ANTIOXIDANT ACTIVITY AND POLYPHENOLIC CONTENT OF THE BULGARIAN WILD HERB Cistus incanus L. STORED UNDER DIFFERENT CONDITIONS

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ABSTRACT

Present study investigates the effect of storage (at room temperature and at a frozen state) of Cistus incanus leaves and its hard-coated seeds on the total polyphenol content and the antioxidant capacity in view of the extraction carried out in 50 % ethanol-in water solution. A correlation between the polyphenol content and the antioxidant activity of the leaves stored at room temperature is elucidated. The frozen stored material generates better extraction efficiency and keeps a very high antioxidant activity.

This investigation demonstrates that Cistus incanus from Strandja Mountain, Bulgaria is an important source of polyphenols and antioxidant activity. The latter is present in leaves stored for a couple of years. The results from this study contribute to better understanding the extraction process of this non-traditional Bulgarian herb.

Keywords: Cistus incanus, Strandja, antioxidant capacity, polyphenol content, tannins, total dry residue, frozen state storage.

INTRODUCTION

Cistus incanus and other species of Cistaceae family are used in the Mediterranean folk medicine since hundreds of years as a general remedy for treatment various diseases [1]: skin diseases, rheumatism, fever and diarrhea. The small evergreen shrub is widespread in Strandja Mountain, Bulgaria [2], but it has not been used as a natural remedy neither in the past nor now. Data from recent studies show that Cistus extracts have pharmacologically beneficial effects on inflammatory or infectious diseases and confirm their strong gastric anti-ulcer activity, gastroprotective effects [3], antimicrobial [4], antitumor [5], antiviral, anti-inflammatory, hypotensive, skin care, antispasmodic activities [6], in-vitro cytotoxic activity of Cistus creticus [7] and Cistus monspeliensis [8] against several human leukemia cell lines. It is also reported [9] that Cistus incanus and Cistus monspeliensis leaves extracts show antibacterial and antifungal activities.

The research interest towards active compounds, especially polyphenols, of a natural source (herbs, seeds, fruits, vegetables) has greatly increased in recent years [10, 11]. Phenolic compounds, principally flavonoids and phenolic acids, are the main antioxidants in human diet. The estimated daily total dietary intake has increased from 20 mg to 1 g [12]. For that reason, plants containing natural antioxidants are used as an important source of new drugs formulations. Some of Cistus species are well-known around the world and they are studied in details. Cistus incanus infusions and products containing extracts are notable examples for such polyphenol-rich food supplements because many research studies have demonstrated that the main
biologically active components of the wild herb refer to polyphenolic compounds such as gallic acid, rutin, five different flavonoid glycones (some of them based on quercetin, kaempferol, and glycoside compounds belonging to the flavonol family), flavan-3-ols as well as catechin, epicatechin [13, 14], galloecchin, galloecchin-3-gallate together with the oligomeric procyanidin B1 and B3 [9]. In addition, proanthocyanidins and genetically related dihydroflavonol [13] and shikimic acid, dimer prodelphinidin [15], and other polyphenols [16] are found in Cistus incanus extracts. For that reason, a few companies offer worldwide “Hairy Rockrose” tea, essential oils, antiseptic sprays, tablets and capsules. There is information on the chemical composition of Cistus incanus in the literature but no data is reported on the kinetics of its extraction. It is well recognized that phenolic compounds extraction depends on multiple factors referring to the samples chemical nature, particles size, storage conditions and periods. Furthermore, it depends on the quantification methods chosen, the standards applied, interferences presence [17].

The antioxidant properties of the sub-endemic species Cistus incanus are not yet studied at our longitudes. Thus, its beneficial properties could differ due to the tendency to polymorphism or photochemical composition alteration because of the different environmental factors, conditions and seasonality. Cistus incanus is not treated as a medicinal plant in Bulgaria and its resources can be used indefinitely. Besides, the hard-coated seeds of the plant are never examined. Their eventual antioxidant power could make them a new source of potential bioactive compounds.

The aim of this study is to explore the antioxidant potential and the medicinal benefits of 50 % ethanol extracts of Cistus incanus L. stored under different conditions (even for some years) in order to reveal opportunities for the plant to be used in herbal supplements production by Bulgarian nutraceutical companies.

EXPERIMENTAL

Materials and methods

All analyses were conducted with 50 % ethanol-in-water solution. The total phenolic content (TPC) was determined by the method of Folin Ciocalteau [22] referring to measurement of samples spectral absorption at 765 nm. The antioxidant activity (AOA) was examined spectrophotometrically [19] using DPPH assay at 517 nm. The tannins concentration was measured by titration with a potassium permanganate solution [24]. The total dry residue (TDR) was found gravimetrically [25]. The procedure included evaporation of the liquid phase of 10 ml extract, drying of pressed solid phase to a constant weight in an oven at 105°C. The loss on drying (LOD) and the total ash (TA) were determined also gravimetrically [26, 27]. Aiming this samples of 2.0 g of the powdered drugs were dried to a constant mass and subsequently ignited.

Chemical reagents

The ethanol (96 %) used was provided by Valerus, Bulgaria. HPLC grade methanol, sodium carbonate (> 99 %), gallic acid anhydride (> 99 %) and sulfuric acid (98 %) were supplied by Merck, Germany, while the Folin-Ciocalteau reagent (2N solution), 2,2-diphenyl-1-picrylhydrazyl (DPPH), indigo carmine, potassium permanganate (0,1N aqueous solution, F = 0,9985) were supplied by Sigma Aldrich, Germany. Deionized water obtained by Elix70C Gulfstream-Merck water deionizer was used in all experiments.

Plant samples

Young wild Cistus incanus leaves and hard-coated Cistus incanus seeds collected in August 2014 were used for this study. The herb was gathered by the end of the flowering period in a region close to village Bulgaria (Tsarevo municipality) following the conservation rules in respect to the biodiversity of the National Park Strandja, Bulgaria. They were recognized by the experienced biologists working in the park. The leaves and the hard-coated seeds were dried at room temperature (the state is designated as RTS) and kept in dry atmosphere until further use. Some the leaves collected were stored at -18°C in a freezer (the state is designated as FS). They were dried for a week prior to examination. All samples used were stored for a period of two years.

Sample preparation and extraction conditions

The samples were studied two years after preparation. They were grounded in a grinder and sieved. A fraction of a particle size of 0,5 - 2,0 mm was used for
the experiments carried out. Samples of 2g (± 0,0005 g) dried leaves (LOD = 9.27 %, TA = 5.69 %), dried hard-coated seeds (LOD = 10.2 %, TA = 3.80 %) and dried frozen leaves (LOD = 10.0 %) of Cistus incanus were mixed with 50 % ethanol-in-water solution at a solid-to-liquid ratio $\zeta = 0,05$ g ml$^{-1}$. The extraction temperature used was 25°C (± 2). The samples were mixed in a laboratory shaker (model THUS 2) using a rotation speed of 320 rpm. Each point of the kinetic curve was obtained independently under identical conditions referring to the liquid volume, the temperature, the solid–to-liquid ratio, the shaking, but at different duration of the extraction procedure. The exhausted raw material was carefully pressed, while the extract was filtered through cotton and Whatman No. 1 filter paper and analyzed immediately following appropriate dilutions.

**Antioxidant activity by the DPPH method**

The DPPH method is the most commonly used one for antioxidant activity quantification (AOA). It is introduced by Brand-Williams, Cuvelier, and Berset [18] and later modified by Sánchez-Moreno, Larrauri, and Saura-Calixto [19]. DPPH solutions show high absorption at 517 nm due to the deep violet color. The absorbance gradually disappears as a result of discoloration depending stoichiometrically on the degree of free radicals reduction. The remaining DPPH measured after a certain time corresponds inversely to the antioxidants scavenging ability in respect to free radicals.

One thousand microliters of ethanol extracts of varying concentrations were added to 4 mL of 0.004 % methanol solution of DPPH. After an incubation period of one hour at room temperature, the absorbance was measured at 517 nm against a blank sample. All spectrophotometric data was acquired using a UV-VIS-spectrophotometer (T60UV/VIS ver. 1.0) using a 10 mm path length cuvette. The antioxidant activity defined as the extract concentration required neutralizing 50 % of the free radicals present (IC$_{50}$) was calculated on the ground of the correlation between the extract concentration (ml L$^{-1}$) and the inhibition observed (C/I, %). The graph was obtained using extracts of a varying concentration (0.05 μg ml$^{-1}$ - 0.25 μg ml$^{-1}$).

The scavenging ability of the tested samples in respect to the free radicals present was calculated using the formula (Yen & Duh) [20]:

$$IC \% = \left( \frac{A_o - A_a}{A_o} \right) \times 100$$  \hspace{1cm} (1)

where $A_o$ was the average absorbance value of the blank sample, $A_a$ was the average absorbance value AOA, while IC was the inhibition capacity (in %). After recalculation, the results were presented as IC$_{50}$ values (μg ml$^{-1}$).

**Total polyphenolic assay obtained by the Folin-Ciocalteu method**

The Folin-Ciocalteu method (FCM) is usually applied to assess the total phenolic content of the plant extracts [21]. The Folin-Ciocalteu phenol reagent is used.

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$^{-1}$ with 50 % ethanol; this was done in case of all analyses) and 1.58 ml of deionized water. A minute later 0.3 ml of a 20 % Na$_2$CO$_3$ solution was added to the tube. The samples were kept in dark for two hours and then the absorbance was measured at 765 nm against the reagent blank [22].

The results were calculated as gallic acid equivalents using a standard curve: Abs = 1,016.x ($R^2 = 0.9984$) obtained with standard solutions of gallic acid (0.1 mg ml$^{-1}$- 1.0 mg ml$^{-1}$). The total phenolic content of the Cistus incanus extracts was expressed as milligrams of Gallic acid equivalents per gram dry weight (mg GAE/g dw) using the following expression:

$$C = C_{ga} \times V_e \times F / M$$  \hspace{1cm} (2)

where $C$ was the total phenolic content (mg GAE/g dw), $C_{ga}$ was the concentration of galic acid (mg ml$^{-1}$), $V$ was the volume of the solvent used (L), $F$ was the dilution coefficient of the sample, while $M$ was the mass of the sample (g).

**Tannins content**

The antifungal and antiviral activity of Cistus incanus extracts is associated with the presence of condensed tannins (i.e. proanthocyanidins). Tannins are high molecular weight phenolic compounds comprised of gallic ester of flavan-3-ol polymers [23].
The total water-soluble tannins content (T) was determined following the corresponding European pharmacopoeia method and its subsequent modifications [24]. An aliquot of 50 ml boiling water was added to 2 g of powdered drug and the mixture was heated in a water bath under frequent stirring. The liquid was allowed to stand for a few minutes and carefully filtered through cotton in 250 ml flask. A portion of new 50 ml of boiling water was added to the sample and the procedure described above was applied. This sequence continued until the reaction of the filtrate with 10 g L\(^{-1}\) NH\(_4\)Fe(SO\(_4\)) solution gave a negative reaction, i.e. no blue-violet color was observed. After cooling, the filtrates were diluted with deionized water to 250 ml. 25 ml of the sample extract were mixed with 25 ml of an indigo carmine solution and 750 ml deionized water. Then, the blue solution was titrated with 0.02 mol L\(^{-1}\) potassium permanganate until golden-yellow coloration. In addition a mixture of 25 ml of the indigo carmine solution and 750 ml of deionized water was titrated as blank. The calculations based on the average value (n = 3) obtained were carried out on the ground of the equation:

\[
T(\%) = \left( V - V_o \right) \times 0.004157 \times 100 / M \times 250
\]  

(3)

where \(V\) is the volume of 0.1 N water solution of KMnO\(_4\) for sample titration (ml), \(V_o\) is the volume of 0.1 N water solution of KMnO\(_4\) for titration of the blank sample (ml), the value of 0.004157 refers to the tannins equivalent in 1 ml of 0.1 N water solution of KMnO\(_4\), \(M\) is the mass of the sample (g), the figure of 250 stands for the volume of volumetric flask (ml), while the figure of 100 is introduced to obtain the tannins content in %.

**Extracts total dry residue**

The total dry residue of the extracts was determined in accordance with the corresponding European pharmacopoeia method and its modifications [25]. Rapidly exhausted drug and 10 ml of the extract examined were introduced to flat-bottomed dishes. The samples were dried at 105°C in an oven (“Robotica”, Velingrad) to a constant mass and subsequently cooled in a desiccator under anhydrous silica gel R and weighted. The results were presented as grams of a dry mass.

**Loss on drying and total ash**

The loss on drying and the total ash of the selected species were evaluated gravimetrically following the corresponding European pharmacopoeia method. About 2.0 g of the powdered drug were dried at 105°C in an oven (Memmert 100-800, Germany) to a constant mass [26] or ignited in a muffle furnace (Linn High Term, VMK, Germany) at 600°C ± 25°C.

It is worth noting that the total ash content determination refers to a process of mineralization. The residues after A sample is completely burnt and the residues obtained consist mostly of metal oxides, metal salts containing Na\(^+\), K\(^+\) and Ca\(^{2+}\). They include also trace minerals required by molecules like chlorophyll and hemoglobin [27].

**RESULTS AND DISCUSSION**

Detailed literature research on phenolic compounds present in *Cistus incanus* leaves and hard-coated seeds is carried out. There is no data referring to the selected drugs antioxidant activity and polyphenolic content. The effect of the storage conditions and prolongation on *Cistus incanus* leaves antioxidant power and the polyphenols content has also not been studied.

It is recognized that the extracted polyphenols yield depends on the chemical composition and physical characteristics of the samples but also on the type of the solvents and their polarity. According to literature data [28] the extraction of *Cistus incanus* is usually carried out with a mixture of water with methanol or ethanol. The latter is a good solvent for polyphenols extraction. It is safe and non-toxic compared to other solvents and that is the reason to use it in the present study.

**Total dry residue**

The total dry residue (TDR) of *Cistus incanus* leaves stored at room temperature in a solid and liquid state is subjected to extraction. The increase of TDR in the liquid phase (the extract) corresponds to TDR decrease in the solid phase. The kinetics of the process is examined. The results are expressed in grams dry weight against extraction time. The kinetic curves obtained are illustrated in Fig. 1.
The water presence (9.27 %) and the total mineral content (5.69 %) are not recalculated and hence the presence of volatile substances is quite probable. The probable yield of the mass extracted in grams of dry extract per liter is calculated because it is an essential parameter in correspondence with extracts industrial production. As shown by Fig.1 the kinetic curves are characterized by an initial steep section corresponding to the dissolution of the readily available substances on the samples surface, a second one most probably explained by the simultaneous dissolution of the substances left on the surface and those from the samples bulk (the mixed zone control) and a plateau reached approximately at the 80th min, where the total dry residue quantity is 10.86 g L\(^{-1}\). The latter increases slowly until the 220th min. The value reached refers to 11.55 g L\(^{-1}\). The further prolongation of the experiments until the 390th min leads to TDR of 12.15 g L\(^{-1}\) or 10.8 % increase when compared to that at the 80th min. The values presented provide to conclude that 80 min is the optimal extraction time in relation to the yield obtained. The last section of the kinetics curves corresponds to the complete consumption of the substance on the surface and that from the internal pores. The equilibrium reached can be affected by eventual extraction of poorly soluble compounds under the conditions provided or by the incomplete extraction performed in this examination.

**Antioxidant capacity**

The thorough AOA kinetic study of *Cistus incanus* leaves extracts is performed only with leaves stored at RTS. Standard curves are obtained in accordance with free radicals decrease by DPPH. The interpretation of the DPPH assay results requires the introduction of the half maximal inhibitory concentration (IC\(_{50}\)). It is defined as the inhibitor concentration reduced to one half. Thus, the lower IC\(_{50}\) value corresponds to better expressed antioxidant properties. Fig.2 presents the variation of the inhibition capacity at 50 % of IC\(_{50}\) (expressed as an extract concentration in the range of 368.74 μg ml\(^{-1}\) - 120.17 μg ml\(^{-1}\)). The plateau is reached approximately at the 80th min (136.43 μg ml\(^{-1}\) as in the case of the kinetic curve shown in Fig. 1. The best result is obtained at 220th min referring to a concentration of 120.17 μg ml\(^{-1}\). The latter, which can reduce 50 % of the free stable DPPH radicals is 6.7 times lower than that of IC\(_{50}\) of *Cistus incanus* essential oil reported in 2013 by Loizzo et al. [29] as equal to 814.70 μg ml\(^{-1}\).

The lower values of IC\(_{50}\) values indicate higher AOA or higher DPPH radical scavenging power. It is evident from Fig.3 that the best results for AOA are obtained for extracts from leaves stored at FS, followed by those from leaves stored at RT and hard-coated seeds. It can be also seen that the best results are obtained in case of 220 min extraction. It is worth noting that the results obtained for other periods of extraction are in fact quite close.
The TPC kinetics of *Cistus incanus* (stored at RTS, FS) leaves extracts and *Cistus incanus* (stored at RTS) hard-coated seeds extract is followed. The results are presented in Fig. 4. and Fig. 5. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the DPPH reagent. FCM actually measures the total reduction capacity of the sample [30].

The amounts of total polyphenols in RTS stored leaves extracts vary from 41.73 mg GAE/g dry weight up to 98.69 mg GAE/g dry weight. The lowest phenolic content values as well as AOA are detected at 5th min, while the highest - at 220th min as shown in Fig. 4. There is another maximum at the 80th min which refers to 89.28 mg GAE/g dw.

On the other hand, the extract from FS leaves reaches a stable value after 180 min. The value of the total polyphenols content found is 110.66 mg GAE/g dw (11.07 %) or with 10.9 % higher than that obtained for 220 min shaking of the extracts of RTS stored *Cistus* leaves (Fig. 5). The analysis [31] of an extract of the „pink rockrose“ (CYSTUS® 052 manufactured by *Cistus incanus* PANDALIS) shows 26% content of polyphenols.
and less than 2% presence of monomers (gallic acid, epigallocatechin, catechin and epicatechin).

This assay is specific not only for polyphenols but for 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 [19]. It can be concluded that Bulgarian Cistus incanus has a lot of potential to reveal.

**Tannins**

Tannins are a subfamily of polyphenols having the ability to precipitate proteins. Tannins have been used since ancient times in tannery industry and have important cosmetic applications. Traditionally Cistus species have been used as antidiarrheic remedy revealed through shrinkage of intestine mucous membranes and decrease of mucous secretions. It is well established that tannins exhibit antioxidant activity. Tannins do not act just as primary antioxidants, which donate hydrogen atom or electrons. They also play the role of secondary antioxidants. Tannins have the ability to chelate metal ions such as Fe (II) and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation [32]. On the other hand differences in solubility are likely to affect their biological functions [33]. There is a significant amount present in the total dry matter, i.e.
5.71% of water-soluble tannins of *Cistus incanus* leaves stored at RTS. The value obtained is comparable to those reported for species such as *Cistus ladanifer* and *Cistus populifolius* whose values refer to 6.8% and 8.2%, respectively [34]. The concentration of tannins in plants is not only species-specific but it also depends on soil fertility and pH, light intensity, plant age or temperature stress [35]. All these factors determine the different tannins’ concentrations of the plant species and make their comparison complicated.

**Correlation between AOA and TPC**

Generally, extracts of a high polyphenol content exhibit also a high antioxidant activity. The correlation between AOA and TPC of *Cistus incanus* leaves stored at RTS is calculated using MATLAB. The results are presented in Fig. 6. The curve obtained is described by the following equation:

\[ Y = -0.0817X + 0.5197 \]  
\[ (R^2 = 0.667) \]

where: \( Y \) – AOA, g per litter; \( X \) – TPC, g GAE per litter.

The antioxidant activity of phenolics is mainly due to their redox properties which allow them to act as reducing agents and hydrogen donors. They may also have a metal chelating potential [36]. The results suggest that 67% of the antioxidant capacity of *Cistus incanus* extracts is due to the contribution of phenolic compounds (\( R^2 = 0.667 \)). It can be concluded that the antioxidant activity of the plant extracts is not limited to phenolics. It may also be due to the presence of other antioxidant secondary metabolites as volatile oils, carotenoids, and vitamins.

**CONCLUSIONS**

The investigation reported shows that *Cistus incanus* from Strandja Mountain, Bulgaria and its hard-coated seeds are important sources of polyphenols. The plant leaves have also a significant antioxidant activity. The extraction yield of polyphenols from leaves stored at FS is higher and discharge of these high-added value components in 50% ethanolic solution is faster than that of the extracts of the leaves stored at RTS. This means that freezing even for some years provides good storage conditions of plants rich in polyphenols and antioxidants such as a *Cistus incanus*. Further studies are required to isolate and identify the chemical compounds that contribute to the total antioxidant activity. This study is an initial step to be followed by further optimization of the extraction process.

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