in summer or winter leaves but in its stalks (woods parts), buds and hard-coated seeds. The results showed that 30% ethanol in aqueous extracts gave the highest content of total polyphenols and flavanoids, albeit with prolonged extraction. Additionally, the antioxidant activities were well correlated with the content of the extracted bioactives.

This study is an initial step of the extraction evaluation of *Cistus incanus*. A further optimization is possible and necessary for total process evaluation for example - decreasing the size of particles, changing the extraction method or increasing the extraction temperature and all that to decrease the obtained long extraction time in respect with increasing costs.

Our results provided better understanding of the high value antioxidant potential of the Bulgarian *Cistus incanus* to be applied in the food, cosmetic, and drug fields.

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