Content variability of bioactive secondary metabolites in Hypericum perforatum L.

Alessandra Carrubba*[1], Silvia Lazzara[2], Antonio Giovino[2], Giuseppe Ruberto[3], Edoardo Napoli[3]

[1] Dept. of Agricultural, Food and Forest Sciences (D/SAAF), University of Palermo; Viale delle Scienze, Build 4, Entr. L, 90128 Palermo, Italy. Email: alessandra.carrubba@unipa.it

[2] Council for Agricultural Research and Economics, Research Centre for Plant Protection and Certification (CREA-DC), SS 113 km 245.500, 90011, Bagheria (PA), Italy. Email: silvia.lazzara@crea.gov.it; antonio.giovino@crea.gov.it

[3] Institute of Biomolecular Chemistry, National Research Council (ICB-CNR), Via Paolo Gaifami 18, 95126 Catania, Italy. Email: giuseppe.ruberto@icb.cnr.it; edoardo.napoli@icb.cnr.it

*: corresponding author

Abstract

St John's Wort (*Hypericum perforatum* L.; *Hypericaceae*) is a perennial medicinal herb widespread and largely used in folk medicine inside the Mediterranean basin. Many bioactive compounds have been identified within its extracts. Under a pharmacological point of view, the most important of them belong to the chemical classes of naphthodianthrones, phloroglucinols and polyphenols. Many factors have been claimed responsible for the phytochemical variability in *Hypericum perforatum*, such as genotype, geographical origin, harvesting stage and age of the plants. Yet, when harvested plant material is addressed to the industry, the standardization of the active ingredients over cultivation years is a crucial issue. With the aim to detect the stability over years and genotypes of several bioactive *Hypericum* compounds, seven *Hypericum* biotypes retrieved from different Italian geographical areas were cultivated in 2015 and 2016, and their aerial flowering parts were analyzed. Naphthodianthrones (hypericin and its biosynthetic precursors), phloroglucinols (hyperforin and adhyperforin), and main polyphenols were determined by HPLC-DAD analysis. The results were statistically evaluated through ANOVA, and the stability over cultivation years of the tested genotypes was assessed. In rather all the examined metabolites, the ANOVA revealed a remarkable effect of both factors "year" (Y) and "provenance" (P), but the occurrence of significant "Y x P" interactions evidenced that the effect of climatic variability was often different according to the genotype. The evaluation of the stability level between years evidenced that only one biotype out of seven exhibited constantly higher-than-average amounts of rather all identified metabolites.

Keywords: *Hypericum perforatum*; St John's Wort; cultivation; phytochemical variability; secondary metabolites

1. Introduction

St John's Wort (*Hypericum perforatum* L.; *Hypericaceae*) is a perennial herb widespread and often sub-spontaneous inside the Mediterranean basin. According to the European Pharmacopoeia (IX ed.), the drug of *Hypericum* is represented by the dried flowered tops of the plant, that in the Mediterranean areas are traditionally used to prepare ointments endowed with a lenitive and wound-healing action. Due to its wide range of pharmacological activities, including antidepressant, antiviral, and antibacterial effects, *Hypericum perforatum* is one of the most consumed medicinal plants in the world (Linde, 1996), and its extracts are extensively used as phytopharmaceuticals and nutraceuticals. The active constituents of *H. perforatum* have been reviewed by several papers (Patočka, 2003; Napoli et al., 2018); the identified compounds belong to the chemical families of naphthodianthrones (Fig. 1A), phloroglucinols (Fig. 1B), flavonols (Fig. 1C), cinnamic acids (Fig. 1D), flavanols (Fig. 1E), and biflavonoids (Fig. 1F), along with a number of "minor" compounds. Naphthodianthrones, including hypericin and related compounds (pseudohypericin, protohypericin and protopseudohypericin) are typical of the genus *Hypericum* (Patočka, 2003), and represent the best known and most studied components of *H. perforatum* extracts. Although hypericins were found in many *Hypericum* species, they are more abundant in *H. perforatum* (Napoli et al., 2018), that for commercial purposes, should contain not less than 0.08% hypericins calculated as hypericin (WHO, 2002). Hypericin is a potent natural photosensitizing agent useful in photodynamic therapy (PDT) (Napoli et al., 2018), but also possesses several other pharmacological properties, including antimicrobial, anticancer, and anti-inflammatory effects (Jendželovská et al., 2016).

Phloroglucinol derivatives are contained in *H. perforatum* within the range 0.2-4% (EMEA, 2009). Among them, hyperforin, a light-sensitive and unstable compound (Napoli et al., 2018), has been claimed responsible for several herb-drug interactions recorded during treatments with *H. perforatum* (Chrubasik-Hausmann et al., 2019), and its presence in high amounts is considered an

important clue to confirm the actual presence of *H. perforatum* in herbal preparations (Raclariu et al., 2017).

Besides naphthodianthrones and phloroglucinols, *H. perforatum* extracts deal with many other bioactive compounds, including phenolic acids and a broad range of flavonoids. Research has shown that phenolic compounds play a crucial role in the many properties of *Hypericum* herb, being often involved in synergistic mechanisms with the other plant constituents, such as hypericins and hyperforin (Butterweck et al., 2000, 2003).

Many polyphenols detected in *Hypericum* species belong to the flavonols chemical class. In general, flavonols are thought to play an important role as co-effectors for improving the biopharmaceutical properties of hypericins (Jürgenliemk and Nahrstedt, 2002). In *H. perforatum*, most of them are glycosides of quercetin (Tatsis et al., 2007), among which quercetin-3-*O*-rutinoside (rutin), myricitrin, quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), and quercitrin. Myricetin and quercetin were found to have antitumoral *in vitro* activity, showing a good potential for the therapy of prostate cancer (Chaudhary and Willett, 2006). Quercetin was typically found in the highest concentrations in both St. John's Wort and certain foods (especially onions and apples) (Chaudhary and Willett, 2006). In *H. perforatum,* rutin, hyperoside, quercetin, and quercitrin, were retrieved in higher amount in leaves than in stems (quercitrin was found in the leaves only) by Dresler et al. (2018), and hyperoside was found as majoritary component (17.7 mg g^{-1}) in *H. perforatum* extracts by Jürgenliemk and Nahrstedt (2002).

In analytical studies of *Hypericum* extracts, many cinnamic acids were also detected: 3-*O*-caffeoylquinic acid, *p*-coumaroilquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid) and *p*-coumaric acid among the others were the most cited (Napoli et al., 2018). Quantitative results from literature are highly variable, but there is general agreement in assessing that, among *Hypericum* species, *H. perforatum* is the one containing the highest amounts of phenolic acids (Pilepić et al., 2013). Many

of these compounds have been isolated from various plant sources, and research demonstrated their significant antioxidant, antibacterial, and anti-inflammatory activity (Liu et al., 2020).

Among flavan-3-ols, catechins are widely studied, due to their acknowledged antioxidant, as well as anticarcinogen, cardiopreventive, anti-microbial, anti-viral, and neuro-protective properties (Aron and Kennedy, 2008). Although retrievable in a large quantity of plants, they are very common in tea leaves (Pietta, 2000). Their presence in several *Hypericum* taxa was found to vary according to the species, being represented in *H. perforatum* in rather low amount (about 0.02 g kg^{-1}) (Napoli et al., 2018).

Finally, biflavonoids are common in plants, where they are generally claimed to have a protective activity against UV radiation, as well as against predators, insects and fungi (Gontijo et al., 2016). In *H. perforatum*, two compounds belonging to this chemical group were detected, i.e., the apigenin dimers biapigenin and amentoflavone, present in a total amount of about 4.74 g kg^{-1} d.m. (Berghöfer and Hölzl, 1987, 1989; Chaudhary and Willett, 2006; Tatsis et al., 2007; Napoli et al., 2018). The amount of biapigenin is generally much higher (more than ten-fold) than amentoflavone (Silva et al., 2005; Michler et al., 2011; Napoli et al., 2018); however, the latter compound showed a significant antidepressant action, and has been therefore addressed to many specific studies (Michler et al., 2011; Yu et al., 2017).

Due to the great interest paid by pharmaceutical industry to those compounds, much research has been conducted to explore the factors responsible for their presence in *H. perforatum* chemical composition. Although the biosynthesis of the major active compounds relies on genetical basis (Rizzo et al., 2020), many factors actually play a role in gene expression, ultimately giving rise to a large amount of intra-specific variability. The geographical provenance of the analyzed biotypes is often acknowledged as one of the most significant factors inducing variability (Božin et al., 2013; Bagdonaité, 2012), but relevant variability was also found in neighboring populations, due to

intraspecific polymorphism (Mártonfi et al., 2001). This large inherent variability poses a serious issue to the standardization of plant material from wild populations; for this reason - and for many other environmental, economic and technical considerations (Bruni and Sacchetti, 2009; Canter et al., 2005; Carrubba and Catalano, 2009) -, specialized *Hypericum* cultivation is considered the most reliable option for industrial supply. The cultivation of selected genotypes, by means of finely-tuned cropping protocols, may indeed be determinant in assessing plant composition, and within certain limits, cultivation is acknowledged as a practice able to standardize metabolite content in plant (Carrubba and Catalano, 2009). Yet, variations in plant metabolic pattern cannot be excluded even in the same geographical site, due to the unpredictable climatic conditions occurring in different years. Of course, a higher stability in the biosynthesis and storage of one, or more, selected compounds, is a welcome trait when decisions about cultivation are taken. Pluhar et al. (2002) partitioned cultivated *Hypericum* into more or less "stable" biotypes, on the basis of the variability of their active compounds throughout an estimated 2-3 years plant cultivation cycle. Although interesting, these results involved repeated observations on the same stands, and therefore did not allow to distinguish the effects due to the climatic variability from those caused by the increased age of plants. Hence, it seemed worth to assess how much these bioactive compounds can vary among different *H. perforatum* provenances with the sole effect of year climatic pattern, i.e., in subsequent newly seeded cultivations in the same location of the same genetic material.

With this purpose, we put in cultivation in two consecutive years (2015 and 2016) seeds of *Hypericum perforatum* obtained from different areas of Italy (table 1; figure 2). The flowered tops collected in both years (always from 1-year-old plants) were analyzed to evaluate the presence of the major biologically active compounds, and the comparison between either cultivation years allowed to obtain information about the phytochemical stability of the studied genotypes.

2. Results and discussion

The analyses confirmed the presence of many biologically active compounds, belonging to the chemical groups of naphthodianthrones, phloroglucinols, flavonols, cinnamic acids, flavan-3-ols, and dimers. The ANOVA carried out on the different detected compounds (tables 2-6) proved that significant variations occurred between years (Y factor always significant), but highly significant effects showed also up according to genotypes (P), as well as, in most cases, according to the interaction of both factors (YxP).

As a whole, the total amount (mg g^{-1} d.m.) of the studied secondary metabolites was higher in 2015 than in 2016, but important variations in their relative amounts were evidenced. For example, unlike the trend evidenced for the total metabolites amount, the content in naphthodianthrones and phloroglucinols was significantly higher in 2016 than in 2015.

Along the timespan interested by plants' cultivation (March-June; figure 3), rainfall amount was similar in both years (179.6 mm in 2015 and 164 mm in 2016). Otherwise, the extreme temperature values reached rather different values (5.79 to 28.83 °C in 2015, and 6.96 to 29.82 °C in 2016), as well as the corresponding thermal sums (2102.18 °C in 2015, and 2202.35 °C in 2016; Tbase 0°C). Furthermore, a definitely contrasting pattern could be observed in the trend of temperatures: in 2015, Tmax values were constantly increasing from the second half of March to the first half of May, thereafter showing a marked decrease until the first ten days of June. Contrastingly, in 2016, maximum temperatures throughout March and April were much higher than in 2015 (peak values in April 21.5 °C in 2015 vs. 26.6 °C in 2016), then dropped to about 20 °C in the first days of May, and thereafter continuously increased until the end of the trial. That means, *Hypericum* plants experienced in 2015 lower temperatures in the first vegetative growth stages and after the onset of floral budding stage (about May 20th in both years) until full flowering (June 30th), and higher temperatures in the month preceding the floral budding stage; the opposite occurred in 2016. Since,

in *Hypericum*, secondary metabolites content is expected to increase after the flowering stage (Southwell and Bourke, 2001; Couceiro et al., 2006), some observed variations in plants' metabolites content between the two years can be reasonably attributed to such varied maximum temperatures throughout the reproductive fraction of plant growth stages.

The major detected naphthodianthrones, as well as the sum of their measured values, exhibited at ANOVA significant differences both according to years (Y) and provenances (P), and pseudohypericin was the only compound not showing also a significant YxP interaction (table 2). In both trial years, hypericin and pseudohypericin represented the majority of naphthodianthrones detected in all provenances (more than 99% in 2015 and about 73% in 2016).

In *H. perforatum,* the effects exerted by the temperature on the biosynthesis and accumulation of hypericins have been studied by several Authors, with contrasting results according to the experimental conditions. High temperatures (30°C) were claimed responsible for a decrease in hypericin content in 45-days old plantlets of *H. perforatum* (Yao et al., 2019); contrastingly, in older greenhouse-grown *H. perforatum* plants, increasing temperatures from 24 to 32°C (Odabas et al., 2009), or from 25 to 30°C (Couceiro et al., 2006), caused hypericin and pseudohypericin contents to increase. In our work, total naphthodianthrones content in 2016 was rather double than in 2015 (10.4 vs. 5.9 mg g^{-1} d.m.), demonstrating the crucial influence of the higher temperatures experienced by plants in the blooming stage. However, a strong effect of genotype was also found, as many of the tested genotypes showed contrasting values in both years (figure S1). In the first year, hypericin values ranged from 1.72 (PFR-TN) to 5.73 (PFR-CG) mg g^{-1} d.m., and from 1.67 (PFR-SI) to 5.29 (PFR-CG) in 2016. The provenance PFR-CG showed in both years the highest content in hypericin and total naphthodianthrones, also demonstrating the highest stability; oppositely, PFR-TN and PFR-SI showed in both years the lowest values, expressing a high stability but in the lower positions. All other provenances showed a variable behavior, with alternate low and high responses in the two years.

Likewise, the amounts of hyperforin and adhyperforin expressed a significant variability between years and provenances (table 3). Hyperforin content was on average 35.3 mg g^{-1} d.m. in 2015, and 39.5 mg g^{-1} d.m. in 2016. In both years, hyperforin was the most abundant phloroglucinol detected (on average, 87-88% of total phloroglucinols), whereas the mean values of adhyperforin were 5.6 (2015) and 5.1 (2016) (figure S2). Yet, a strong variability among provenances showed up. In the first trial year, hyperforin values ranged between 19.6 (PFR-NA) and 64.0 (PFR-AG). In 2016, the highest hyperforin amount was detected in the provenance PFR-VI (41.3), and the lowest in the provenance PFR-SI (30.0). A good stability across years (figure S2) was found in the provenances PFR-PM and PFR-AG (hyperforin) and only in PFR-PM for adhyperforin. The provenances PFR-TN and PFR-SI expressed in both years a hyperforin amount lower than the year average, hence being positioned in the 3rd quadrant of the graph.

The major detected flavonols (myricitrin, myricetin derivatives, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin, and quercitrin) were found in 2015 in almost double quantity than in 2016, adding up to 19.0 mg g^{-1} and 9.2 mg g^{-1}, respectively (figure S3). Because of this large difference between years, the "year" effect accounts for more than 82% of total experimental variability concerning total flavonols content (table 4). As shown, the ANOVA evidenced significant differences for all variables (including each detected flavonol) and experimental factors (Y and P, both alone and in interaction). Differently from our results, a constraining effect of high temperatures on flavonols content was found by Yao et al. (2019), who retrieved in *H. perforatum* plantlets a significantly decreasing flavonols amount with increasing temperatures from 15 to 22 to 30 °C. Notwithstanding, in our work, despite the large difference between years, the different identified flavonols kept the same relative proportions, allowing to deduce that the involved biosynthetic pathways were all blocked by high temperatures in a similar manner, and no preferential synthesis and storage occurred in any of the compounds.

A marked distinction may be observed (fig. S3-d) between stable high-yielding genotypes (PFR-CG and PFR-TN) and stable low-yielding genotypes (PFR-AG and PFR-VI), whereas the other three accessions exhibited a variable behavior between years. Among flavonols, myricitrin was practically absent from the tested samples. In both years, the most abundant flavonol was quercetin-3-*O*-galactoside (hyperoside; fig. S3-a), accounting for more than 40% of all identified flavonols. Quercetin-3-*O*-glucoside (isoquercitrin) was the second most represented flavonol (about 23% of all identified flavonols), followed by quercitrin (fig. S3-c; 14.6% in both years), quercetin-3-*O*-rutinoside (rutin; 11% in 2015 and 8.4% in 2016), and quercetin (fig. S3-b; 6.2% in 2015 and 3.8% in 2016). As evidenced in the graphs in figure S3, however, the mean values over years are not always representative of the actual behavior of biotypes, and wide deviations from the respective mean values can be observed in each biotype. Five biotypes out of 7 (PFR-AG, PFR-CG, PFR-VI, PFR-PM, and PFR-NA – interestingly, all biotypes from Sicily and Southern Italy) did not reveal any presence of rutin (only represented in less than 0.4 mg g^{-1} d.m. in 2015 in the PFR-PM biotype), whereas the same compound was found in appreciable amounts in the two biotypes from Northern Italy (PFR-TN and PFR-SI). A similar result was obtained by Scotti et al. (2019), who noticed the absence of rutin in the majority of analyzed samples coming from Spain, whereas this compound was always detected in samples from other provenances (including China). This result is consistent with the hypothesis that a no-rutin metabolic pathway can have been selected in warmer areas, but of course, the actual existence of rutin-based chemotypes must be confirmed by further research.

Only one of the studied biotypes (PFR-CG) showed a good stability level in the accumulation of hyperoside (fig. S3-a), quercetin (fig. S3-b), and quercitrin (fig. S3-c); the biotypes from Trento (PFR-TN) and Naples (PFR-NA) expressed high and stable yields of quercetin (fig. S3-b) and quercitrin (fig. S3-c), whereas the biotype from Siena (PFR-SI) could be classed as a low-yielding *H. perforatum* concerning the detected amounts of hyperoside (fig. S3-a) and quercetin (fig. S3-b).

The cinnamic acids (Fig. 1d) found in the examined *H. perforatum* provenances were 3-*O*-caffeoylquinic acid, *p*-coumaroilquinic acid, 5-*O*-caffeoylquinic acid and p-coumaric acid. The two caffeoylquinic acids (3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid) were always the most abundant, averaging between 87 and 97% of total retrieved cinnamic acids. All of them exhibited significant differences at ANOVA (table 5) for both factors "provenance" and "year". In the pooled two-year analysis, significant differences showed also up in the interaction "PxY" for all of them, with the only exception of *p*-coumaroilquinic acid, where the factors "Y" and "P" showed an additive effect.

As shown in figure S4-a, 3-*O*-caffeoylquinic acid in the first year averaged a much higher value than in 2016 (1.6 vs. 0.4 mg g^{-1} d.m.). Inside provenances, PFR-CG (2.5 mg g^{-1} d.m.) and PFR-VI (2.1 mg g^{-1} d.m.) reached the highest values in 2015, whereas PFR-CG and PFR-PM (both with 0.7 mg g^{-1} d.m.) ranked first in 2016. Hence, only one provenance (PFR-CG) was stable for high values of 3-*O*-caffeoylquinic acid, whereas PFR-AG, PFR-SI and PFR-NA were also stable, but for lowest values.

Also 5-*O*-Caffeoylquinic acid (fig. S4-c) reached in 2015 a higher amount than in 2016, with mean values of 0.7 and 0.3 mg g^{-1} d.m., respectively. It exhibited a good inter-annual stability level in four provenances out of seven, but only two of them (PFR-VI and PFR-NA) had consistently high values, whereas the other two (PFR-AG and PFR-SI), in both years ranked in the lowest positions. *P*-coumaroilquinic acid (fig. S4-b) and *p*-coumaric acid were found in lower amounts. The first accounted for 3.8-10% of total cinnamic acids in 2015 and 2.3-12% in 2016. *p*-Coumaric acid was instead found in very low amounts (on average 0.01 mg g^{-1} d.m. in 2015 and 0.002 in 2015, being in this second year totally absent in two out of seven provenances, namely PFR-CG and PFR-TN. Increased amount of cinnamic acids was found in plantlets of *H. perforatum* grown at decreasing temperatures (30°C, 22°C, and 15°C) (Yao et al., 2019).

Catechin content in the studied *Hypericum* accessions exhibited at ANOVA significant variations due to both provenance (P) and year (Y), as well as to their interaction (PxY) (table 6). The factor Y, however, was able to explain more the 50% of experimental variability. In 2015, catechin content averaged 0.7 mg g^{-1}, a markedly higher value than in 2016 (0.2 mg g^{-1}). Among biotypes (fig. S5-a), four biotypes out of seven showed a fairly high stability in years, being PFR-VI and PFR-CG stable on high values, and PFR-TN and PFR-AG stable on low ones.

As concerns biflavonoids, in this work, biapigenin and amentoflavone achieved a total amount of 6.8 mg g^{-1} in 2015 and 4.1 mg g^{-1} in 2016. The most abundant of them was always biapigenin, being amentoflavone always detected in amounts lower than 0.3 mg g^{-1}. At the ANOVA (table 6), the two compounds and their cumulated amount showed a highly significant effect of both factors Year (Y) and Provenance (P); the YxP interaction was not significant in the amentoflavone content, allowing to assess that the effect on this compound of year and genotype was mostly exerted additively. As biapigenin represented the majority of total dimers amount, the stability assessment for biapigenin (fig S5-b) is very similar to that of total dimers (fig. S5-d). The biotype PFR-AG, and, to a lesser extent, the biotype PFR-CG revealed as the most stable for high yield levels, whereas PFR-TN and PFR-PM could be allocated as low-yielding genotypes.

3. Conclusions.

It is well known that plant-derived products deal with a typical and wide variability, due to many genetic and environmental factors involved in the biosynthetic and storage processes of their secondary metabolites (Sangwan et al., 2001). Hence, from the phytochemical point of view, the final outcome of *Hypericum* cultivation is often unpredictable. When cultivation is meant for pharmaceutical supply, this represents a serious issue. Among the causes of phytochemical variability of cultivated plants, genetic plant features are undoubtedly very important. Indeed, many active

compounds have been found to vary according to the species, and within species, according to lower-order taxon. From here, the frequent advice to put in cultivation only plant genotypes with selected and well assessed quality characteristics (Couceiro et al., 2006; Lazzara et al., 2020). Anyway, in cultivated plants, also additional sources of variability (including e.g., soil characteristics, water availability, competing organisms such as weeds, pests, and insects) exert significant effects on plant biochemical features.

In this work, we have compared the amount of several active metabolites in *H. perforatum* according to genotypes and year of cultivation. As expected, significant differences showed up among genotypes, stressing the occurrence of a high variability due to the genetic background of cultivated plants. However, attention was also focused on the "stability" level showed throughout years by the different accessions. This approach allowed to partition the tested biotypes based on their tendency to maintain consistently high –or low- levels of the metabolites of interest, or conversely, to express a variable response between years. Only one among the tested biotypes (PFR-CG) exhibited constantly higher-than-average amounts of all identified metabolites, with the only exception of hyperforin and amentoflavone.

4. Experimental

4.1 General experimental procedures. Polyphenols quantitative analyses were carried out on a HPLC instrument equipped with a binary pump and a photodiode array detector (Thermo Scientific, Italy), using a reverse-phase column (Gemini C_{18}, 250 x 4.6 mm, 5 µm particle size, Phenomenex, Italy) equipped with a guard column (Gemini C_{18}, 4 x 3.0 mm, 5 µm particle size, Phenomenex, Italy). Naphthodianthrones and phloroglucinols quantitative analyses were carried out on a similar HPLC instrument (Hitachi Chromaster). Chromatographic runs were performed using the same column of polyphenols. In order to unambiguously identify the chromatographic signals and/or to confirm peak

assignments, a series of HPLC/ESI/MS analyses were performed on a significant number of samples. The HPLC apparatus used was the same described above, whilst ESI mass spectra were acquired by a Thermo Scientific Exactive Plu Orbitra MS (Thermo Fisher Scientific, Inc., Milan, Italy).

4.2 Plant material. A collection activity of seeds of *Hypericum perforatum* from different areas of Italy (table 1; figure 2) was carried out in 2014 and 2015. In 2015 and 2016, established plants obtained from the collected seeds were disposed in a catalogue field, renewed every year, located within the facilities of CREA-DC in Bagheria (PA, Italy; 38°05'25" N-13°31'08" E). Climatic data (maximum, minimum and mean air temperatures, and rainfall amount) during the timespan of *Hypericum* plants growth (March-June) were measured through a meteorological station near to the experimental site. After transplant, plant grew quickly, reaching the floral budding stage in the last ten days of May, and the flowering phase in early June in both years. At full-flowering time, the flowered tops (15-20 cm) were collected, and after cutting, plant samples were stored in paper bags and dried at 20-25 °C in the dark for further analyses.

4.3. Analytical determinations. The dried flowers collected from the different treatments were finely crushed and aliquots (1 g) of powder were extracted with 20 mL of ethanol for 72 hours under gently continuous stirring, avoiding light exposure due the photo sensibility of some of the metabolites of interest. The resulting deep red colored suspensions were filtered on PTFE 0,45 μ filters (PALL Corporation), put into 2mL amber vials and sent to analytical determinations.

Hypericum polyphenols (Fig. 1) were eluted with the following gradient of B (formic acid, 2.5% solution in acetonitrile) in A (2.5% solution of formic acid in water): 0 min: 5 % B; 10 min: 15 % B; 30 min: 25 % B; 35 min: 30 % B; 50 min: 90 % B; then kept for 7 min at 100 % B. The solvent flow rate was 1 mL/min. Quantifications were carried out using the corresponding reference substances or

adequate analogous at 330 or 350 nm. Naphthodianthrones and phloroglucinols quantitative analyses were carried out on a similar HPLC instrument (Hitachi Chromaster). Chromatographic runs were performed using the same column of polyphenols and were carried out with the following gradient of B (acetonitrile) in A (ammonium acetate 20 mM in water): 0 min: 50 % B; 25 min: 50% B; 35 min: 10 % B; 45 min: 90 % B; 50 min: 50 % B (Tawaha et al., 2010). The solvent flow rate was 1 mL/min. Quantifications were run at 290 nm for phloroglucinols (Fig. 1), with hyperforin as reference substance and at 590 nm for naphthodianthrones (Fig. 1) using hypericin and protohypericin as standards. All analyses were carried out in triplicate.

4.4 Statistical data management. At the end of the second trial year (2016), a dataset of 42 individual samples (7 accessions x 2 years x 3 repetitions) was available. Data obtained in both years were submitted to ANOVA by means of the statistical package Minitab 17.0. The ANOVA carried out on pooled two-years data showed the occurrence of significant differences both between years and among the interactions "Year x Provenance". Hence, the analysis was repeated separately for both years. Stability of chemical features of the examined biotypes was determined through graphical assessment, by plotting the values of the selected compounds obtained in each biotype in the two years on the two axes of an X-Y graph, along with the average values across both years, +/- their respective standard deviations. The graphical representation (reported as Supplementary Material, figures S1 to S5) allows individuating which provenance showed a higher "stability"; all biotypes that in both years showed a higher-than-average content are located in the top right section of the graph (1st quadrant), whereas those having in both years a lower-than-average content are symmetrically placed in the bottom left position (3rd quadrant). When biotypes revealed an "unstable" behavior, i.e., they were dealing with contrasting values in both years, they are situated in the top left and bottom right positions (2nd or 4th quadrant, respectively).

References

Aron P.M., Kennedy J.A. 2008. Flavan-3-ols: Nature, occurrence and biological activity. Mol. Nutr. Food Res., 52: 79 –104. doi: 10.1002/mnfr.200700137

Bagdonaitė E., Mártonfi P., Repčák M., Labokas J. 2012. Variation in concentrations of major bioactive compounds in *Hypericum perforatum* L. from Lithuania. Ind. Crops Prod. 35, 302–308.

Berghöfer R., Hölzl J. 1987. Biflavonoids in *Hypericum perforatum*; Part 1. Isolation of I3, II8-biapigenin. Planta Med., 53, 2: 216-217. doi: 10.1055/s-2006-962676.

Berghöfer R., Hölzl J. 1989. Isolation of I3', II8-Biapigenin (Amentoflavone) from *Hypericum perforatum*. Planta Med., 55, 91

Božin B., Kladar N., Grujić N., Anačkov G., Samojlik I., Gavarić N., Srđenović Čonić B. 2013. Impact of origin and biological source on chemical composition, anticholinesterase and antioxidant properties of some St. John's Wort species (*Hypericum* spp., Hypericaceae) from the Central

Balkans. Molecules, 18, 11733-11750. doi: 10.3390/molecules181011733

Butterweck V., Jürgenliemk G., Nahrstedt A., Winterhof, H. 2000. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. Planta Med. 66, 3–6.

Butterweck V., Christoffel V., Nahrstedt A., Petereit F., Spengler B., Winterhoff H. 2003. Step by step removal of hyperforin and hypericin: activity profile of different *Hypericum* preparations in behavioral models. Life Sci., 73, 627–639. doi: 10.1016/S0024-3205(03)00314-X

Carrubba A., Catalano C. 2009. Essential oil crops for sustainable agriculture - A review. In: Lichtfouse, E. (Ed), Climate change, intercropping, pest control and beneficial microorganisms, Springer, Dijon, pp.137-188. doi: 10.1007/978-90-481-2716-0_8

Chaudhary A., Willett K.L. 2006. Inhibition of human cytochrome CYP1 enzymes by flavonoids of St.

John's wort. Toxicology, 217: 194–205.

Chrubasik-Hausmann S., Vlachojannis J., McLachlan A.J. 2019. Understanding drug interactions with St John's wort (*Hypericum perforatum* L.): impact of hyperforin content. J. Pharm. Pharmacol., 71: 129–138. doi: 10.1111/jphp.12858

Couceiro M.A., Afreen F., Zobayed S.M.A., Kozai T. 2006. Variation in concentrations of major bioactive compounds of St. John's wort: Effects of harvesting time, temperature and germplasm. Plant Sci., 170: 128–134. doi:10.1016/j.plantsci.2005.08.011

Dresler S., Kováčik J., Strzemski M., Sowa I., Wójciak-Kosiorc M. 2018. Methodological aspects of biologically active compounds quantification in the genus *Hypericum*. J. Pharmac. Biomed. Anal., 155: 82–90. doi: 10.1016/j.jpba.2018.03.048

EMEA, European Medicines Agency. 2009. Committee on Herbal Medicinal Products (HMPC). Assessment Report on *Hypericum perforatum* L., Herba. Doc. Ref.: EMA/HMPC/101303/2008. [https://www.ema.europa.eu/en/documents/herbal-report/assessment-report-hypericum perforatum-l-herba_en.pdf].

Gontijo V.S., dos Santos M.H., Viegas C. 2017. Biological and chemical aspects of natural biflavonoids from plants: A brief review. Mini-Rev. Med. Chem., 17: 834-862.

Jendželovská Z., Jendželovský R., Kuchárová B., Fedoročko P. 2016. Hypericin in the light and in the dark: two sides of the same coin. Front. Plant Sci. 6 (7), 560.

Jürgenliemk G., Nahrstedt A. 2002. Phenolic compounds from *Hypericum perforatum*. Planta Med., 68: 88-91.

Lazzara S., Carrubba A., Napoli E. 2020. Variability of hypericins and hyperforin in Hypericum species from the Sicilian flora. Chem. Biodiv., 17, e190059610. doi: 1002/cbdv.201900596

Linde, K., Ramirez G., Mulrow C.D., Pauls A., Weidenhammer W., Melchart D. 1996. St John's Wort for depression—An overview and meta-analysis of randomised clinical trials. BMJ 313, 253–258.

Liu W., Li J., Zhang X., Zu Y., Yang Y., Liu W., Xu Z., Gao H., Sun X., Jiang X., Zha Q. 2020. Current advances in naturally occurring caffeoylquinic acids: Structure, bioactivity, and synthesis. J. Agric. Food Chem., 68, 10489−10516. doi: 10.1021/acs.jafc.0c03804

Mártonfi P., Repčák M., Ciccarelli D., Garbari F. 2001. *Hypericum perforatum* L. - chemotype without rutin from Italy. Biochem. System. Ecol., 29: 659–661.

Michler H., Laakmann G., Wagner H. 2011. Development of an LC-MS method for simultaneous quantitation of Amentoflavone and Biapigenin, the minor and major biflavones from Hypericum perforatum L., in human plasma and its application to real blood. Phytochem. Anal. 22: 42-50. doi: 10.1002/pca.1249.

Nahrstedt A., Butterweck V. 1997. Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. Pharmacopsychiat., 30 (Supplement): 129 – 134.

Nahrstedt A., Butterweck V. 2010. Lessons learned from herbal medicinal products: The Example of St. John's Wort. J. Nat. Prod., 73: 1015–1021

Napoli E., Siracusa L., Ruberto G., Carrubba A., Lazzara S., Speciale A., Cimino F., Saija A., Cristani M. 2018. Phytochemical profiles, phototoxic and antioxidant properties of eleven *Hypericum* species: a comparative study. Phytochemistry, 152: 162-173.

Odabas M.S., Raduğienë J., Camas N., Janulis V., Ivanauskas L., Çırak C. 2009. The quantitative effects of temperature and light intensity on hyperforin and hypericins accumulation in *Hypericum perforatum* L. J. Med. Plants Res., 3(7): 519-525.

Patočka J. 2003. The chemistry, pharmacology, and toxicology of the biologically active constituents of the herb *Hypericum perforatum* L. J. Appl. Biomed., 1: 61–70.

Pietta P.G. 2000. Flavonoids as antioxidants. J. Nat. Prod., 63: 1035-1042. doi: 10.1021/np9904509.

Pilepić K.H., Maleš Ž., Crkvenčić M. 2013. Quantitative analysis of total flavonoids and total phenolic acids in thirty Hypericum taxa. Nat. Prod. Com., 8 (3): 347-349.

Pluhár Zs., Bernáth J., Neumayer É. 2002. Morphological, production biological and chemical diversity of St. John's Wort (*Hypericum perforatum* L.). Acta Hort. 576: 33-40.

Raclariu A.C., Paltinean R., Vlase L., Labarre A., Manzanilla V., Ichim M.C., Crisan G., Krag Brysting A., de Boer H. 2017. Comparative authentication of *Hypericum perforatum* herbal products using DNA metabarcoding, TLC and HPLC-MS. Scientific Reports, 7: 1291. doi: 10.1038/s41598-017-01389-w.

Rizzo P., Altschmied L., Ravindran B.M., Rutten T., D'Auria J.C. 2020. The biochemical and genetic basis for the biosynthesis of bioactive compounds in *Hypericum perforatum* L., one of the largest medicinal crops in Europe. Genes 11, 1210. doi: 10.3390/genes11101210

Sangwan N.S., Farooqi A.H.A., Shabih F., Sangwan R.S., 2001. Regulation of essential oil production in plants. Plant Growth Regulation, 34: 3–21.

Scotti F., Löbel K., Booker A., Heinrich M., 2019. St. John's Wort (*Hypericum perforatum*) Products - How variable is the primary material? Front. Plant Sci. 9: 1973. doi: 10.3389/fpls.2018.01973

Silva B.A., Ferreres F., Malva J.O., Dias A.C.P. 2005. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. Food Chem., 90: 157–167. doi: doi:10.1016/j.foodchem.2004.03.049

Southwell I.A., Bourke C.A. 2001. Seasonal variation in hypericin content of Hypericum perforatum L. (St. John's Wort). Phytochemistry, 56: 437.441.

Stevinson C., Ernst E. 1999. *Hypericum* for depression. An update of the clinical evidence. European Neuropsychopharmacology, 9: 501 –505.

Sun P., Kang T., Xing H., Zhang Z., Yang D., Zhang J., Paré P.W., Li M. 2019. Phytochemical changes in aerial parts of *Hypericum perforatum* at different harvest stages. Rec. Nat. Prod. 13, 1–9.

Tatsis E.C., Boeren S., Exarchou V., Troganis A.N., Vervoort J., Gerothanassis I.P. 2007. Identification of the major constituents of *Hypericum perforatum* by LC/SPE/NMR and/or LC/MS. Phytochemistry, 68: 383–393.

Tawaha K., Gharaibeh M., El-Elimat T., Alali F.Q. 2010. Determination of hypericin and hyperforin content in selected Jordanian *Hypericum* species. Ind. Crops Prod. 32: 241-245.

WHO, World Health Organization. 2002. *Herba Hyperici.* WHO monographs on selected medicinal plants, vol. 2: 149-171. WHO, Geneva.

Yao Y., Kang T., Jin L., Liu Z., Zhang Z., Xing H., Sun P., Li M. 2019. Temperature-dependent growth and hypericin biosynthesis in *Hypericum perforatum*. Plant Phys. Biochem., 139: 613–619. doi: 10.1016/j.plaphy.2019.04.012.

Yu S., Yan H., Zhang L., Shan M., Chen P., Ding A., Li S.F.Y. 2017. A Review on the phytochemistry, pharmacology, and pharmacokinetics of amentoflavone, a naturally-occurring biflavonoid. Molecules, 22, 299. doi:10.3390/molecules22020299.

Zdunic G., Godjevac D., Savikin K., Petrovic S. 2017. Comparative analysis of phenolic compounds in seven *Hypericum* species and their antioxidant properties. Nat. Prod. Com. 12, 1805–1811.

TABLES

Table 1 - Geographical provenance of the studied *H. perforatum* accessions.

#	Code	Provenance
1	PFR-AG	Cammarata (AG)
2	PFR-CG	Capo Gallo (PA)
3	PFR-VI	Vicaretto (PA)
4	PFR-PM	P. Marcato - Castelbuono (PA)
5	PFR-SI	Siena (SI)
6	PFR-TN	Trento (TN)
7	PFR-NA	Napoli (NA)

Table 2. Results of ANOVA for the content in major naphthodianthrones in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Hyp	PsHyp	PrPsHyp	PrHyp	Total
2015-2016						
Provenance (P)	6	*** (75.5%)	** (25.4%)	*** (11.3%)	*** (12.6%)	*** (33.7%)
Year (Y)	1	** (1.9%)	*** (26.2%)	*** (77.7%)	*** (75.0%)	*** (46.4%)
P x Y	6	*** (17.5%)	n.s. (16.3%)	*** (11.0%)	*** (12.4%)	*** (12.8%)
Error	28					
Total	41					
2015						
Provenance (P)	6	***	n.s.	***	***	***
Error	14					
Total	20					
2016						
Provenance (P)	6	***	***	***	***	***
Error	14					
Total	20					

[a] Hyp=hypericin; PsHyp=pseudohypericin; PrPsHyp=protopseudohypericin; PrHyp=protohypericin

Table 3. Results of ANOVA for the content in major phloroglucinols in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Hypf	AdHypf	Total
2015-2016				
Provenance (P)	6	*** (46.4%)	*** (38.4%)	*** (45.1%)
Year (Y)	1	*** (3.9%)	** (1.4%)	*** (2.2%)
P x Y	6	*** (45.5%)	*** (56.8%)	*** (48.7%)
Error	28			
Total	41			
2015				
Provenance (P)	6	***	***	***
Error	14			
Total	20			
2016				
Provenance (P)	6	***	***	***
Error	14			
Total	20			

[a]Hypf=hyperforin; AdHypf=adhyperforin; total=hyperforin + adhyperforin

Table 4. Results of ANOVA for the content in myricetin derivatives, quercetin-3-*O*-rutinoside, Quercetin-3-*O*-galactoside, Quercetin-3-*O*-glucoside, Quercetin, Quercitrin, and total flavonols detected in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Myrdv	Q3Orut	Q3Ogal	Q3Oglu	Quercet	Quercit	Total flavonols
2015-2016								
Provenance (P)	6	*** (44.9%)	*** (77.7%)	*** (31.8%)	*** 37.3%)	*** (3.4%)	*** 32.2%)	*** (7.1%)
Year (Y)	1	*** (10.0%)	*** (6.9%)	*** (57.6%)	*** (48.8%)	*** (93.3%)	*** (52.0%)	*** (82.4%)
P x Y	6	*** (44.9%)	*** (15.3%)	*** (9.1%)	*** (13.4%)	*** (2.1%)	*** (14.8%)	*** (9.7%)
Error	28							
Total	41							
2015								
Provenance (P)	6	***	***	***	***	***	***	***
Error	14							
Total	20							
2016								
Provenance (P)	6	n.s.	***	***	***	***	***	***
Error	14							
Total	20							

[a] Myrdv=myricetin derivatives; Q3Orut=quercetin-3-*O*-rutinoside; Q3Ogal=quercetin-3-*O*-galactoside; Q3Oglu=quercetin-3-*O*-glucoside; Quercet=quercetin; Quercit=quercitrin; total flavonols=myricitrin + myricetin derivatives + quercetin-3-*O*-rutinoside + quercetin-3-*O*-galactoside + quercetin-3-*O*-glucoside + quercetin + quercitrin

Table 5. Results of ANOVA for the content in major cinnamic acids derivates in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor	DF	3-O-CQ	*p*-CouQ	5-O-CQ	*p*-Cou	Total
2015-2016						
Provenance (P)	6	*** (18.2%)	** (11.5%)	*** (43.6%)	*** (15.2%)	*** (18.4%)
Year (Y)	1	** (75.4%)	*** (76.1%)	*** (33.2%)	*** (61.0%)	*** (76.7%)
P x Y	6	*** (6.2%)	n.s. (9.3%)	*** (22.0%)	*** (14.2%)	*** (4.6%)
Error	28					
Total	41					
2015						
Provenance (P)	6	***	***	***	***	***
Error	14					
Total	20					
2016						
Provenance (P)	6	***	***	***	***	***
Error	14					
Total	20					

[a] 3-O-CQ=3-*O*-Caffeoylquinic acid; *p*-CouQ=*p*-Coumaroilquinic acid; 5-O-CQ=5-*O*-Caffeoylquinic acid; *p*-Cou=*p*-Coumaric acid; total=3-O-CQ + *p*-CouQ + 5-O-CQ + *p*-Cou

Table 6. Results of ANOVA for the content in catechin, biapigenin, amentoflavone and total dimers in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Ctchin	Bpgn	Amtfl	Total dimers
2015-2016					
Provenance (P)	6	*** (27.7%)	*** (34.1%)	*** (33.0%)	*** (34.3%)
Year (Y)	1	*** (52.4%)	*** (43.5%)	*** (43.5%)	*** (43.9%)
P x Y	6	*** (18.9%)	*** (21.3%)	n.s. (7.2%)	*** (20.8%)
Error	28				
Total	41				
2015					
Provenance (P)	6	***	***	*	***
Error	14				
Total	20				
2016					
Provenance (P)	6	***	***	***	***
Error	14				
Total	20				

[a] Ctchin=catechin; Bpgn=biapigenin; Amtfl=amentoflavone; total dimers=biapigenin + amentoflavone

Content variability of bioactive secondary metabolites in Hypericum perforatum L.

Alessandra Carrubba*[1], Silvia Lazzara[2], Antonio Giovino[2], Giuseppe Ruberto[3], Edoardo Napoli[3]

[1] Dept. of Agricultural, Food and Forest Sciences (D/SAAF), University of Palermo; Viale delle Scienze, Build 4, Entr. L, 90128 Palermo, Italy. Email: alessandra.carrubba@unipa.it

[2] Council for Agricultural Research and Economics, Research Centre for Plant Protection and Certification (CREA-DC), SS 113 km 245.500, 90011, Bagheria (PA), Italy. Email: silvia.lazzara@crea.gov.it; antonio.giovino@crea.gov.it

[3] Institute of Biomolecular Chemistry, National Research Council (ICB-CNR), Via Paolo Gaifami 18, 95126 Catania, Italy. Email: giuseppe.ruberto@icb.cnr.it; edoardo.napoli@icb.cnr.it

*: corresponding author

Abstract

St John's Wort (*Hypericum perforatum* L.; *Hypericaceae*) is a perennial medicinal herb widespread and largely used in folk medicine inside the Mediterranean basin. Many bioactive compounds have been identified within its extracts. Under a pharmacological point of view, the most important of them belong to the chemical classes of naphthodianthrones, phloroglucinols and polyphenols. Many factors have been claimed responsible for the phytochemical variability in *Hypericum perforatum*, such as genotype, geographical origin, harvesting stage and age of the plants. Yet, when harvested plant material is addressed to the industry, the standardization of the active ingredients over cultivation years is a crucial issue. With the aim to detect the stability over years and genotypes of several bioactive *Hypericum* compounds, seven *Hypericum* biotypes retrieved from different Italian geographical areas were cultivated in 2015 and 2016, and their aerial flowering parts were analyzed. Naphthodianthrones (hypericin and its biosynthetic precursors), phloroglucinols (hyperforin and adhyperforin), and main polyphenols were determined by HPLC-DAD analysis. The results were statistically evaluated through ANOVA, and the stability over cultivation years of the tested genotypes was assessed. In rather all the examined metabolites, the ANOVA revealed a remarkable effect of both factors "year" (Y) and "provenance" (P), but the occurrence of significant "Y x P" interactions evidenced that the effect of climatic variability was often different according to the genotype. The evaluation of the stability level between years evidenced that only one biotype out of seven exhibited constantly higher-than-average amounts of rather all identified metabolites.

Keywords: *Hypericum perforatum*; St John's Wort; cultivation; phytochemical variability; secondary metabolites

1. Introduction

St John's Wort (*Hypericum perforatum* L.; *Hypericaceae*) is a perennial herb widespread and often sub-spontaneous inside the Mediterranean basin. According to the European Pharmacopoeia (IX ed.), the drug of *Hypericum* is represented by the dried flowered tops of the plant, that in the Mediterranean areas are traditionally used to prepare ointments endowed with a lenitive and wound-healing action. Due to its wide range of pharmacological activities, including antidepressant, antiviral, and antibacterial effects, *Hypericum perforatum* is one of the most consumed medicinal plants in the world (Linde, 1996), and its extracts are extensively used as phytopharmaceuticals and nutraceuticals. The active constituents of *H. perforatum* have been reviewed by several papers (Patočka, 2003; Napoli et al., 2018); the identified compounds belong to the chemical families of naphthodianthrones (Fig. 1A), phloroglucinols (Fig. 1B), flavonols (Fig. 1C), cinnamic acids (Fig. 1D), flavanols (Fig. 1E), and biflavonoids (Fig. 1F), along with a number of "minor" compounds. Naphthodianthrones, including hypericin and related compounds (pseudohypericin, protohypericin and protopseudohypericin) are typical of the genus *Hypericum* (Patočka, 2003), and represent the best known and most studied components of *H. perforatum* extracts. Although hypericins were found in many *Hypericum* species, they are more abundant in *H. perforatum* (Napoli et al., 2018), that for commercial purposes, should contain not less than 0.08% hypericins calculated as hypericin (WHO, 2002). Hypericin is a potent natural photosensitizing agent useful in photodynamic therapy (PDT) (Napoli et al., 2018), but also possesses several other pharmacological properties, including antimicrobial, anticancer, and anti-inflammatory effects (Jendželovská et al., 2016).

Phloroglucinol derivatives are contained in *H. perforatum* within the range 0.2-4% (EMEA, 2009). Among them, hyperforin, a light-sensitive and unstable compound (Napoli et al., 2018), has been claimed responsible for several herb-drug interactions recorded during treatments with *H. perforatum* (Chrubasik-Hausmann et al., 2019), and its presence in high amounts is considered an

important clue to confirm the actual presence of *H. perforatum* in herbal preparations (Raclariu et al., 2017).

Besides naphthodianthrones and phloroglucinols, *H. perforatum* extracts deal with many other bioactive compounds, including phenolic acids and a broad range of flavonoids. Research has shown that phenolic compounds play a crucial role in the many properties of *Hypericum* herb, being often involved in synergistic mechanisms with the other plant constituents, such as hypericins and hyperforin (Butterweck et al., 2000, 2003).

Many polyphenols detected in *Hypericum* species belong to the flavonols chemical class. In general, flavonols are thought to play an important role as co-effectors for improving the biopharmaceutical properties of hypericins (Jürgenliemk and Nahrstedt, 2002). In *H. perforatum*, most of them are glycosides of quercetin (Tatsis et al., 2007), among which quercetin-3-*O*-rutinoside (rutin), myricitrin, quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), and quercitrin. Myricetin and quercetin were found to have antitumoral *in vitro* activity, showing a good potential for the therapy of prostate cancer (Chaudhary and Willett, 2006). Quercetin was typically found in the highest concentrations in both St. John's Wort and certain foods (especially onions and apples) (Chaudhary and Willett, 2006). In *H. perforatum,* rutin, hyperoside, quercetin, and quercitrin, were retrieved in higher amount in leaves than in stems (quercitrin was found in the leaves only) by Dresler et al. (2018), and hyperoside was found as majoritary component (17.7 mg g^{-1}) in *H. perforatum* extracts by Jürgenliemk and Nahrstedt (2002).

In analytical studies of *Hypericum* extracts, many cinnamic acids were also detected: 3-*O*-caffeoylquinic acid, *p*-coumaroilquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid) and *p*-coumaric acid among the others were the most cited (Napoli et al., 2018). Quantitative results from literature are highly variable, but there is general agreement in assessing that, among *Hypericum* species, *H. perforatum* is the one containing the highest amounts of phenolic acids (Pilepić et al., 2013). Many

of these compounds have been isolated from various plant sources, and research demonstrated their significant antioxidant, antibacterial, and anti-inflammatory activity (Liu et al., 2020).

Among flavan-3-ols, catechins are widely studied, due to their acknowledged antioxidant, as well as anticarcinogen, cardiopreventive, anti-microbial, anti-viral, and neuro-protective properties (Aron and Kennedy, 2008). Although retrievable in a large quantity of plants, they are very common in tea leaves (Pietta, 2000). Their presence in several *Hypericum* taxa was found to vary according to the species, being represented in *H. perforatum* in rather low amount (about 0.02 g kg^{-1}) (Napoli et al., 2018).

Finally, biflavonoids are common in plants, where they are generally claimed to have a protective activity against UV radiation, as well as against predators, insects and fungi (Gontijo et al., 2016). In *H. perforatum*, two compounds belonging to this chemical group were detected, i.e., the apigenin dimers biapigenin and amentoflavone, present in a total amount of about 4.74 g kg^{-1} d.m. (Berghöfer and Hölzl, 1987, 1989; Chaudhary and Willett, 2006; Tatsis et al., 2007; Napoli et al., 2018). The amount of biapigenin is generally much higher (more than ten-fold) than amentoflavone (Silva et al., 2005; Michler et al., 2011; Napoli et al., 2018); however, the latter compound showed a significant antidepressant action, and has been therefore addressed to many specific studies (Michler et al., 2011; Yu et al., 2017).

Due to the great interest paid by pharmaceutical industry to those compounds, much research has been conducted to explore the factors responsible for their presence in *H. perforatum* chemical composition. Although the biosynthesis of the major active compounds relies on genetical basis (Rizzo et al., 2020), many factors actually play a role in gene expression, ultimately giving rise to a large amount of intra-specific variability. The geographical provenance of the analyzed biotypes is often acknowledged as one of the most significant factors inducing variability (Božin et al., 2013; Bagdonaité, 2012), but relevant variability was also found in neighboring populations, due to

intraspecific polymorphism (Mártonfi et al., 2001). This large inherent variability poses a serious issue to the standardization of plant material from wild populations; for this reason - and for many other environmental, economic and technical considerations (Bruni and Sacchetti, 2009; Canter et al., 2005; Carrubba and Catalano, 2009) -, specialized *Hypericum* cultivation is considered the most reliable option for industrial supply. The cultivation of selected genotypes, by means of finely-tuned cropping protocols, may indeed be determinant in assessing plant composition, and within certain limits, cultivation is acknowledged as a practice able to standardize metabolite content in plant (Carrubba and Catalano, 2009). Yet, variations in plant metabolic pattern cannot be excluded even in the same geographical site, due to the unpredictable climatic conditions occurring in different years. Of course, a higher stability in the biosynthesis and storage of one, or more, selected compounds, is a welcome trait when decisions about cultivation are taken. Pluhar et al. (2002) partitioned cultivated *Hypericum* into more or less "stable" biotypes, on the basis of the variability of their active compounds throughout an estimated 2-3 years plant cultivation cycle. Although interesting, these results involved repeated observations on the same stands, and therefore did not allow to distinguish the effects due to the climatic variability from those caused by the increased age of plants. Hence, it seemed worth to assess how much these bioactive compounds can vary among different *H. perforatum* provenances with the sole effect of year climatic pattern, i.e., in subsequent newly seeded cultivations in the same location of the same genetic material.

With this purpose, we put in cultivation in two consecutive years (2015 and 2016) seeds of *Hypericum perforatum* obtained from different areas of Italy (table 1; figure 2). The flowered tops collected in both years (always from 1-year-old plants) were analyzed to evaluate the presence of the major biologically active compounds, and the comparison between either cultivation years allowed to obtain information about the phytochemical stability of the studied genotypes.

2. Results and discussion

The analyses confirmed the presence of many biologically active compounds, belonging to the chemical groups of naphthodianthrones, phloroglucinols, flavonols, cinnamic acids, flavan-3-ols, and dimers. The ANOVA carried out on the different detected compounds (tables 2-6) proved that significant variations occurred between years (Y factor always significant), but highly significant effects showed also up according to genotypes (P), as well as, in most cases, according to the interaction of both factors (YxP).

As a whole, the total amount (mg g^{-1} d.m.) of the studied secondary metabolites was higher in 2015 than in 2016, but important variations in their relative amounts were evidenced. For example, unlike the trend evidenced for the total metabolites amount, the content in naphthodianthrones and phloroglucinols was significantly higher in 2016 than in 2015.

Along the timespan interested by plants' cultivation (March-June; figure 3), rainfall amount was similar in both years (179.6 mm in 2015 and 164 mm in 2016). Otherwise, the extreme temperature values reached rather different values (5.79 to 28.83 °C in 2015, and 6.96 to 29.82 °C in 2016), as well as the corresponding thermal sums (2102.18 °C in 2015, and 2202.35 °C in 2016; Tbase 0°C). Furthermore, a definitely contrasting pattern could be observed in the trend of temperatures: in 2015, Tmax values were constantly increasing from the second half of March to the first half of May, thereafter showing a marked decrease until the first ten days of June. Contrastingly, in 2016, maximum temperatures throughout March and April were much higher than in 2015 (peak values in April 21.5 °C in 2015 vs. 26.6 °C in 2016), then dropped to about 20 °C in the first days of May, and thereafter continuously increased until the end of the trial. That means, *Hypericum* plants experienced in 2015 lower temperatures in the first vegetative growth stages and after the onset of floral budding stage (about May 20th in both years) until full flowering (June 30th), and higher temperatures in the month preceding the floral budding stage; the opposite occurred in 2016. Since,

in *Hypericum*, secondary metabolites content is expected to increase after the flowering stage (Southwell and Bourke, 2001; Couceiro et al., 2006), some observed variations in plants' metabolites content between the two years can be reasonably attributed to such varied maximum temperatures throughout the reproductive fraction of plant growth stages.

The major detected naphthodianthrones, as well as the sum of their measured values, exhibited at ANOVA significant differences both according to years (Y) and provenances (P), and pseudohypericin was the only compound not showing also a significant YxP interaction (table 2). In both trial years, hypericin and pseudohypericin represented the majority of naphthodianthrones detected in all provenances (more than 99% in 2015 and about 73% in 2016).

In *H. perforatum,* the effects exerted by the temperature on the biosynthesis and accumulation of hypericins have been studied by several Authors, with contrasting results according to the experimental conditions. High temperatures (30°C) were claimed responsible for a decrease in hypericin content in 45-days old plantlets of *H. perforatum* (Yao et al., 2019); contrastingly, in older greenhouse-grown *H. perforatum* plants, increasing temperatures from 24 to 32°C (Odabas et al., 2009), or from 25 to 30°C (Couceiro et al., 2006), caused hypericin and pseudohypericin contents to increase. In our work, total naphthodianthrones content in 2016 was rather double than in 2015 (10.4 vs. 5.9 mg g^{-1} d.m.), demonstrating the crucial influence of the higher temperatures experienced by plants in the blooming stage. However, a strong effect of genotype was also found, as many of the tested genotypes showed contrasting values in both years (figure S1). In the first year, hypericin values ranged from 1.72 (PFR-TN) to 5.73 (PFR-CG) mg g^{-1} d.m., and from 1.67 (PFR-SI) to 5.29 (PFR-CG) in 2016. The provenance PFR-CG showed in both years the highest content in hypericin and total naphthodianthrones, also demonstrating the highest stability; oppositely, PFR-TN and PFR-SI showed in both years the lowest values, expressing a high stability but in the lower positions. All other provenances showed a variable behavior, with alternate low and high responses in the two years.

Likewise, the amounts of hyperforin and adhyperforin expressed a significant variability between years and provenances (table 3). Hyperforin content was on average 35.3 mg g^{-1} d.m. in 2015, and 39.5 mg g^{-1} d.m. in 2016. In both years, hyperforin was the most abundant phloroglucinol detected (on average, 87-88% of total phloroglucinols), whereas the mean values of adhyperforin were 5.6 (2015) and 5.1 (2016) (figure S2). Yet, a strong variability among provenances showed up. In the first trial year, hyperforin values ranged between 19.6 (PFR-NA) and 64.0 (PFR-AG). In 2016, the highest hyperforin amount was detected in the provenance PFR-VI (41.3), and the lowest in the provenance PFR-SI (30.0). A good stability across years (figure S2) was found in the provenances PFR-PM and PFR-AG (hyperforin) and only in PFR-PM for adhyperforin. The provenances PFR-TN and PFR-SI expressed in both years a hyperforin amount lower than the year average, hence being positioned in the 3rd quadrant of the graph.

The major detected flavonols (myricitrin, myricetin derivatives, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin, and quercitrin) were found in 2015 in almost double quantity than in 2016, adding up to 19.0 mg g^{-1} and 9.2 mg g^{-1}, respectively (figure S3). Because of this large difference between years, the "year" effect accounts for more than 82% of total experimental variability concerning total flavonols content (table 4). As shown, the ANOVA evidenced significant differences for all variables (including each detected flavonol) and experimental factors (Y and P, both alone and in interaction). Differently from our results, a constraining effect of high temperatures on flavonols content was found by Yao et al. (2019), who retrieved in *H. perforatum* plantlets a significantly decreasing flavonols amount with increasing temperatures from 15 to 22 to 30 °C. Notwithstanding, in our work, despite the large difference between years, the different identified flavonols kept the same relative proportions, allowing to deduce that the involved biosynthetic pathways were all blocked by high temperatures in a similar manner, and no preferential synthesis and storage occurred in any of the compounds.

A marked distinction may be observed (fig. S3-d) between stable high-yielding genotypes (PFR-CG and PFR-TN) and stable low-yielding genotypes (PFR-AG and PFR-VI), whereas the other three accessions exhibited a variable behavior between years. Among flavonols, myricitrin was practically absent from the tested samples. In both years, the most abundant flavonol was quercetin-3-*O*-galactoside (hyperoside; fig. S3-a), accounting for more than 40% of all identified flavonols. Quercetin-3-*O*-glucoside (isoquercitrin) was the second most represented flavonol (about 23% of all identified flavonols), followed by quercitrin (fig. S3-c; 14.6% in both years), quercetin-3-*O*-rutinoside (rutin; 11% in 2015 and 8.4% in 2016), and quercetin (fig. S3-b; 6.2% in 2015 and 3.8% in 2016). As evidenced in the graphs in figure S3, however, the mean values over years are not always representative of the actual behavior of biotypes, and wide deviations from the respective mean values can be observed in each biotype. Five biotypes out of 7 (PFR-AG, PFR-CG, PFR-VI, PFR-PM, and PFR-NA – interestingly, all biotypes from Sicily and Southern Italy) did not reveal any presence of rutin (only represented in less than 0.4 mg g^{-1} d.m. in 2015 in the PFR-PM biotype), whereas the same compound was found in appreciable amounts in the two biotypes from Northern Italy (PFR-TN and PFR-SI). A similar result was obtained by Scotti et al. (2019), who noticed the absence of rutin in the majority of analyzed samples coming from Spain, whereas this compound was always detected in samples from other provenances (including China). This result is consistent with the hypothesis that a no-rutin metabolic pathway can have been selected in warmer areas, but of course, the actual existence of rutin-based chemotypes must be confirmed by further research.

Only one of the studied biotypes (PFR-CG) showed a good stability level in the accumulation of hyperoside (fig. S3-a), quercetin (fig. S3-b), and quercitrin (fig. S3-c); the biotypes from Trento (PFR-TN) and Naples (PFR-NA) expressed high and stable yields of quercetin (fig. S3-b) and quercitrin (fig. S3-c), whereas the biotype from Siena (PFR-SI) could be classed as a low-yielding *H. perforatum* concerning the detected amounts of hyperoside (fig. S3-a) and quercetin (fig. S3-b).

The cinnamic acids (Fig. 1d) found in the examined *H. perforatum* provenances were 3-*O*-caffeoylquinic acid, *p*-coumaroilquinic acid, 5-*O*-caffeoylquinic acid and p-coumaric acid. The two caffeoylquinic acids (3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid) were always the most abundant, averaging between 87 and 97% of total retrieved cinnamic acids. All of them exhibited significant differences at ANOVA (table 5) for both factors "provenance" and "year". In the pooled two-year analysis, significant differences showed also up in the interaction "PxY" for all of them, with the only exception of *p*-coumaroilquinic acid, where the factors "Y" and "P" showed an additive effect.

As shown in figure S4-a, 3-*O*-caffeoylquinic acid in the first year averaged a much higher value than in 2016 (1.6 vs. 0.4 mg g^{-1} d.m.). Inside provenances, PFR-CG (2.5 mg g^{-1} d.m.) and PFR-VI (2.1 mg g^{-1} d.m.) reached the highest values in 2015, whereas PFR-CG and PFR-PM (both with 0.7 mg g^{-1} d.m.) ranked first in 2016. Hence, only one provenance (PFR-CG) was stable for high values of 3-*O*-caffeoylquinic acid, whereas PFR-AG, PFR-SI and PFR-NA were also stable, but for lowest values.

Also 5-*O*-Caffeoylquinic acid (fig. S4-c) reached in 2015 a higher amount than in 2016, with mean values of 0.7 and 0.3 mg g^{-1} d.m., respectively. It exhibited a good inter-annual stability level in four provenances out of seven, but only two of them (PFR-VI and PFR-NA) had consistently high values, whereas the other two (PFR-AG and PFR-SI), in both years ranked in the lowest positions. *P*-coumaroilquinic acid (fig. S4-b) and *p*-coumaric acid were found in lower amounts. The first accounted for 3.8-10% of total cinnamic acids in 2015 and 2.3-12% in 2016. *p*-Coumaric acid was instead found in very low amounts (on average 0.01 mg g^{-1} d.m. in 2015 and 0.002 in 2015, being in this second year totally absent in two out of seven provenances, namely PFR-CG and PFR-TN. Increased amount of cinnamic acids was found in plantlets of *H. perforatum* grown at decreasing temperatures (30°C, 22°C, and 15°C) (Yao et al., 2019).

Catechin content in the studied *Hypericum* accessions exhibited at ANOVA significant variations due to both provenance (P) and year (Y), as well as to their interaction (PxY) (table 6). The factor Y, however, was able to explain more the 50% of experimental variability. In 2015, catechin content averaged 0.7 mg g^{-1}, a markedly higher value than in 2016 (0.2 mg g^{-1}). Among biotypes (fig. S5-a), four biotypes out of seven showed a fairly high stability in years, being PFR-VI and PFR-CG stable on high values, and PFR-TN and PFR-AG stable on low ones.

As concerns biflavonoids, in this work, biapigenin and amentoflavone achieved a total amount of 6.8 mg g^{-1} in 2015 and 4.1 mg g^{-1} in 2016. The most abundant of them was always biapigenin, being amentoflavone always detected in amounts lower than 0.3 mg g^{-1}. At the ANOVA (table 6), the two compounds and their cumulated amount showed a highly significant effect of both factors Year (Y) and Provenance (P); the YxP interaction was not significant in the amentoflavone content, allowing to assess that the effect on this compound of year and genotype was mostly exerted additively. As biapigenin represented the majority of total dimers amount, the stability assessment for biapigenin (fig S5-b) is very similar to that of total dimers (fig. S5-d). The biotype PFR-AG, and, to a lesser extent, the biotype PFR-CG revealed as the most stable for high yield levels, whereas PFR-TN and PFR-PM could be allocated as low-yielding genotypes.

3. Conclusions.

It is well known that plant-derived products deal with a typical and wide variability, due to many genetic and environmental factors involved in the biosynthetic and storage processes of their secondary metabolites (Sangwan et al., 2001). Hence, from the phytochemical point of view, the final outcome of *Hypericum* cultivation is often unpredictable. When cultivation is meant for pharmaceutical supply, this represents a serious issue. Among the causes of phytochemical variability of cultivated plants, genetic plant features are undoubtedly very important. Indeed, many active

compounds have been found to vary according to the species, and within species, according to lower-order taxon. From here, the frequent advice to put in cultivation only plant genotypes with selected and well assessed quality characteristics (Couceiro et al., 2006; Lazzara et al., 2020). Anyway, in cultivated plants, also additional sources of variability (including e.g., soil characteristics, water availability, competing organisms such as weeds, pests, and insects) exert significant effects on plant biochemical features.

In this work, we have compared the amount of several active metabolites in *H. perforatum* according to genotypes and year of cultivation. As expected, significant differences showed up among genotypes, stressing the occurrence of a high variability due to the genetic background of cultivated plants. However, attention was also focused on the "stability" level showed throughout years by the different accessions. This approach allowed to partition the tested biotypes based on their tendency to maintain consistently high –or low- levels of the metabolites of interest, or conversely, to express a variable response between years. Only one among the tested biotypes (PFR-CG) exhibited constantly higher-than-average amounts of all identified metabolites, with the only exception of hyperforin and amentoflavone.

4. Experimental

4.1 General experimental procedures. Polyphenols quantitative analyses were carried out on a HPLC instrument equipped with a binary pump and a photodiode array detector (Thermo Scientific, Italy), using a reverse-phase column (Gemini C_{18}, 250 x 4.6 mm, 5 µm particle size, Phenomenex, Italy) equipped with a guard column (Gemini C_{18}, 4 x 3.0 mm, 5 µm particle size, Phenomenex, Italy). Naphthodianthrones and phloroglucinols quantitative analyses were carried out on a similar HPLC instrument (Hitachi Chromaster). Chromatographic runs were performed using the same column of polyphenols. In order to unambiguously identify the chromatographic signals and/or to confirm peak

assignments, a series of HPLC/ESI/MS analyses were performed on a significant number of samples. The HPLC apparatus used was the same described above, whilst ESI mass spectra were acquired by a Thermo Scientific Exactive Plu Orbitra MS (Thermo Fisher Scientific, Inc., Milan, Italy).

4.2 Plant material. A collection activity of seeds of *Hypericum perforatum* from different areas of Italy (table 1; figure 2) was carried out in 2014 and 2015. In 2015 and 2016, established plants obtained from the collected seeds were disposed in a catalogue field, renewed every year, located within the facilities of CREA-DC in Bagheria (PA, Italy; 38°05'25" N-13°31'08" E). Climatic data (maximum, minimum and mean air temperatures, and rainfall amount) during the timespan of *Hypericum* plants growth (March-June) were measured through a meteorological station near to the experimental site. After transplant, plant grew quickly, reaching the floral budding stage in the last ten days of May, and the flowering phase in early June in both years. At full-flowering time, the flowered tops (15-20 cm) were collected, and after cutting, plant samples were stored in paper bags and dried at 20-25 °C in the dark for further analyses.

4.3. Analytical determinations. The dried flowers collected from the different treatments were finely crushed and aliquots (1 g) of powder were extracted with 20 mL of ethanol for 72 hours under gently continuous stirring, avoiding light exposure due the photo sensibility of some of the metabolites of interest. The resulting deep red colored suspensions were filtered on PTFE 0,45 μ filters (PALL Corporation), put into 2mL amber vials and sent to analytical determinations.

Hypericum polyphenols (Fig. 1) were eluted with the following gradient of B (formic acid, 2.5% solution in acetonitrile) in A (2.5% solution of formic acid in water): 0 min: 5 % B; 10 min: 15 % B; 30 min: 25 % B; 35 min: 30 % B; 50 min: 90 % B; then kept for 7 min at 100 % B. The solvent flow rate was 1 mL/min. Quantifications were carried out using the corresponding reference substances or

adequate analogous at 330 or 350 nm. Naphthodianthrones and phloroglucinols quantitative analyses were carried out on a similar HPLC instrument (Hitachi Chromaster). Chromatographic runs were performed using the same column of polyphenols and were carried out with the following gradient of B (acetonitrile) in A (ammonium acetate 20 mM in water): 0 min: 50 % B; 25 min: 50% B; 35 min: 10 % B; 45 min: 90 % B; 50 min: 50 % B (Tawaha et al., 2010). The solvent flow rate was 1 mL/min. Quantifications were run at 290 nm for phloroglucinols (Fig. 1), with hyperforin as reference substance and at 590 nm for naphthodianthrones (Fig. 1) using hypericin and protohypericin as standards. All analyses were carried out in triplicate.

4.4 Statistical data management. At the end of the second trial year (2016), a dataset of 42 individual samples (7 accessions x 2 years x 3 repetitions) was available. Data obtained in both years were submitted to ANOVA by means of the statistical package Minitab 17.0. The ANOVA carried out on pooled two-years data showed the occurrence of significant differences both between years and among the interactions "Year x Provenance". Hence, the analysis was repeated separately for both years. Stability of chemical features of the examined biotypes was determined through graphical assessment, by plotting the values of the selected compounds obtained in each biotype in the two years on the two axes of an X-Y graph, along with the average values across both years, +/- their respective standard deviations. The graphical representation (reported as Supplementary Material, figures S1 to S5) allows individuating which provenance showed a higher "stability"; all biotypes that in both years showed a higher-than-average content are located in the top right section of the graph (1st quadrant), whereas those having in both years a lower-than-average content are symmetrically placed in the bottom left position (3rd quadrant). When biotypes revealed an "unstable" behavior, i.e., they were dealing with contrasting values in both years, they are situated in the top left and bottom right positions (2nd or 4th quadrant, respectively).

References

Aron P.M., Kennedy J.A. 2008. Flavan-3-ols: Nature, occurrence and biological activity. Mol. Nutr. Food Res., 52: 79 –104. doi: 10.1002/mnfr.200700137

Bagdonaitė E., Mártonfi P., Repčák M., Labokas J. 2012. Variation in concentrations of major bioactive compounds in *Hypericum perforatum* L. from Lithuania. Ind. Crops Prod. 35, 302–308.

Berghöfer R., Hölzl J. 1987. Biflavonoids in *Hypericum perforatum*; Part 1. Isolation of I3, II8-biapigenin. Planta Med., 53, 2: 216-217. doi: 10.1055/s-2006-962676.

Berghöfer R., Hölzl J. 1989. Isolation of I3', II8-Biapigenin (Amentoflavone) from *Hypericum perforatum*. Planta Med., 55, 91

Božin B., Kladar N., Grujić N., Anačkov G., Samojlik I., Gavarić N., Srđenović Čonić B. 2013. Impact of origin and biological source on chemical composition, anticholinesterase and antioxidant properties of some St. John's Wort species (*Hypericum* spp., Hypericaceae) from the Central

Balkans. Molecules, 18, 11733-11750. doi: 10.3390/molecules181011733

Butterweck V., Jürgenliemk G., Nahrstedt A., Winterhof, H. 2000. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. Planta Med. 66, 3–6.

Butterweck V., Christoffel V., Nahrstedt A., Petereit F., Spengler B., Winterhoff H. 2003. Step by step removal of hyperforin and hypericin: activity profile of different *Hypericum* preparations in behavioral models. Life Sci., 73, 627–639. doi: 10.1016/S0024-3205(03)00314-X

Carrubba A., Catalano C. 2009. Essential oil crops for sustainable agriculture - A review. In: Lichtfouse, E. (Ed), Climate change, intercropping, pest control and beneficial microorganisms, Springer, Dijon, pp.137-188. doi: 10.1007/978-90-481-2716-0_8

Chaudhary A., Willett K.L. 2006. Inhibition of human cytochrome CYP1 enzymes by flavonoids of St.

John's wort. Toxicology, 217: 194–205.

Chrubasik-Hausmann S., Vlachojannis J., McLachlan A.J. 2019. Understanding drug interactions with St John's wort (*Hypericum perforatum* L.): impact of hyperforin content. J. Pharm. Pharmacol., 71: 129–138. doi: 10.1111/jphp.12858

Couceiro M.A., Afreen F., Zobayed S.M.A., Kozai T. 2006. Variation in concentrations of major bioactive compounds of St. John's wort: Effects of harvesting time, temperature and germplasm. Plant Sci., 170: 128–134. doi:10.1016/j.plantsci.2005.08.011

Dresler S., Kováčik J., Strzemski M., Sowa I., Wójciak-Kosiorc M. 2018. Methodological aspects of biologically active compounds quantification in the genus *Hypericum*. J. Pharmac. Biomed. Anal., 155: 82–90. doi: 10.1016/j.jpba.2018.03.048

EMEA, European Medicines Agency. 2009. Committee on Herbal Medicinal Products (HMPC). Assessment Report on *Hypericum perforatum* L., Herba. Doc. Ref.: EMA/HMPC/101303/2008. [https://www.ema.europa.eu/en/documents/herbal-report/assessment-report-hypericum perforatum-l-herba_en.pdf].

Gontijo V.S., dos Santos M.H., Viegas C. 2017. Biological and chemical aspects of natural biflavonoids from plants: A brief review. Mini-Rev. Med. Chem., 17: 834-862.

Jendželovská Z., Jendželovský R., Kuchárová B., Fedoročko P. 2016. Hypericin in the light and in the dark: two sides of the same coin. Front. Plant Sci. 6 (7), 560.

Jürgenliemk G., Nahrstedt A. 2002. Phenolic compounds from *Hypericum perforatum*. Planta Med., 68: 88-91.

Lazzara S., Carrubba A., Napoli E. 2020. Variability of hypericins and hyperforin in Hypericum species from the Sicilian flora. Chem. Biodiv., 17, e190059610. doi: 1002/cbdv.201900596

Linde, K., Ramirez G., Mulrow C.D., Pauls A., Weidenhammer W., Melchart D. 1996. St John's Wort for depression—An overview and meta-analysis of randomised clinical trials. BMJ 313, 253–258.

Liu W., Li J., Zhang X., Zu Y., Yang Y., Liu W., Xu Z., Gao H., Sun X., Jiang X., Zha Q. 2020. Current advances in naturally occurring caffeoylquinic acids: Structure, bioactivity, and synthesis. J. Agric. Food Chem., 68, 10489−10516. doi: 10.1021/acs.jafc.0c03804

Mártonfi P., Repčák M., Ciccarelli D., Garbari F. 2001. *Hypericum perforatum* L. - chemotype without rutin from Italy. Biochem. System. Ecol., 29: 659–661.

Michler H., Laakmann G., Wagner H. 2011. Development of an LC-MS method for simultaneous quantitation of Amentoflavone and Biapigenin, the minor and major biflavones from Hypericum perforatum L., in human plasma and its application to real blood. Phytochem. Anal. 22: 42-50. doi: 10.1002/pca.1249.

Nahrstedt A., Butterweck V. 1997. Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. Pharmacopsychiat., 30 (Supplement): 129 – 134.

Nahrstedt A., Butterweck V. 2010. Lessons learned from herbal medicinal products: The Example of St. John's Wort. J. Nat. Prod., 73: 1015–1021

Napoli E., Siracusa L., Ruberto G., Carrubba A., Lazzara S., Speciale A., Cimino F., Saija A., Cristani M. 2018. Phytochemical profiles, phototoxic and antioxidant properties of eleven *Hypericum* species: a comparative study. Phytochemistry, 152: 162-173.

Odabas M.S., Raduğienë J., Camas N., Janulis V., Ivanauskas L., Çırak C. 2009. The quantitative effects of temperature and light intensity on hyperforin and hypericins accumulation in *Hypericum perforatum* L. J. Med. Plants Res., 3(7): 519-525.

Patočka J. 2003. The chemistry, pharmacology, and toxicology of the biologically active constituents of the herb *Hypericum perforatum* L. J. Appl. Biomed., 1: 61–70.

Pietta P.G. 2000. Flavonoids as antioxidants. J. Nat. Prod., 63: 1035-1042. doi: 10.1021/np9904509.

Pilepić K.H., Maleš Ž., Crkvenčić M. 2013. Quantitative analysis of total flavonoids and total phenolic acids in thirty Hypericum taxa. Nat. Prod. Com., 8 (3): 347-349.

Pluhár Zs., Bernáth J., Neumayer É. 2002. Morphological, production biological and chemical diversity of St. John's Wort (*Hypericum perforatum* L.). Acta Hort. 576: 33-40.

Raclariu A.C., Paltinean R., Vlase L., Labarre A., Manzanilla V., Ichim M.C., Crisan G., Krag Brysting A., de Boer H. 2017. Comparative authentication of *Hypericum perforatum* herbal products using DNA metabarcoding, TLC and HPLC-MS. Scientific Reports, 7: 1291. doi: 10.1038/s41598-017-01389-w.

Rizzo P., Altschmied L., Ravindran B.M., Rutten T., D'Auria J.C. 2020. The biochemical and genetic basis for the biosynthesis of bioactive compounds in *Hypericum perforatum* L., one of the largest medicinal crops in Europe. Genes 11, 1210. doi: 10.3390/genes11101210

Sangwan N.S., Farooqi A.H.A., Shabih F., Sangwan R.S., 2001. Regulation of essential oil production in plants. Plant Growth Regulation, 34: 3–21.

Scotti F., Löbel K., Booker A., Heinrich M., 2019. St. John's Wort (*Hypericum perforatum*) Products - How variable is the primary material? Front. Plant Sci. 9: 1973. doi: 10.3389/fpls.2018.01973

Silva B.A., Ferreres F., Malva J.O., Dias A.C.P. 2005. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. Food Chem., 90: 157–167. doi: doi:10.1016/j.foodchem.2004.03.049

Southwell I.A., Bourke C.A. 2001. Seasonal variation in hypericin content of Hypericum perforatum L. (St. John's Wort). Phytochemistry, 56: 437.441.

Stevinson C., Ernst E. 1999. *Hypericum* for depression. An update of the clinical evidence. European Neuropsychopharmacology, 9: 501 –505.

Sun P., Kang T., Xing H., Zhang Z., Yang D., Zhang J., Paré P.W., Li M. 2019. Phytochemical changes in aerial parts of *Hypericum perforatum* at different harvest stages. Rec. Nat. Prod. 13, 1–9.

Tatsis E.C., Boeren S., Exarchou V., Troganis A.N., Vervoort J., Gerothanassis I.P. 2007. Identification of the major constituents of *Hypericum perforatum* by LC/SPE/NMR and/or LC/MS. Phytochemistry, 68: 383–393.

Tawaha K., Gharaibeh M., El-Elimat T., Alali F.Q. 2010. Determination of hypericin and hyperforin content in selected Jordanian *Hypericum* species. Ind. Crops Prod. 32: 241-245.

WHO, World Health Organization. 2002. *Herba Hyperici.* WHO monographs on selected medicinal plants, vol. 2: 149-171. WHO, Geneva.

Yao Y., Kang T., Jin L., Liu Z., Zhang Z., Xing H., Sun P., Li M. 2019. Temperature-dependent growth and hypericin biosynthesis in *Hypericum perforatum*. Plant Phys. Biochem., 139: 613–619. doi: 10.1016/j.plaphy.2019.04.012.

Yu S., Yan H., Zhang L., Shan M., Chen P., Ding A., Li S.F.Y. 2017. A Review on the phytochemistry, pharmacology, and pharmacokinetics of amentoflavone, a naturally-occurring biflavonoid. Molecules, 22, 299. doi:10.3390/molecules22020299.

Zdunic G., Godjevac D., Savikin K., Petrovic S. 2017. Comparative analysis of phenolic compounds in seven *Hypericum* species and their antioxidant properties. Nat. Prod. Com. 12, 1805–1811.

TABLES

Table 1 - Geographical provenance of the studied *H. perforatum* accessions.

#	Code	Provenance
1	PFR-AG	Cammarata (AG)
2	PFR-CG	Capo Gallo (PA)
3	PFR-VI	Vicaretto (PA)
4	PFR-PM	P. Marcato - Castelbuono (PA)
5	PFR-SI	Siena (SI)
6	PFR-TN	Trento (TN)
7	PFR-NA	Napoli (NA)

Table 2. Results of ANOVA for the content in major naphthodianthrones in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Hyp	PsHyp	PrPsHyp	PrHyp	Total
2015-2016						
Provenance (P)	6	*** (75.5%)	** (25.4%)	*** (11.3%)	*** (12.6%)	*** (33.7%)
Year (Y)	1	** (1.9%)	*** (26.2%)	*** (77.7%)	*** (75.0%)	*** (46.4%)
P x Y	6	*** (17.5%)	n.s. (16.3%)	*** (11.0%)	*** (12.4%)	*** (12.8%)
Error	28					
Total	41					
2015						
Provenance (P)	6	***	n.s.	***	***	***
Error	14					
Total	20					
2016						
Provenance (P)	6	***	***	***	***	***
Error	14					
Total	20					

[a] Hyp=hypericin; PsHyp=pseudohypericin; PrPsHyp=protopseudohypericin; PrHyp=protohypericin

Table 3. Results of ANOVA for the content in major phloroglucinols in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Hypf	AdHypf	Total
2015-2016				
Provenance (P)	6	*** (46.4%)	*** (38.4%)	*** (45.1%)
Year (Y)	1	*** (3.9%)	** (1.4%)	*** (2.2%)
P x Y	6	*** (45.5%)	*** (56.8%)	*** (48.7%)
Error	28			
Total	41			
2015				
Provenance (P)	6	***	***	***
Error	14			
Total	20			
2016				
Provenance (P)	6	***	***	***
Error	14			
Total	20			

[a]Hypf=hyperforin; AdHypf=adhyperforin; total=hyperforin + adhyperforin

Table 4. Results of ANOVA for the content in myricetin derivatives, quercetin-3-*O*-rutinoside, Quercetin-3-*O*-galactoside, Quercetin-3-*O*-glucoside, Quercetin, Quercitrin, and total flavonols detected in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Myrdv	Q3Orut	Q3Ogal	Q3Oglu	Quercet	Quercit	Total flavonols
2015-2016								
Provenance (P)	6	*** (44.9%)	*** (77.7%)	*** (31.8%)	*** 37.3%)	*** (3.4%)	*** 32.2%)	*** (7.1%)
Year (Y)	1	*** (10.0%)	*** (6.9%)	*** (57.6%)	*** (48.8%)	*** (93.3%)	*** (52.0%)	*** (82.4%)
P x Y	6	*** (44.9%)	*** (15.3%)	*** (9.1%)	*** (13.4%)	*** (2.1%)	*** (14.8%)	*** (9.7%)
Error	28							
Total	41							
2015								
Provenance (P)	6	***	***	***	***	***	***	***
Error	14							
Total	20							
2016								
Provenance (P)	6	n.s.	***	***	***	***	***	***
Error	14							
Total	20							

[a] Myrdv=myricetin derivatives; Q3Orut=quercetin-3-*O*-rutinoside; Q3Ogal=quercetin-3-*O*-galactoside; Q3Oglu=quercetin-3-*O*-glucoside; Quercet=quercetin; Quercit=quercitrin; total flavonols=myricitrin + myricetin derivatives + quercetin-3-*O*-rutinoside + quercetin-3-*O*-galactoside + quercetin-3-*O*-glucoside + quercetin + quercitrin

Table 5. Results of ANOVA for the content in major cinnamic acids derivates in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor	DF	3-O-CQ	*p*-CouQ	5-O-CQ	*p*-Cou	Total
2015-2016						
Provenance (P)	6	*** (18.2%)	** (11.5%)	*** (43.6%)	*** (15.2%)	*** (18.4%)
Year (Y)	1	** (75.4%)	*** (76.1%)	*** (33.2%)	*** (61.0%)	*** (76.7%)
P x Y	6	*** (6.2%)	n.s. (9.3%)	*** (22.0%)	*** (14.2%)	*** (4.6%)
Error	28					
Total	41					
2015						
Provenance (P)	6	***	***	***	***	***
Error	14					
Total	20					
2016						
Provenance (P)	6	***	***	***	***	***
Error	14					
Total	20					

[a] 3-O-CQ=3-*O*-Caffeoylquinic acid; *p*-CouQ=*p*-Coumaroilquinic acid; 5-O-CQ=5-*O*-Caffeoylquinic acid; *p*-Cou=*p*-Coumaric acid; total=3-O-CQ + *p*-CouQ + 5-O-CQ + *p*-Cou

Table 6. Results of ANOVA for the content in catechin, biapigenin, amentoflavone and total dimers in the MeOH extract. In brackets, the amount of experimental variability (%) explained by each factor.

Factor[a]	DF	Ctchin	Bpgn	Amtfl	Total dimers
2015-2016					
Provenance (P)	6	*** (27.7%)	*** (34.1%)	*** (33.0%)	*** (34.3%)
Year (Y)	1	*** (52.4%)	*** (43.5%)	*** (43.5%)	*** (43.9%)
P x Y	6	*** (18.9%)	*** (21.3%)	n.s. (7.2%)	*** (20.8%)
Error	28				
Total	41				
2015					
Provenance (P)	6	***	***	*	***
Error	14				
Total	20				
2016					
Provenance (P)	6	***	***	***	***
Error	14				
Total	20				

[a] Ctchin=catechin; Bpgn=biapigenin; Amtfl=amentoflavone; total dimers=biapigenin + amentoflavone