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RESEARCH

Canopy openness, proportion of deciduous trees and topsoil C/N ratio drive the yield, but their effect on the polyphenol content of medicinal plants is species-specific

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Abstract

Forests and woodlands are the major source of wild medicinal plants worldwide. In our study, we aimed to identify the factors influencing the yield and polyphenol content of *Aegopodium podagraria* L., *Galium aparine* L., *Rubus fruticosus* L., *Rubus idaeus* L., *Stachys sylvatica* L. and *Urtica dioica* L., the common and abundant medicinal plant species in the study areas. We showed that European temperate forests are potentially an important source of the medicinal resources. Light availability, controlled by canopy cover, proportion of deciduous trees and stand basal area were the most important factors positively influencing both abundance and quality of medicinal plants. The C/N ratio and pH of the topsoil were the most important factors positively influencing the content of phenolic compounds. The phenolic content was highly species-specific and varied according to local environmental conditions. A high proportion of deciduous species and a high canopy openness increased the yield and quality of medicinal plants by ensuring high light availability. Plants with high total polyphenol content should also be sought on biologically active (non-acidic) soils with a high C/N ratio. Our results can be used to guide forest management in areas where harvesting of understory medicinal plants is an important provisioning ecosystem service. In many cases a forest management scenario friendly to medicinal plants may require only a minor changes in forest management intensity, as cultivation or enhanced growth of MD plants can take place in intensively thinned forests and cleared forest patches, without competing with timber production.

Keywords DrFOREST · Secondary metabolites · Ethnomedicinal plants · Soil properties · Light availability · Ecosystem services

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Introduction

People have harvested forest plants for medicinal purposes for millennia and forests and woodlands are still the major source of wild medicinal plants (MD) worldwide (Rao et al. 2004). Medicinal plants are those that possess therapeutic properties or beneficial pharmacological effects (Namdeo 2018) and provide resources used in traditional and modern medicine, including discoveries of new plant-derived drugs. Throughout the world, about 50,000 to 70,000 plant species, belonging to several hundred genera, are considered medicinal, which represents about 12-17% of the world's flora (Rao et al. 2004; Koleva et al. 2021). However, only 28,000 plant species are mentioned in official pharmacopoeias (Willis 2017). In Europe, the share of MD species in the total flora was estimated at 1,200–1,300 plant species out of the 9,875 species native to the continent (Kalusová et al. 2017).

Due to the low production cost in nature, universal availability, low side effects and high effectiveness in treatment of many diseases, plant-derived remedies are commonly used worldwide. In effect of growing popularity, the global trade of plant-derived medicinal products, which in 2010 was worth 60 billion US dollars (USD), is expected to grow to 5 trillion USD before 2050 (Zahra et al. 2020), which may increase the pressure on wild growing medicinal plants, negatively influencing their conservation status (Chen et al. 2016; Brinckmann et al. 2022) and functioning of forest ecosystems (Ticktin 2004).

Medicinal plants owe their healing properties mainly to secondary metabolites. These substances, e.g. hydrocarbons, terpenoids, phenolic compounds, alkaloids and many others, are produced by plants to remain competitive in interactions with other organisms and to control their own physiological processes (Bravo 2009; Teoh 2016). Of the more than 50,000 types of secondary metabolites discovered in plants, the largest group is made up of chemical compounds containing at least one cyclic carbon ring with a hydroxyl group, known as aromatics, including polyphenols, the organic compounds with multiple phenolic groups, also referred to as phenolics, which are highly valued for their health-promoting and healing properties.

Despite of the high number of papers studying the effect of environmental factors (e.g.: pH, nutrients, light conditions) on species richness and biomass of forest plants (Liang et al. 2016; Fu et al. 2017; Landuyt et al. 2020), the knowledge regarding the role of these factors for the production of secondary metabolites in general, and polyphenols in particular, still remains scarce in wild plants (Stamp 2003), and is mainly confined to economically important and cultivated species. Many papers report the polyphenol content of the selected medicinal or food plant species from specific regions (Silva et al. 2007) or countries (Singh et al. 2016; Aryal et al. 2019; Koleva et al. 2021; Laanet et al. 2023) but without paying any attention to environmental factors modulating content of secondary metabolites. They report that the concentration of phenolics in plants varies greatly between plant species and even between plant organs within the same organism (Silva et al. 2007; Stankovic et al. 2011; López-Laredo et al. 2012; Koleva et al. 2021). The review on polyphenol content of agricultural plants (Heimler et al. 2017) revealed no effect of environmental or management factors, with the exception for the negative effect of high nitrogen supply. Li et al. (Li et al. 2020), Pant et al. (Pant et al. 2021) and Ncube et al. (Ncube et al. 2012) in their reviews found that several environmental factors (e.g. light and ultraviolet radiation, moisture, temperature, salinity of the soil, ozone) may influence content of secondary metabolites (including polyphenols) in plants. However, they reported positive or negative effects, depending on plant species, type of the compound and plant organ. There are very few studies on the factors influencing the content of biologically active compounds in forest MD plants. Naud et al. (2010) examined four North American MD plant species growing under canopy of forests managed in different manner. They found that under conditions limiting plant growth (low pH, light and nutrient availability) more biologically active substances are allocated in rhizoms and roots but the total content of active components in total plant biomass positively depended on the same factors as plant growth (Naud et al. 2010). Such non-conclusive results may be caused by the fact that the major effect in triggering the production and accumulation of secondary metabolites can be driven not by average environmental conditions but by their maxima or minima, i.e. environmental stresses such as extremely high or low temperatures, drought, salinity, and heavy metals concentration (Herms and Mattson 1992; Pant et al. 2021; Endara et al. 2023).

Despite the high popularity of herbal medicines, very few medicinal plants are cultivated. Globally, only 3,227 taxa of MD plants are harvested from various cultivation systems (Brinckmann et al. 2022), which is mere 12% of the species officially recognized as medicinal by pharmacopoeia (Willis 2017) or 5% of all species used globally for healing purposes by both traditional or modern medicine (Koleva et al. 2021). In Europe, 90% of native MD species are still harvested from wild ecosystems, often from forests (Balunas and Kinghorn 2005), and three European countries: Germany, Poland and Spain were in 2010–2019 among the top ten exporters of medicinal and aromatic plants in the world, with the mean total annual export on the level of 45,000 t (Sucholas et al. 2021). The share of plant medicinal resources collected from nature in Europe varies from 30 to 50% in Hungary up to close to 100% of the country harvest in Albania and Turkey (Lange 1998). Taking into account the significant share of medicinal resources harvested in wild, especially in forest ecosystems (Rao et al. 2004), the quantification of forest characteristics that determine the potential harvest and the content of biologically active compounds in wild plants is of high importance. Therefore, in this study we aimed to identify those forest characteristics that significantly modify species cover (as a proxy for potential biomass available for harvest) and content of the polyphenols in medicinal plants.

We tested the following main hypotheses: (i) MD plants are more light demanding than other understory plant species; (ii) the content of polyphenols in MD plants is higher in open forests (with low canopy cover and low basal area); (iii) due to stress caused by low nutrient availability (Herms and Mattson 1992; Reshi et al. 2023), the content of polyphenols in MD plants is higher on soils with low pH and high C/N ratio of the topsoil layer; (iv) because pathogen load and herbivory attack rates are lower in mixed forests, compared to monocultures (Hantsch et al. 2013; Damien et al. 2016), the content of polyphenols in MD plants is lower in mixed forests than in monocultures.

Methods

Study area

We conducted our research in three mature forest (MAT) sites located in Belgium (TREEWEB (TW), Germany (Hainich National Park) and Poland (Białowieża Forest), and three relatively young experimental forests (EXP) located in Belgium in Zedelgem and Gedinne and in Germany (Kaltenborn; Online Resource 1). The MAT sites were established as permanent research platforms according to the methodological approach developed by the FunDivEUROPE project, with the main aim to study the influence of forest diversity on functioning of mature forest ecosystems (Baeten et al. 2013). The basic principle of that approach was the selection of forest plots along a gradient of tree diversity levels at each site (from single species up to five species mixtures) with simultaneous minimization of environmental variability among the plots, thus representing a comparative study design. The plots in MAT sites are 30 m × 30 m and are surrounded by a minimum 10 m wide buffer zone of similar forest composition and structure to avoid edge effects. The study sites in Białowieża Forest (eastern Poland) and in Hainich National Park (central Germany) were established in 2011 during the implementation of the FunDivEUROPE project (Baeten et al. 2013). The TREEWEB study site (north-western Belgium) was established in 2014 following the same procedure of plot selection, assuring their compatibility with the FunDivEUROPE sites (De Groote et al. 2017). The EXP sites were established on post-agricultural land (Zedelgem, north-eastern Belgium and Kaltenborn, central Germany) in 2010 and 2003/2004 (respectively), and on cleared forest site (Gedinne, southern Belgium) in 2010, to study the effect of tree species diversity on ecosystem multifunctionality. They consist of monocultures and mixtures of tree species planted on an environmentally homogeneous site, with all species combinations replicated and randomly distributed in space (Scherer-Lorenzen et al. 2007; Verheyen et al. 2013; Online Resource 1).

Vegetation, stand and soil data

In June-July 2020 and 2021 we carried out vegetation surveys in all three mature forests and in the Kaltenborn experimental site, to record all plant species and visually estimate their percentage cover, including estimation of the tree canopy closure. To allow comparisons with a historical vegetation surveys of the FunDivEUROPE exploratory sites, we followed the survey method proposed by Ampoorter et al. (Ampoorter et al. 2016): each study plot (30 m x 30 m) was subdivided in nine subplots of 10 m × 10 m. In the southwestern, central and northeastern subplots, the understory was surveyed in a 5 m × 5 m quadrat placed at fixed location (see Fig. 1 in Ampoorter et al. 2016). The vegetation for Zedelgem and Gedinne experimental sites was surveyed in July-August 2018 by the local site managers, using the similar methodological approach, however, plots were divided into four subplots and in each subplot vegetation was surveyed in the center of the subplot on the 4.5 m x 4.5 m quadrat. For all sites we combined the data from quadrats to calculate an average cover of each plant species per study plot.

The plot-level data characterizing forest stands and soils in Białowieża Forest (MAT) and Hainich National Park (MAT) were collected during the FunDivEUROPE project (2011–2014) and are available at the DRYAD data portal (Scherer-Lorenzen et al. 2023). Tree diameters (at breast height) at these sites were re-measured in 2018. Tree diameters were used to calculate stem basal area of each tree species and used to calculate the share of each tree species at plot level. The same data was used to estimate the proportion of deciduous trees at plot level. Soil characteristics: C/N ratio and nitrogen concentration were measured in 2012 by (Dawud et al. 2016) according to method described in their paper. Soil parameters in Gedinne (EXP), Zedelgem (EXP), Kaltenborn (EXP) and TREEWEB (MAT) were obtained using the same method in 2015–2018.

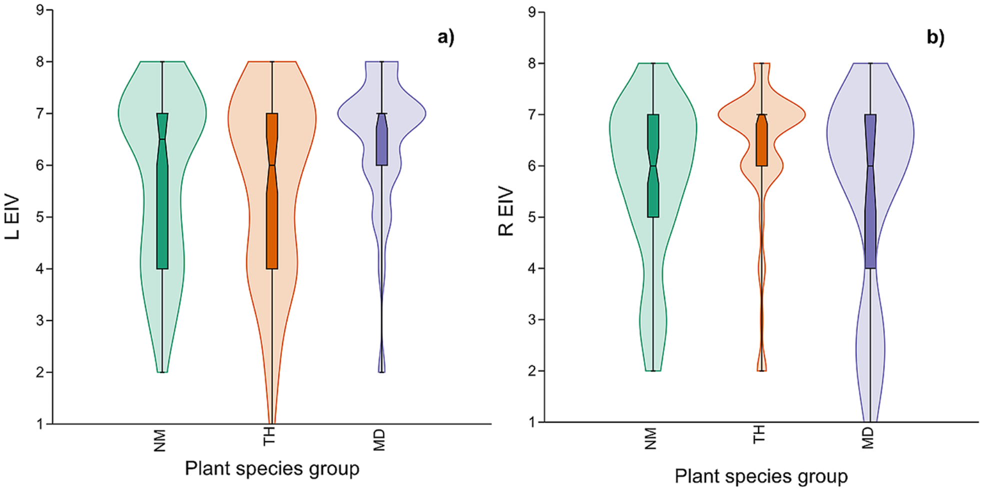

Fig. 1 Ellenberg's ecological indicator value for (a) light and (b) reaction (pH) of non-medical (NM), traditionally herb (TH) and medicinal (MD) plant species recorded on study plots in mature forests: (Białowieża Forest (Poland), Hainich National Park (Germany) and TREEWEB (Belgium) and in experimental sites: Kaltenborn (Germany), Zedelgem, and Gedinne (Belgium)

Sampling of medicinal plants

We studied six forest plant species common to European mixed deciduous forests, including three MD species: *Rubus fruticosus* L. (Rosaceae), *Rubus idaeus* L. (Rosaceae) and *Urtica dioica* L. (Urticaceae), and three species used in traditional medicine (TH): *Aegopodium podagraria* L. (Apiaceae), *Galium aparine* L. (Rubiaceae) and *Stachys sylvatica* L. (Lamiaceae). The bramble *R. fruticosus* and raspberry *R. idaeus* are common species of open forests. They are rich in anthocyanins, flavonol glycosides, and other phenolics (Oszmiański et al. 2015). Their leaf phenolic composition is similar to that of their fruits: they are rich in ellagitannins and chlorogenic acid (Ferlemi and Lamari 2016), known for high antioxidant properties, which may have a potential effects in preventing oxidative related diseases, such as cardiovascular diseases (Larrosa et al. 2010). Despite their high medicinal value bramble leaves are just a byproducts of harvesting its fruits and are traded in much lower amounts than fruits (Ferlemi and Lamari 2016). The common nettle *U. dioica* is one of the most commonly used wild plants of temperate climate. It serves as ingredient of food as well as herbal, medicinal and cosmetic raw material. It contains high amounts of alkaloids, sesquiterpenoids, flavonoids, triterpenoids, sphingolipids, phenols, sterols, vitamins and minerals, which results in antiproliferative, anti-inflammatory, antioxidant, analgesic, anti-infectious and hypotensive properties (Bhusal et al. 2022).

Information on healing properties of plant species used in traditional medicine is much scarcer and often based on traditional knowledge rather than on the thorough medical examinations. Consecutively, their biomass is harvested mostly for the own use. The biologically active compounds of goutweed *A. podagraria* has not been fully determined. It contains some polyacetylenes (falcarinol and falcarindiol), phenols, mono- and sesquiterpenes and coumarins (Orav et al. 2010), which possess anti-inflammatory and antimicrobial properties. The tinctures and extracts from goutweed may potentially be used in the treatment of gout and metabolic diseases including type 2 diabetes (Jakubczyk et al. 2020). *G. aparine* has been used to reduce infection and inflammation, to treat wounds, burns, and skin diseases. It contains significant amount of polyphenols, mainly cryptochlorogenic acid, asperulosidic acid, rutin and quercetin, known for their antioxidant properties (Laanet et al. 2023). The least used TH species is hedge woundwort *S. sylvatica*. It is characterized by very low content of alkaloids but contains flavonoids, phenolics and diterpens and is used to treat inflammation of the salivary glands, varicose veins and swelling (Kanjevac et al. 2022). It possesses also diuretic, digestive, emmenagogue, antispasmodic, anti-inflammatory, sedative and tonic properties, and in Iran is used as disinfectant, anti-spasmodic and for treatment of wounds (Tomou et al. 2020).

Prior to the collection of plant samples for biochemical analyses we divided all non-woody understory plant species, recorded during vegetation surveys, into three categories (Online Resource 2): (1) medicinal plants (MD) – plant species that possess therapeutic properties or exert beneficial pharmacological effects on the human body (Namdeo 2018) and that were approved for official use in phytotherapy in European countries (see European Pharmacopoeia and European Medicines Agency). (2) Traditional herbal plants (TH) – plant species reported by Broda & Mowszowicz (Broda and Mowszowicz 1985) as being used for healing in traditional or folklore medicine but not recognized as official MD plants. (3) Non-medicinal species (NM) – plants not classified as either MD or TH.

The six species for chemical analyses were selected based on the criterion of frequency (present at eight or more plots per site), cover (higher than 5% at the majority of the study plots) and species conservation status (species with high conservation value: protected, threatened or vulnerable were excluded). Out of them, two MD species were present and abundant in more than one site: *R. fruticosus* in TW (MAT), Zedelgem (EXP) and Gedinne (EXP), and *U. dioica* in Białowieża Forest (MAT) and Zedelgem (EXP). The other four species were harvested only at single study sites (see Online resource 3 for list of samples). None of the MD or TH plant species in Hainich National Park (MAT) and Kaltenborn (EXP) (both in Germany) fulfilled the criteria for collection – they were present on too few plots, at too low abundance or their conservation status was too high for sampling. To obtain high content of biologically active substances in plant tissues, all plants were harvested before flowering or at the initial phase of flowering.

The entire above-ground part of plants were harvested in June-July 2021 by shearing with pruning shears (see Online resource 1 for exact dates of sampling at each site). To avoid the effect of local variability in environmental conditions and substantial damage to plant populations, single shoots of the target plant species were sampled from the entire plot area (up to 20–30 individuals per plot). We tied the harvested plants in small bundles, labeled them with species name, site name, plot number, and date of collection, and hung them in a shady airy place until they dried. After drying, plant bundles were packed in labelled paper bags, and stored in dry room till processing in the laboratory.

Biochemical analysis

Depending on the plant part used as herbal raw material in phytomedicine, we used for analysis the whole aerial parts of *S. sylvatica* and *G. aparine* and only leaves of *R. ideaus*, *U. dioica*, *A. podagraria* and *R. fruticosus*. Samples (0.2 g) of dry plant material of each species, finely ground using a kitchen blender, were extracted with 5 ml of 70% methanol (v/v) in an ultrasonic bath at 60 °C for 0.5 h. The extract was filtered through 0.45 µm Chromafil membrane (Machery-Nagel, Germany). Plant extracts were assessed qualitatively and quantitatively using the UHPLC-DAD-MS/MS method. The analysis was performed using the UHPLC-3000 RS system (Dionex, Germering, Germany) with a diode-array detection (DAD) and an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Bremen, Germany). Separation was performed on a Zorbax SB-C18 column (150 × 2.1 mm, 1.9 µm) (Agilent, Santa Clara, California, USA). The mobile phase consisted of 0.1% HCOOH in water (A) and 0.1% HCOOH in MeCN (B) using the following gradients: 0–60 min, 5–40% B. To 0.9 ml of each extract sample 0.1 ml of 0,1% of formic acid was added. The aliquot of 5 µl of extract was injected. The LC eluate was introduced into the ESI interface without splitting, and compounds were analysed in the negative ion modes with the following settings: nebuliser pressure of 40 psi; drying gas flow rate of 9 l/min; nitrogen gas temperature of 300C^{o}; and a capillary voltage of 4.5 kV. The mass scan ranged from 100 to 2200 m/z. UV spectra were recorded in the range of 200–400 nm. Compounds were identified by comparing their retention time, UV and mass spectra, by comparison with literature data (Oszmiański et al. 2015; Ilina et al. 2020; Kim et al. 2020; Laanet et al. 2023). The parameters of the phenolic compounds identification in each sample are provided in Online resource 4. The quantity of polyphenols was calculated using the regression parameters of the calibration curves: ellagic acid (0.5 mg/ml) was used for ellagic acid, ellagic acid derivatives and ellagitannins determination and chlorogenic acid (1 mg/ml) was used for hydroxycinnamic acid derivatives and flavonoids determination. Chromatograms were recorded at 254 and 325 nm (see sample chromatograms in Online resource 5).

For four plant species (three MD: *R. fruticosis*, *R. ideaus*, *U. dioica* and one TH: *G. aparine*) we also assessed the total phenolic content (TPC). Samples of the remaining two TH species (*A. podagraria* and *S. sylvatica*) were lost due to technical reasons. Samples of ground dry plant material (10 mg of *G. aparine* and *U. doica*; 5 mg of *R. fruticosus* and *R. ideaus*) were extracted with 1 ml of 70% methanol (v/v) in an ultrasonic bath at 60 °C for 0.5 h. The TPC was determined using the Folin-Ciocalteu assay according to Skowrońska et al. (2021). In a 96-well plate, 40 µl of plant extract, 75 µl of 15% Folin-Ciocalteu reagent, and 85 µl of Na_2CO_3 were added (160 µl of distilled water was added for the background). A calibration curve was established using gallic acid dissolved in 75% methanol as a standard at concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/ml. The plate was incubated for 15 min at 45 °C. Absorbance was measured at 765 nm, using a BioTek microplate reader (Highland Park, Winooski, VT, USA). The TCP in plant extracts was calculated to gallic acid.

Statistical analysis

All analyses were carried out in R software version 2023.09.1 (R Core Team 2023) in R Studio environment (Posit Team 2023). A non-parametric two-tailed (Wilcoxon) Mann-Whitney U test was used to examine whether MD, NM and TH plants differ significantly by their ecological requirements. The expert-based Ellenberg's Indicator Values (EIV) for light, soil reaction (pH), temperature, moisture and nutrients were used as a proxy of species ecological requirements (Ellenberg and Leuschner 2010).

We used MD plant percentage cover as a proxy of their potential biomass available for harvesting. Before assessing the relationship between MD plant available biomass and environmental factors, we pooled together the quadrats

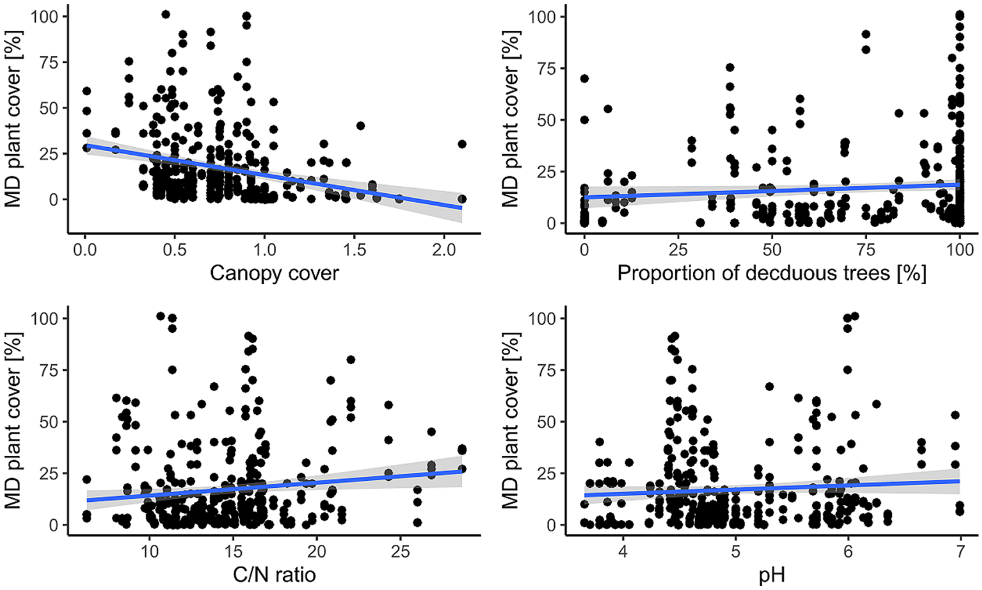

Fig. 2 Relationships between the percentage cover of medicinal plants and site characteristics at the study plots (each point corresponds to exact study plot in exact study site): total tree crown cover [%], proportion of deciduous trees in the stand [%], pH of the topsoil (0–10 cm layer) and C/N ratio, assessed using generalized linear model (see Table 1 for model summary)

surveyed at the same plot and calculated average plant cover. To assess factors affecting MD species cover and content of polyphenols in the analyzed species, we used generalized linear models, which were evaluated using Akaike's Information Criterion (AIC). The models were built separately to assess factors influencing: (i) potential biomass available for harvesting (expressed as species percentage cover) of all MD species recorded (one model), (ii) the content of each detected polyphenol type in each plant species (19 models – see Online Resource 6 for full description) and (iii) the total polyphenol content in three MD (*R. fruticosus*, *R. ideaus*, *U. dioica*) and one TH (*G. aparine*) species (four models). In each case we started with a full model, with all available soil and stand characteristics (stand diversity (categorical: monoculture/mixed), proportion of deciduous trees in the stand (percentage of the total basal area per plot), total cover of trees and shrubs (percentage), total stand basal area (m^2ha^{-1}), pH (of the topsoil 10 cm), C/N ratio (of the topsoil 10 cm). Then, the collinear variables were excluded based on the variance inflation factors, and the full model was reduced to minimize AICc, using the MuMIn::dredge() function (Bartoń 2023) and the criterion of $\Delta AICc > 2$ to select the most complex model within the set of all models. The results of the final models were visualized using package *effects* (Fox et al. 2022).

Results

Out of 193 understory plant species recorded during the surveys, 82 species (42%) did not possess any medical properties (NM species). Seventy-nine species (41%) were recognized as traditional herb (TH) plants and 32 species (17%) as MD species.

Table 1 The model explaining the percentage cover of medicinal plants in mature forests (Białowieża Forest (Poland), Hainich National Park (Germany) and TREEWEB (Belgium) and in experimental sites: Kaltenborn (Germany), Zedelgem and Gedinne (Belgium)); CovTot – total tree crown cover, DeciProp – proportion of deciduous trees in stands, pH of the topsoil (0–10 cm) and C/N ratio

Variable	Estimate	SE	t	Pr(>|t|)
(Intercept)	-7.1473	12.4064	-0.576	0.565
CovTot	-16.5038	3.1491	-5.241	2.76e-07
DeciProp	0.1062	0.0321	3.304	0.001
pH	3.2619	1.6948	1.925	0.055
C/N	0.8672	0.2997	2.894	0.004

The analysis of Ellenberg's ecological indicator values (EIV) revealed that MD species were more light demanding than TH and NM species (Mann-Whitney test; $U = 835.5$, $p = 0.028$ and $U = 1006$, $p = 0.063$, respectively; Fig. 1a). TH species were typical for less acid soils than NM and MD species (Mann-Whitney test; $U = 2352.5$, $p = 0.004$ and $U = 821.5$, $p = 0.040$, respectively; Fig. 1b). The other EIVs (temperature, moisture and nutrients) did not show significant differences between the studied groups of plant species.

Factors affecting the cover of medicinal plants

The biomass of MD plants potentially available for harvesting, expressed by their percentage cover, was negatively affected by the total tree crown cover and positively by the proportion of deciduous trees in the stand and soil C/N ratio. Influence of the pH of the top soil layer (0–10 cm) was at the verge of significance (Fig. 2; Table 1).

Table 2 Effects of environmental factors (SR – stand diversity (monoculture – mixed); pH – reaction of the topsoil layer (0–10 cm); C/N ratio (0–10 cm); BA – total basal area of trees; DP – proportion of deciduous trees in the stand) on the content of polyphenols (CGA – chlorogenic acid; DICQA – 3,4-dicaffeoylquinic acids (two isomers); QHR – quercetin hexosyl-rutinoside; EA – ellagic acid; S – sanguiin (H2 and H6); CMA – caffeoylmalic acid) in samples of *Aegopodium podagraria*, *Galium aparine*, *Rubus fruticosus* and *Urtica dioica*, revealed by generalized linear models. Polyphenol content in *Stachys sylvatica* and *Rubus idaeus* was not explained by any of the studied environmental factors. N/A – the polyphenol was not detected in the sample; see Online Resource 6 for full estimates of the models

Species	CGA	DICQA(I)	DICQA(II)	CMA	QHR	EA	SH2	SH6
Aegopodium podagraria	SR ↗	SR ↗ pH ↗ C/N ↗	No significant effect	N/A	N/A	N/A	N/A	N/A
Galium aparine	BA ↘ C/N ↗	N/A	N/A	N/A	BA ↘	N/A	N/A	N/A
Rubus fruticosus	N/A	N/A	N/A	N/A	N/A	C/N ↘	DP ↗ pH ↗	DP ↗ pH ↗
Urtica dioica	SR ↘ BA ↘	N/A	N/A	SR ↘ pH ↗	N/A	N/A	N/A	N/A

Factors affecting the content of individual polyphenols

Among the studied environmental factors stand diversity (monoculture – mixed), pH of the topsoil layer (0–10 cm), C/N ratio and total basal area of trees per plot affected significantly the content of polyphenols in four out of the six sampled plant species. The content of 3,4-dicaffeoylquinic acid (DICQA) in *A. podagraria*, sanguiin H2 (SH2) and sanguiin H6 (SH6) in *R. fruticosus*, and caffeoylmalic acid (CMA) in *U. dioica* increased with increasing pH of the soil. The content of CGA in *U. dioica*, and CGA and a flavonoid- quercetin hexosyl-rutinoside (QHR) in *G. aparine* decreased with increasing stand basal area. The content of SH2 and SH6 in *R. fruticosus* increased with the proportion of deciduous trees in the stand. The effects of stand diversity and C/N ratio were not consistent. The content of CGA and DICQA in *A. podagraria* was higher in mixed stands than in monocultures but the content of CGA and CMA in *U. dioica* was lower in mixed stands than in monocultures. Increase in C/N ratio positively affected content of CGA in *G. aparine* and DICQA in *A. podagraria* but negatively content of ellagic acid (EA) in *R. fruticosus* (Table 2, Online Resource 6). The content of polyphenols in *S. sylvatica* (acetylated apigenine dihexoside, acetylated chrysoeriol *p*-kumaroilo-dihexoside, CGA, chrysoeriol dihexoside, stachysponoside-acetylated chrysoeriol dihexoside, verbascoside/isoverbascoside) and *R. idaeus* (EA, ellagic acid pentoside, SH6) was not explained by any of the studied environmental factors (Online Resource 6).

Table 3 The model explaining the total content of polyphenols in *Galium aparine* sampled in the experimental sites of Gedinne and Zegeldem (Belgium) as a function of the proportion of deciduous trees in stand (DeciProp) and the total cover of tree crowns (CovTot)

Variable	Estimate	SE	T	Pr(>|t|)
(Intercept)	1.8110	1.02808	1.762	0.106
DeciProp	0.0281	0.0107	2.632	0.023
CovTot	-2.3765	0.8008	-2.968	0.013

Factors affecting the total polyphenol content

The total polyphenol content (TPC) in *G. aparine* was positively affected by proportion of deciduous trees in the stand and negatively by the total cover of tree crowns (Table 3; Fig. 3). In *R. fruticosus* TPC decreased with increasing C/N ratio (Table 4; Fig. 4). TPC in *U. dioica* was negatively affected by stand basal area and total tree crown cover and was higher in monocultures than in mixed stands (Table 5; Fig. 5). TPC in *R. idaeus* was not explained by any of the environmental factors studied.

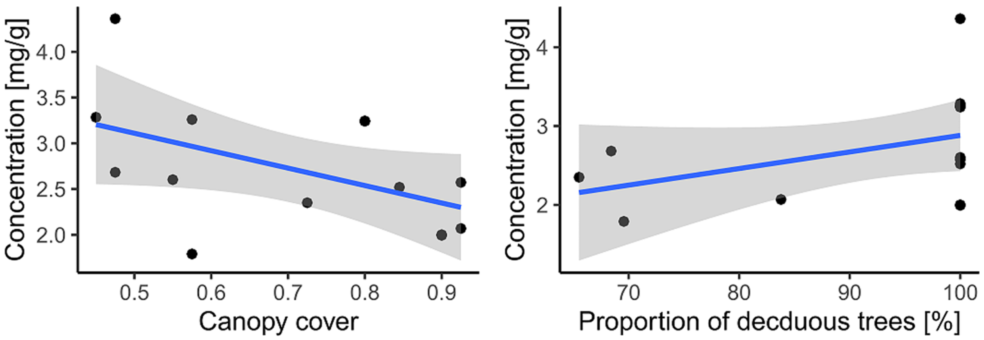

Fig. 3 Prediction of the total content of polyphenols in *Galium aparine* [mg/g of dry mass], based on the proportion of deciduous trees in stand and the total cover of tree crowns (see Table 3 for model summary)

Table 4 The model explaining the total content of polyphenols in *Rubus fruticosus* sampled in experimental site Gedinne and Zegeldem and mature forest site TREEWEB in Belgium, as response to C/N ratio

Variable	Estimate	SE	T	Pr(>|t|)
(Intercept)	11.1522	1.3724	8.126	2.79e-09
C/N	-0.2336	0.0932	-2.506	0.018

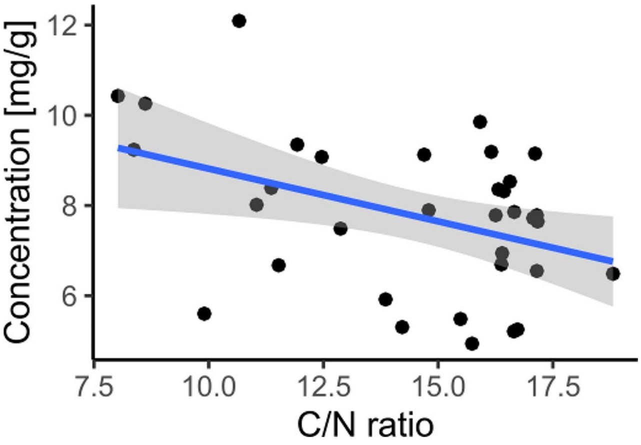

Fig. 4 Prediction of the total content of polyphenols in *Rubus fruticosus* [mg/g of dry mass], based on C/N ratio (see Table 4 for model summary)

Table 5 The model analyzing the total content of polyphenols in *Urtica dioica* sampled in experimental site Zegeldem (Belgium) and mature forest Białowieża Forest (Poland), as response to stand basal area (BA), total cover of tree crowns (CovTot) and stand diversity (SR) – mixed stand effect

Variable	Estimate	SE	T	Pr(>|t|)
(Intercept)	5.8529	0.6063	9.653	9.76e-10
BA	-0.0322	0.0135	-2.374	0.026
CovTot	-2.6064	1.0240	-2.545	0.018
SR	-0.7765	0.3591	-2.162	0.041

Discussion

Our study highlights the importance of European temperate forests as a reservoir of medicinal plants (MD). The proportion of medicinal plants (17%) in the flora of forest ecosystems, found in our study, was considerably higher than the 12–13% estimated by Kalusheva et al. (2017) in the flora of Europe as a whole. On a global scale, the distribution of plant species used by humans overlaps with plant biodiversity hotspots (Pironon et al. 2024), with 25% of drugs used in modern medicine derived from plants growing in rainforests (Shah and Bhat 2019). Our study shows that medicinal plants make up a large proportion of the flora of regular temperate forests outside of biodiversity hotspots, highlighting their importance as potential source of this resource.

Several forest characteristics significantly influenced the quantity (species cover) of MD plants and their quality (content of polyphenols). The cover of MD plants increased with decreasing canopy cover and increasing proportion of deciduous trees in the stand, due to the regulating effect of these two forest characteristics on light availability for the forest understory. A negative interaction between canopy cover and understory plant species cover and biomass is well known for the temperate forests (Verheyen et al. 2012; De Frenne et al. 2015; Landuyt et al. 2020). This interaction is driven by stimulating effect of light availability (and associated temperature) on growth, cover and height of understory plants (Tinya and Ódor 2016; Tinya et al. 2019; Blondeel et al. 2020). The proportion of deciduous trees in mixed stands, which emerged as a significant factor influencing MD abundance, is important for understory plants because in temperate forests they provide high seasonal light availability before canopy leaves develop and shade the forest floor (Neufeld and Young 2014). In this context it should be stressed that the MD plants, due to their higher light demands, may be affected by canopy cover stronger than other functional groups of understory plant species. Medicinal plant cover was also positively influenced by the C/N ratio of the upper soil layer. However, the value of the C/N ratio at the study plots was in the range between 8 and 29, which is typical for biologically active soils with mull or moder type of humus, being optimal or suboptimal for plant growth (Ponge 2013). Our findings on factors influencing MD plant cover are in line with the literature reporting that partial shade and soils rich in organic matter are the most basic common requirements of forest MD plants (Rao et al. 2004) and generally requirements of most of the forest understory plants (e.g. (Landuyt et al. 2020).

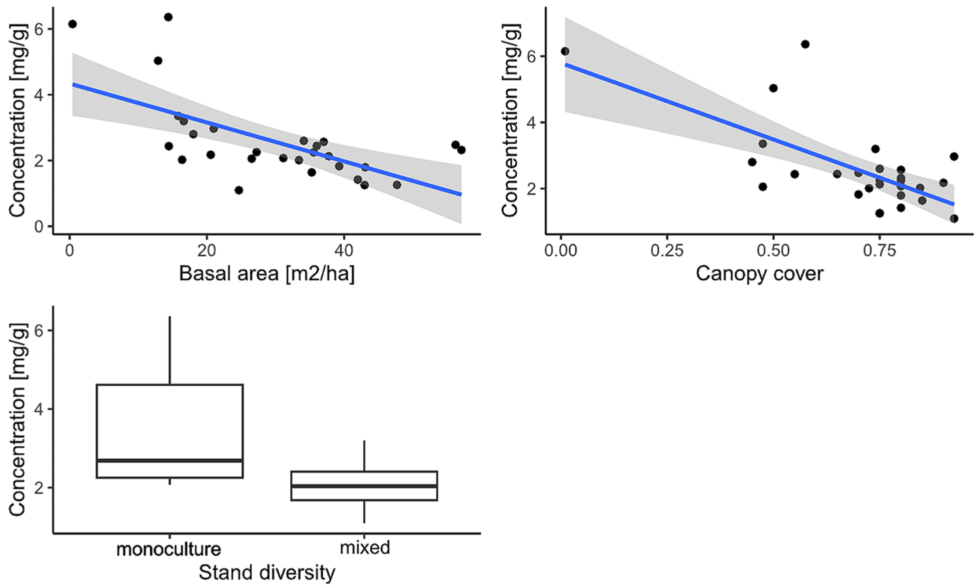

Fig. 5 Prediction of the total content of polyphenols in *Urtica dioica* [mg/g of dry mass], based on stand basal area (BA), total cover of tree crowns (CovTot) and tree diversity (see Table 5 for model summary)

The content of different polyphenol types, even in the same plant species, was often modulated by different factors, making generalizations difficult. For example in *R. fruticosus* the content of ellagic acid was negatively affected by soil C/N ratio, while content of sanguiin was positively affected by proportion of deciduous trees and soil pH. Such differences may reflect the interactions between genetic, ontogenic, morphogenetic and environmental factors, all of which may alter the content of secondary metabolites in plants (Verma and Shukla 2015), triggering biosynthesis of different secondary metabolites and their accumulation in plant tissues (Thoma et al. 2020; Reshi et al. 2023). Experimental studies confirm that content of phenolics is modulated by different factors in different plant species, e.g.: deficit of moisture and sun exposure increases the content of phenolics in *Pteridium arachnoideum* (Alonso-Amelot et al. 2007), exposure to low temperature triggers phenolic production in *Arnica montana* and *Zea mays* (Christie et al. 1994; Pennycooke et al. 2005; Albert et al. 2009), and nutrient deficiency increases content of phenolics in *Ceratonia siliqua* (Kouki and Manetas 2002). However, it must be considered that these plant species were studied under different environmental setups (in different ecosystems) than those covered by our study. We found, that the content of even the same type of polyphenol, the chlorogenic acid (CGA), common to four out of the six forest MD species studied, was affected by different factors (stand diversity, basal area and C/N ratio) in each plant species (see Table 2). Stand diversity, which affected the CGA content in more than one MD species, had an opposite effect in each of them: in *U. dioica* the content of CGA was lower in mixed stands than in monocultures, while in *A. podagraria* lower in monocultures than in mixed stands. Different factors influencing the levels of the same secondary metabolite or the opposite effect of the same factor in different plant species, are consistent with the findings of Pant et al. (2021), that production of secondary metabolites in plants is highly species specific. The effect of environmental factors on the content of phenolics is also context-dependent and can vary greatly depending on local environmental conditions (Ramakrishna and Ravishankar 2011; Radušienė et al. 2012).

The factors affecting the total polyphenol content (TPC) of the MD plants studied were also species-specific. In *G. aparine* the TPC increased with the proportion of deciduous trees in the stand and decreased with increasing canopy cover. The latter factor also influenced the TPC in *U. dioica* in the same way. Both these forest characteristics regulate the amount of light reaching the forest floor: the increasing proportion of deciduous trees increases the amount of light reaching understory in early spring, while decreasing canopy cover increases the amount of light at the forest floor throughout the whole year. Therefore, TPC in *G. aparine* and in *U. dioica* was positively influenced by light availability, which is in line with the studies reporting that light intensity regulates the accumulation and quality of secondary metabolites (Kong et al. 2016; Li et al. 2018, 2020). However, the positive or negative effect of light depends not just on intensity of light but also on its quality, and especially UV light has a positive effect on the synthesis of phenolics (Spitaler et al. 2006). We did not measure light amount reaching forest floor directly, neither any qualitative light characteristics but the more open canopy allows more light to reach the forest floor and direct light contains UV spectrum, which is efficiently filtered by tree canopy (Hovi and Rautiainen 2020).

In *R. fruticosus* TPC decreased with increasing C/N ratio of the topsoil, which may be interpreted as decreasing availability of nitrogen. The importance of nitrogen availability for production of phenolics results from the carbon/nitrogen balance metabolic pathway. In general, plant growth depends on protein synthesis, which competes for common substrate with synthesis of phenolics, thus, this is the classical example of the dilemma of plants: to grow or to defend (Herms and Mattson 1992). The growth processes receive allocation priority in resource-rich environments, while in poorer habitats (characterized by high C/N ratio) production of phenolics, and other carbon-based secondary metabolites is prioritized (see (Nguyen and Niemeyer 2008). There are many papers confirming positive effect of low nitrogen availability on the content of phenolics in crops, vegetables and cultivated fruit plants, summarized by (Heimler et al. 2017) in their review. The experimental evidence of such effect in forest plants is very scarce and suggests rather higher importance of acidity related soil elements (e.g. pH, Al, Zn) than soil fertility expressed e.g. by C/N ratio (Naud et al. 2010). Our findings for *R. fruticosus* are the opposite: C/N ratio had a significant effect on the TPC in this species but pH was insignificant. In case of other studied species none of this two factors had a significant effect on TPC and in case of single polyphenols, their effects were specific to the type of polyphenol and species-specific.

The TPC in *U. dioica* was negatively affected by crown cover and stand basal area, which are often correlated with each other (Korhonen et al. 2007) and limit amount of light reaching forest understory. The importance of light availability for synthesis of secondary metabolites were discussed on the example of *G. aparine*. The higher TPC revealed in monocultures vs. mixed stands is more difficult to interpret but it might be related to the tendency of monocultures to deplete the soils (Liu et al. 2018) and decrease availability of nutrients, which increases allocation of resources for secondary metabolites, discussed on the example of *R. fruticosus*.

We did not find any significant effect of the ambient temperature on polyphenol content in the studied plants. This is inconsistent with the literature reporting that temperature is among environmental factors significantly influencing content of secondary metabolites in plants, including polyphenols (see reviews by Li et al. (2020), Pant et al. (2021) and Ncube et al. (2012). Direct measurement of temperature was carried out in our study, but due to the relatively small distance between plots at each of the study sites (from a few tens of metres to a few tens of kilometres), the differences in mean temperature between plots, were too small to have a significant effect on the content of polyphenols. However, the same temperature datasets, analyzed by Gillerot et al. (2022), revealed significant effect of forest diversity and structure on the buffering of human thermal stress. In our opinion, the lack of a significant effect of temperature on polyphenol accumulation in our study is due to the fact that the production of secondary metabolites is often driven not by average environmental conditions, but by their extremes (Herms and Mattson 1992; Pant et al. 2021), which were not taken into account in our study. It is also worth mentioning that the analysis of the ecological requirements of plant species, based on their temperature ecological indicator values, did not reveal any significant difference between the MD, TH and NM species groups. This result suggests that temperature effects are not different for MD plants compared to the background vegetation in temperate forests.

Very challenging is that the content of individual phenolics in two (*S. sylvatica* and *R. idaeus*) out of the six analyzed species and also the TPC in one (*R. idaeus*) out of the four species studied could not be explained by any environmental factors or stand characteristics considered. This is a further confirmation of the highly species-specific nature of the influence of environmental factors on the production of secondary metabolites in plants (Pant et al. 2021). On the other hand, many phenolics are only metabolized after being induced by stress (e.g. wounding, pathogen or herbivore attack, low or high temperature impulse, UV radiation, etc.) (Dixon and Paiva 1995; Li et al. 2020), whereas such factors were not considered in our study.

Conclusions

We revealed that medicinal plants make up comparatively high portion of the flora of the European temperate forests, making these ecosystems an important source of the herbal resources used in traditional and modern medicine. Light availability was the most important factor positively influencing both effects studied, i.e. quantity and quality of forest MD plants. Therefore, forest managers should keep stands quite open, if non-timber forest products are at stake. The second factor, identified as important for the content of phenolics was soil fertility expressed as C/N ratio and for some types of compounds and some species also pH of the topsoil layer. The concentration of phenolic compounds was higher on less acidic soils and on soils with a higher C/N ratio, which are slightly opposite effects, since soils with a high pH are usually characterized by a lower C/N ratio due to their high biological activity. In general we found that the concentration of phenolics in plant tissues is highly species-specific and can vary greatly depending on local environmental conditions. This raises an important issue from an applied perspective: commercial collectors of herbal resources are paid for the plant biomass, before assessing the content of biologically active compounds. In effect, they are interested in the high biomass yield rather than in the concentration of active compounds, which is impossible to assess in the field. In this respect, the common forest characteristics, that favour a high yield and high quality of MD plants are a high proportion of deciduous species and a high canopy openness, allowing high access of light to the understory. In addition, plants with high total content of polyphenols should be sought for on biologically active (non-acidic) soils with a high C/N ratio, i.e. covered by trees with slowly decomposing litter. Our results can be used to guide forest management in areas where harvesting of understory MD plants is an important provisioning ecosystem service. In many cases a medicinal plant friendly forest management scenario will require only a minor changes in forest management, as cultivation or enhanced growth of MD plants can take place in intensively thinned forests and cleared forest patches, which does not compete with timber production. However, it should be noted that our recommendations are based only on six plant species studied in lowland mesic forests and may not apply to upland and mountain forests or forests growing in extreme environmental conditions (dry, acidic or waterlogged soils), which were not covered by our study.

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Author contributions B.J.: Conceptualization, Data curation, Formal analysis, Funding Acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing; K.S.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing; H.B., Q.P., M.S.-L., K.V.: Funding Acquisition, Methodology, Resources, Writing – review & editing; A.K.: Supervision, Investigation (Lab), Methodology, Resources, Writing – review & editing;

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Data availability The plot-level data characterizing forest stands and soils from Białowieża Forest (Poland) and Hainich (Germany) are available at the DRYAD data portal (Scherer-Lorenzen et al., 2023). The other data will be made available upon request by data providers.

Declarations

Competing interests The authors declare no competing interests.

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