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EXTRACTION OF VALUABLE COMPOUNDS FROM BULGARIAN ST. JOHN'S WORT (*HYPERICUM PERFORATUM L.*). ANTIOXIDANT CAPACITY AND TOTAL POLYPHENOLIC CONTENT

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ABSTRACT

Ethnobotanical reports claim that St. John's Wort has important biological and chemical properties and can be used for the treatment of many diseases. Most studies related to Hypericum perforatum are focused on its antidepressant effects, but it has also been examined for wound healing, antiviral and antimicrobial activity. In this study the experiments are carried out at ambient temperature at constant stirring rate. The solvent is a 50 vol. % water-ethanolic solution, the solid-to-liquid ratio is 1:10 g mL^{-1}, and the average particle size is 0,75 mm. The results show that the antioxidant capacity and the total polyphenolic content are superior compared to those reported by other authors. The liquid extracts have been stored in a refrigerator for 9 months, then they have been examined again and it has been noticed that their antioxidant effect and total polyphenolic content are preserved. After drying at 50°C, storage and regeneration, the quality of the extracts did not change, which is important issue for long term transportation and storage.

Keywords: Hypericum perforatum, extraction, total polyphenolic content, antioxidant activity, total dry, storage, regeneration.

INTRODUCTION

Oxidative damage of biological molecules in the human body is involved in degenerative or pathological processes such as ageing, coronary heart disease, cancer, artheriosclerosis and rheumatism [1]. In the past decade, lots of epidemiological studies have confirmed that intake of exogenous antioxidants is effective in preventing or suppressing such diseases [2, 3]. The use of naturally occurring antioxidants (mainly phenolic compounds) has attracted considerable attention and an increasing interest to prevent oxidative damage [4].

Medicinal plants are widely used in everyday life as part of folk medicinal remedies in the whole world, including Bulgaria. Bulgarian flora is remarkable for its diversity and it is a rich source of medicinal plants - 600 of 3500 plant species are known to be medicinal [5 - 10]. Little is known about the antioxidant potential of Bulgarian medicinal plants and the usefulness of many of them for the modern therapy [11].

Hypericum perforatum L. (St. John's Wort, Hypericaceae) belongs to the genus *Hypericum*, of which there are 400 species worldwide [12]. The flowers are profuse, arranged in branched cymes which bloom from June until September [13]. The plant has a long history of more than 2400 years and has enjoyed a reputation as a wound healer since the fifth century B.C. It can be found in the herbals of Gerard (1597) [14], and Withering (1796) [15].

Gerard [14] a famous English herbalist, referred to a St. John's Wort salve, which he formulated using the herb, as the best and most precious natural wound-healing therapy available.

Among the first most effective pharmaceutical uses of *H. perforatum* in Europe after the 16th century was the use of the distilled oil of the herb as a therapy

for wounds and bruises. It was so effective that it was included in the first official pharmacopoeia of London as Oleum Hyperici [16, 17]. Culpeper (1652) [18] pointed out the unique wound healing properties of the herbal ointment. He also claimed the beneficial properties of the herb against stings and bites of poisonous animals. Recently, the consumption of *H. perforatum*-derived products has increased dramatically, and it is presently one of the most consumed medicinal plants in the world [19, 20]. A survey of the recent literature showed that data on the antioxidant activity of all of the known varieties are rather scarce [21 - 24]. Most recent interest in *H. perforatum* has focused on its antidepressant effects [25 - 27]; however, the herb has shown other activities including antifungal [28 - 31], anti-inflammatory [32, 33], antimycobacterial [34, 35] and antiviral activities [36]. Natural antioxidants are considered to be multifunctional.

The aim of this study is to evaluate the antioxidant activity and the phenolic content of herbal extracts prepared from Bulgarian *Hypericum perforatum* collected from Dabovitsa, Kotlenski Balkan, Bulgaria and from commercial *Hypericum perforatum*. The results have been compared and the stability of the extract solutions has been determined in order to find whether the extracts are suitable for use in cosmetic, pharmaceutical and food products.

EXPERIMENTAL

Materials and methods

Plant material

Wild *Hypericum perforatum* collected from Dabovitsa, Kotlenski Balkan, Bulgaria and cultivated *Hypericum perforatum* from the market, produced by BILEC company, m. Lisichi dupki 5600 Troyan, Bulgaria (batch number L 010615) have been used. The cultivated material consisted of dried and shaded leaves and flowers of St. John's wort. The wild material shoots with full opened flowers were collected in July 2015, and were dried according to the Bulgarian traditional method: bunches of 20 - 25 branches have been tied and suspended in a dry, tempered and ventilated place for 3 months. Then the plant has been grinded with a domestic coffee grinder ENIEM M40. The grinded material has been separated on fractions using a laboratory sieve shaker. The ethanolic extracts have been prepared using plants with average particle size diameter of 0,75 mm.

The dried materials were examined for total polyphenols and antioxidant activity through the Folin-Ciocalteu method (FCM) and the DPPH stable radical scavenging activity.

The extracts have been prepared with 2 g of the dried and grinded herb added to 20 mL ethanol-in-water solution (50 vol. %) and then stirred with a Bio Magnetic stirrer for 2, 5, 10, 15, 30, 45, 60, 90 and 120 minutes in order to determine the kinetics of the process and to find the optimal conditions for the best yield. The extracts have been placed in dark glass bottles and stored in a domestic refrigerator "Whirlpool" at a temperature 0°C - 4°C. Nine months later they have been examined in order to identify the influence of the storage period on the antioxidant activity and total phenolic content.

Chemicals

The solvents used (ethanol and methanol) were of analytical grade (Valerus Ltd, 1592, Sofia, Bulgaria). Gallic acid - anhydrous (Batch: 36245349111/$C_7H_6O_5$/ Assay > 99 %/ and sodium carbonate were from Merck, Germany, DPPH – (2,2- Diphenyl-1-picrylhydrazyl) - from Sigma-Aldrich Co, Germany, Folin & Ciocalteu's phenol reagent – from Sigma- Aldrich, Co., Switzerland.

The measurements were carried out using spectrophotometer PG INSTRUMENTS T60 UV-Vis spectrophotometry (United Kingdom). Bio magnetic stirrer MMS- 3000 (Boeco, Germany), domestic refrigerator Whirlpool, analytical balance – Sartorius analytic and Dryer - Diterm (Robotika, Velingrad, Bulgaria) also have been used.

Measurement of the reduction of the DPPH radical

The reactivity of the extract with DPPH was estimated according to the method described by Brand-Williams, Cuvelier, and Berset [37] later changed by Sánchez-Moreno, Larrauri, and Saura-Calixto [38].

The antioxidant capacity is defined as antiradical activity on a stable form of synthetic product DPPH. Incubation was performed at room temperature, and the absorbance at 517 nm was determined spectrophotometrically. The absorbance gradually disappears as a result of discoloration, which is stoihiometric with respect to the degree of reduction of the free radicals [39].

The antioxidant activity was defined as the

concentration of the extract neutralizing 50 % of the free radicals - IC50 and is calculated by plotting the graph: concentration of the extract (mL L^{-1}) vs inhibition (%) [40].

DPPH scavenging of the tested samples is expressed in % inhibition of free radicals and is calculated using the equation (1) (Yen & Duh) [37]:

$$IC,\% = [(A_{control} - A_{sample}) / A_{control}] \times 100 \quad (1)$$

where

$A_{control}$ - an average of the absorbance of the control sample;

A_{sample} - average value of the absorbance of the samples;

IC- antioxidant capacity or scavenging.

Determination of the total phenolic compounds by the method of Folin-Ciocalteu

The total phenolic content has been determined by the Folin&Ciocalteu's colorimetric method of Singleton et al. (1999) with modifications. The results were expressed as milligrams of gallic acid equivalent per gram of dry weight of the sample. Folin-Ciocalteu method (FCM) is commonly used only to assess the total number of phenolic compounds in plant extracts [41]. FCM actually measures the total reduction capacity of the sample. The determination correlates well with redox and antioxidant capacity of phenolic compounds. Dissociation of the phenol proton to the phenolate anion makes it capable of reducing the FC reagent. FCM is nonspecific for phenolic compounds. Many non-phenolic compounds in fruit, for example, ascorbic acid and saccharides can reduce the amount of reagent [42].

The content of polyphenols in the raw plant material, respectively, resulting from the dry extract, are determined by the equation (2):

$$C_{pph} = (C_{ga} \times V_e) .100 / M, \% \quad (2)$$

where:

C_{ga} - concentration of polyphenols in the extract liquid, defined by the equation of the graph $A = f(C_{ga})$, g L^{-1};

V_e - volume of extractant used in the extraction of solid sample (raw or dry extract), L;

M - mass of the solid sample, g.

The statistical analysis was performed using the Microsoft Office Excel 2013 (Microsoft Corporation) program.

RESULTS AND DISCUSSION

Total phenolic compounds

All the extracts studied show an abundance in polyphenolic compounds (Table 1). The extracts prepared from wild *Hypericum perforatum* exhibit higher concentrations than those prepared from commercial plant. Phenolic compounds have a wide range of polarity and thus, both organic and aqueous extracts show significant phenolic concentrations. It is known that the organic extracts are richer in constituents being in higher quantities, as compared to the aqueous counterparts [43]. For this reason, in our study we have chosen to work with a mixed solvent.

It was found that the total phenolics in the extracts ranged from 10,20 ± 1,23 to 53,84 ± 4,04 mg GAE g^{-1}. Such results are in accordance with literature data. Abdelhadi et al. obtained 21,32 ± 0,02 mg GAE g^{-1} for

Table 1. Total polyphenolic content of fresh ethanolic extracts of wild *Hypericum perforatum* and extracts stored in a refrigerator for 9 months.

Time, min	Fresh extracts TPC, mg GAE g^{-1}dw	Stored extracts TPC, mg GAE g^{-1}dw	Average mg GAE g^{-1}dw
2	9,33	11,07	10,20 ± 1,23
5	10,65	11,69	11,17 ± 0,73
10	12,86	20,69	16,78 ± 5,53
15	27,36	33,43	30,39 ± 4,30
30	31,64	29,56	30,60 ± 1,47
90	47,40	53,60	50,50 ± 4,38
120	50,98	56,69	53,84 ± 4,04

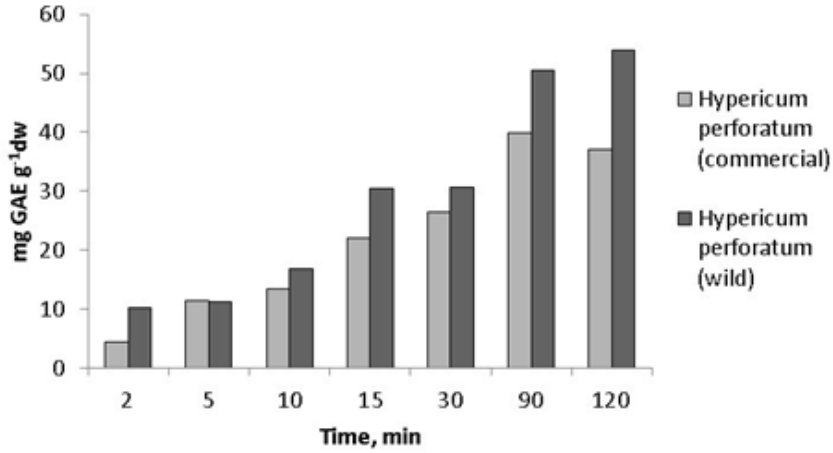

Fig. 1. Total polyphenolic content of wild and commercial *Hypericum perforatum.*

solvent free microwave assisted extraction and only 4,44±0,02 mg GAE g^{-1} for standard method [44].

The stability of the extracts over the time has been evaluated. The results presented in Table 1 confirm that there is no significant difference between the phenolic content of the fresh extracts and those stored for 9 months.

Some differences were noticed in the studies of the wild and cultivated species. Comparing the results of the commercial plant with those obtained for the wild herb, we have determined that the commercial plant contains less phenolic compounds, as shown in Fig. 1.

The higher content of phenolic compounds in the wild herb could be explained with the seasoning and location of gathering as well as the pretreatment processes. It has been noted that the commercial plant possesses more stems than the one collected by us. According to literature the aerial part (Herba Hyperici) must be collected at the beginning or during the blooming period by cutting of the leafy stems including the flowers and the flower buds of about 20 cm from the top. Overblown plants must not be collected [45]. We have collected the herb according to this procedure, but we can not be certain if the commercial plant has been collected accordingly. Observing the commercial dried material, we have noticed that there are too many stems compared to the leaves, flowers and flower buds. A gravimetrical analysis has been carried out in order to determine the ratio of the stems and the leaves and flowers of both commercial and wild plant. According to the experiment 30 % of the commercial plant are stems and 70 % are flowers, leaves and flower buds. At the same time the wild herb possesses 12 % stems and 88 % flowers, leaves and flower buds.

In Fig. 2 are compared the results obtained before and after regeneration of the extracts.

The experimental data illustrated on Fig. 2 show that the TPC of the fresh and dried/regenerated extracts are of the same order. There is certain disaccordance which can be attributed to an accumulation of experimental errors due to the equipment and the disposals. Nevertheless, we may assume that the drying of the extracts does not affect their properties and they may be reused after regeneration without drawbacks. Main advantage of this approach is the possibility to spare storage and transportation space and costs. Furthermore, the dried extracts are suitable to regenerate with different solvents which are acceptable for cosmetic, pharmaceutical, nutritional and drug industries.

Antioxidant activity

The antioxidant activity of the traditional Bulgarian *Hypericum perforatum* ethanolic extracts prepared from wild and commercial species was measured using the chemical assay of scavenging effect on DPPH free radicals. The results obtained are expressed as IC50 values (%) and are reported in Table 2. We have calculated the IC50 values which refer to the smallest concentration of antioxidants necessary for 50 % of reactivity. The lower the IC50 value the more reactive is the compound under study.

DPPH radical scavenging ability is widely used as an index to evaluate the antioxidant potential of medicinal plants. The extent of DPPH radical scavenging

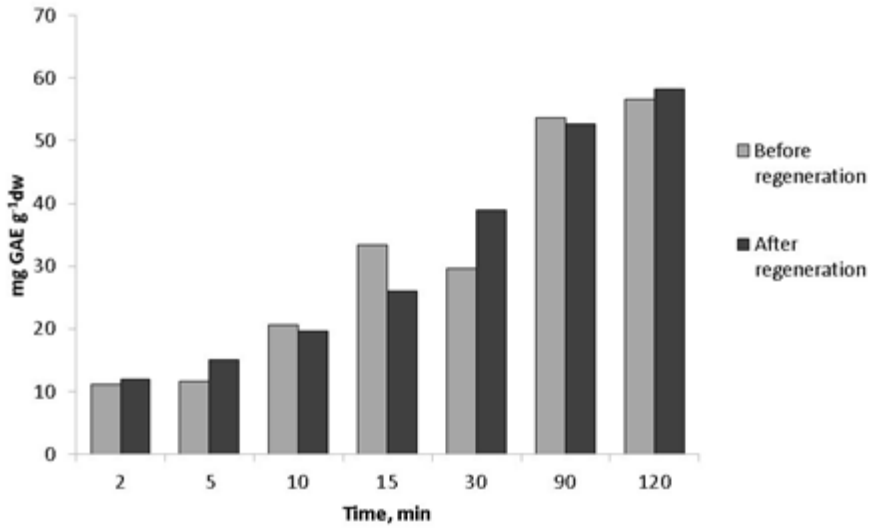

Fig. 2. TPC of the extracts before and after drying/regeneration.

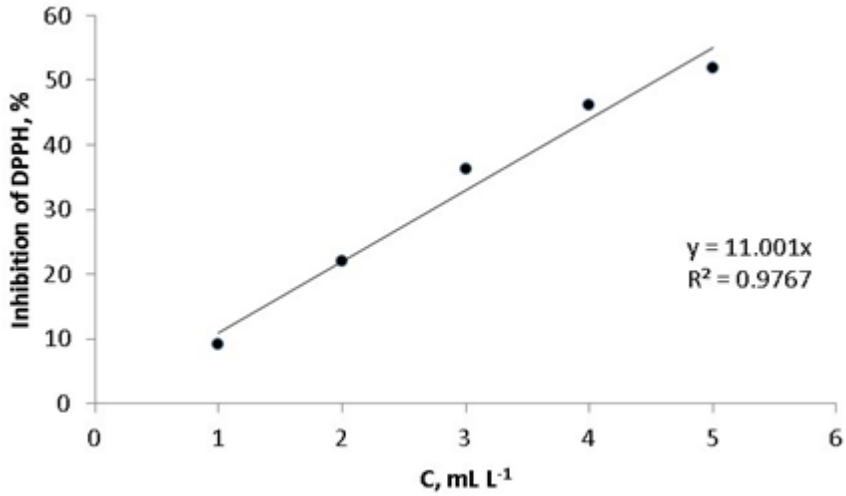

Fig. 3. Inhibition of DPPH vs extract concentration in the sample.

at different concentrations (1 - 5 mL L^{-1}) of *Hypericum perforatum* extracts was measured. The results are presented in Fig. 3.

Obviously the scavenging effect of the plant extracts show a concentration-dependent activity, especially for concentrations below 2 mL L^{-1}. The DPPH radical scavenging activities of extracts therefore increased gradually in a dose dependent manner. DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals. The results revealed that ethanolic extract of the wild plant exhibited the highest radical scavenging activity with 2,50 % followed by the commercial plant extract with 3,82 % (Table 2).

Table 2. Antioxidant activity of ethanolic extracts from wild and commercial *Hypericum perforatum L.*

Time, min	IC50, % (wild plant)	IC50, % (commercial plant)
2	11,15	26,68
5	10,10	10,70
10	6,85	9,64
15	4,88	5,82
30	4,88	4,58
90	2,87	3,38
120	2,50	3,82

Fig. 4. Discoloration of DPPH.

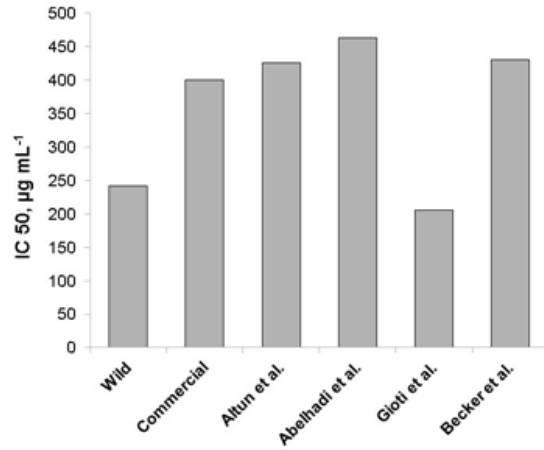

Fig. 5. Comparison between the IC50 (in µg mL^{-1}) values reported by different authors.

Depending on the scavenging power of the concentrations used the purple color of the test solution changes from light purple to yellow. This change is due to the presence of reducers such as compounds with antioxidant properties (Fig. 4). It can be noted that the DPPH solution has a deep purple color and it fades when an antioxidant is present. The increase of the concentration of extracts leads to disappearance of the purple color.

The results (at 120 min) were recalculated in µg mL^{-1} in order to compare the values with those reported by other authors (Fig. 5). It appears that the extracts from the wild herb showed approximately 50% lower IC50 (242,323 µg mL^{-1}) compared to IC50 of the commercial *Hypericum perforatum* extracts (400,499 µg mL^{-1}) indicating that the wild herb extracts are significantly more effective than commercial plant extracts against DPPH radical. Many authors obtained similar results. Altun et al. (2013) [46] obtained 65,22 % inhibition for an extract concentration of 500 µg mL^{-1} for methanolic extracts of Turkish species of *Hypericum perforatum*, Abelhadi et al. (2015) [44] obtained 50 % inhibition for 462,36 µg mL^{-1} for solvent free microwave extraction. The IC50 values determined by Gioti et al. (2009) [43] were comprised between 180 and 230 µg mL^{-1}. Becker et al. (2016) [47] obtained IC50 of 430 µg mL^{-1}. The differences could be attributed to intrinsic factors, mainly genetics and plant parts selected for extractions or extrinsic factors such as climatic factors, processing and storage conditions [48].

The stability of the antioxidants in the extracts had also been examined. The plant extracts were stored in a refrigerator for 9 months. The assays were repeated and no significant changes in the antioxidant activity have been noted as shown in Table 3.

The results summarized in Table 3 show that the antioxidant activity of *Hypericum perforatum* extracts prepared from wild plant are stable when stored in a refrigerator for 9 months. Most values deviate less than 10 % comparing the fresh extracts and those after 9 months of storage. Even though the samples were enclosed in dark glass bottles and stored in a refrigerator, we admit the possibility that because of the sealing some quantity of the solvent may be evaporated during the long conservation period.

Total dry kinetics

The variation of the mass of the initial plant material

Table 3. Antioxidant activity of fresh ethanolic extracts of wild *Hypericum perforatum* and extracts stored in a refrigerator for 9 months.

Time, min	IC 50, % (Fresh extracts),	IC 50, % (After 9 months),
2	11,15	10,73
5	10,10	8,19
10	6,85	6,75
15	4,88	4,49
30	4,88	3,44
90	2,87	2,74
120	2,50	2,49

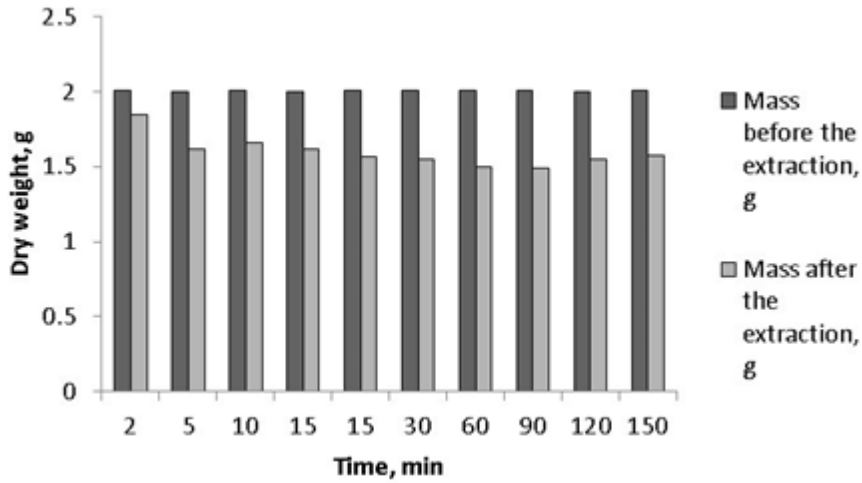

Fig. 6. Weight of the dry plant before and after the extraction from commercial *Hypericum perforatum L.*

vs time was examined gravimetrically after drying at 50°C until constant weight. This experiment was carried out in order to determine the diminishing of the mass of the raw material during the process due to the extracted substance as well as the eliminated water content (initial humidity of the plant). The results are expressed as grams dry weight against extraction time and are presented in Fig. 6.

A typical decrease of the mass of the solid substance due to the extracted active compounds as well as the eliminated water during the drying process was observed. Simultaneously the mass of the dried extracted substances increases as shown in Fig. 7.

The experimental data show a curve with a typical pattern for solid-liquid extraction. The steep part of the curve (approximately till the 20th min) corresponds to the fast kinetics when the leaching of valuable compounds is from the external surface of the plant material. Then the slope decreases which means that the substance is extracted from the interior of the particles where the access of the solvent is more difficult and the transfer to the solid-liquid interface is slower. About 30th min the curve reaches a plateau and the extracted substances decrease with the driving force.

A simple material balance of the initial raw material after processing and drying and the total dry extract gives an idea about the humidity of the plant, calculated as 14 mass % average.

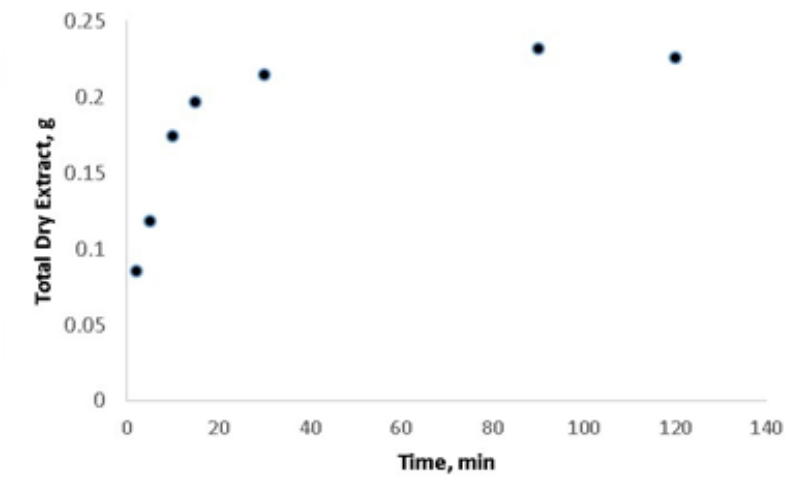

Fig. 7. Total dry yield kinetics.

CONCLUSIONS

A study of the antioxidant activity and polyphenol content of extracts produced from wild *Hypericum perforatum* collected from Dabovitsa, Bulgaria in comparison with commercial *Hypericum perforatum* from the Bulgarian market (produced by BILEC Company, m. Lisichi dupki 5600 Troyan, Bulgaria) has been carried out. The total phenolic content as well as the antioxidant activity of the wild plant is higher than those of the commercial plant. The results have been compared to those reported by other authors and it can be concluded that while the extracts from the commercial plant are in accordance to the majority of other ones reported, those produced from the wild herb exhibit higher antioxidant capacity and total polyphenolic content.

The ethanolic extracts from Bulgarian *Hypericum perforatum* possess high antioxidant activities. They are comparable to methanolic extracts, but ethanol allows the extracts to be used in pharmaceutical, food or cosmetics industries.

The examination of the long term stored liquid extracts reveals no significant change in the antioxidant capacity and total polyphenolic content. Drying and regeneration of the extracts with the same solvent also does not affect their properties. Both facts mentioned above give important indications for economically favorable ways of storage and transportation.

Future analysis on the qualities of the different aerial parts of the plant must be performed in order to identify which are the most useful ones and to prove that the collection of more than 20 cm from the top of the herb is unnecessary and might cause more damage to the environment than profit.

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