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Trends in biological activity research of wild-growing aromatic plants from Central Balkans

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***Abstract*:**

Džamić, A.M., Matejić, J.S., Marin, P.D.: *Trends in biological activity research of wild-growing aromatic plants from Central Balkans. Biologica Nyssana, 7 (2), December 2016: 61-73.*

Flowering plants consists of more than 300.000 species around the world, out of which a small percentage has been sufficiently investigated from phytochemical and biological activity aspects. Plant diversity of the Balkans is very rich, but still poorly investigated. The aim of this paper is survey of current status and trends in research of wild-growing aromatic plants from Central Balkans. Many aromatic plants are investigated from morphological, physiological, ecological, systematic and phytochemical aspects. However, traditionally used medicinal and aromatic plants can also be considered from applicative aspects, concerning their health effects, and from wide range of usage in cosmetics, and as food, agrochemical and pharmaceutical products. In order to achieve all planned objectives, following methodology has been applied: field research, taxonomic authentication and, comparative biologically assayed phytochemical investigations. The total herbal extracts, postdistillation waste (deodorized) extracts, essential oils and individual compounds of some autochthonous plants have been considered as potential source of antibacterial, antifungal, anti-biofilm, antioxidant and cytotoxic agents. In this manuscript, composition of essential oils and extracts were evaluated in a number of species, from the Apiaceae, Lamiaceae, Rosaceae and Asteraceae families. Extracts which were rich in phenols mostly of flavonoids, often showed high antioxidant potential. Also, phenolic compounds identified in essential oils and extracts were mostly responsible for expected antimicrobial activity. Current worldwide demand is to reduce or, if possible, eliminate chemically synthesized food additives. Plant-produced compounds are becoming of interest as a source of more effective and safe substances than synthetically produced antimicrobial agents (as inhibitors, growth reducers or even inactivators) that control growth of microorganisms. Many different pathogens have developed resistance toward synthetic antibiotics and mycotics, so, there is a need for discovering a new antimicrobials. It is worth noting that synergistic effect of components found in essential oils or in various extracts may pay key role in its biological activities.

Key words: Antibacterial, antifungal, antioxidant, cytotoxic activity, plant extracts, essential oil

***Apstrakt*:**

Džamić, A.M., Matejić, J.S., Marin, P.D.: ***Trendovi u istraživanjima biološke aktivnosti divljerastućih aromatičnih biljaka centralnog Balkana. Biologica Nyssana, 7 (2), Decembar 2016: 61-73.***

Broj cvetnica u svetu se procenjuje na više od 300.000 vrsta, od kojih je mali procenat u potpunosti proučen sa fitohemijskog i sa aspekta biološke aktivnosti. Biljni diverzitet Balkana je bogat, ali je i dalje slabo proučen. Cilj ovog rada je pregled trenutnog stanja i trendova u proučavanje samoniklih aromatičnih biljaka sa područja Centralnog Balkana. Mnoge aromatične biljke su proučavane sa morfološkog, fiziološkog, ekološkog, sistematskog i fitohemijskog aspekta. Međutim, lekovite i aromatične biljke koje se koriste u narodnoj medicini mogu se razmatrati i sa stanovišta njihove primenljivosti, imajući u vidu njihov efekat na zdravlje i široku upotrebu u kozmetici, kao hrane, i u agrohemijskim i farmaceutskim proizvodima. U cilju postizanja planiranih zadataka, primenjena je sledeća metodologija istraživanja: terenski rad, taksonomska autentifikacija i komparativna biološka i fitohemijska istraživanja. Ukupni biljni ekstrakti, ekstrakti postdestilacionih ostataka (deodorisani), etarska ulja i pojedinačne komponente autohtonih biljka su razmatrani kao potencijalni izvori antibakterijskih, antifungalnih, anti-biofilm, antioksidativnih i citotoksičnih agensa. U ovom radu prikazan je hemijski sastav etraskih ulja i ekstrakata brojnih vrsta iz familija Apiaceae, Lamiaceae, Rosaceae and Asteraceae. Ekstrakti bogati fenolima, naročito flavonoidima često poseduju visok antioksidativni potencijal. Takođe, fenolne komponente idetifikovane u etarskim uljima i ekstraktima se smatraju odgovornim za antimikrobnu aktivnost. Širom sveta se trenutno radi na smanjenju ili potpunom eliminisanju hemijski sintetisanih aditiva u hrani. Biljni proizvodi su postali značajni kao izvor efikasnijih i bezbednih supstanci nego što su sintetički antimikrobni agensi koji kontrolišu rast mikroorganizama (inhibitori, regulatori rastenja ili inaktivatori rasta). Mnogi različiti patogeni su postali rezistentni na sintetičke antibiotike i mikotike, pa je potrebno pronaći nove efikasne antimikrobne supstance. Potrebno je istaći da sinergistički efekat između komponenti u etarskom ulji ili različitim ekstraktima može imati ključni efekat za njegovu biološku aktivnost.

Ključne reči: antibakterijska, antifungalna, antioksidativna, citotoksična aktivnost, biljni ekstrakti, etarska ulja

Introduction

Plant kingdom, with over 300.000 higher species, represents a source of new chemical agents for active pharmaceutical ingredients and lead compounds. Only a small percent 5% to15% of these plants have been chemically and pharmacologically investigated. Approximately 10.000 to 15.000 of the world's plants have documented medicinal uses and roughly 150-200 have been incorporated in western medicine (Krause & Tobin, 2013). The Balkan flora is not only the richest in Europe, but comprises also many endemics. According to contemporary assessments, vascular flora of the Balkans comprises almost 8.000 taxa (Stevanović et al., 2007). In flora of Sebia 3.562 species and subspecies are recorded and among them 32.12% are protected (Igić et al., 2010). Plants which contain bioactive compounds are often categorised as medicinal or poison plants, and their consumation should have adverse or beneficial result which often depends on the dose of taking. Secondary metabolites are small organic molecules and specific biologically active compouds that are produced by plants. They pay important role in the function of plant organisms and are important for long-term survival and defence. Nowadays, it is known that production of secondary metabolites are the rule rather than exception. Secondary metabolites represent essential source for discovery of a novel compounds because of great diversity of their structures (Bernhoft, 2010). Modern approach adopted to explore plant and its bioactive constituents involve interdisciplinary work in botany, pharmacognosy, chemistry and toxicology (Hostettmann et al., 1995) and should take following steps:

1. selection, collection, botanical identification, preparation of plant material
2. extraction with suitable solvents and preliminary analysis
3. biological and pharmacological screening of crude extracts
4. chromatographic separation of pure bioactive constituents guided by bioassay
5. structure determination
6. analyses and pharmacological profile of pure compounds
7. toxicological testing

If plants show no signs of being attacked by pests and neither has pieces eaten out of the leaves, there is a good chance that some metabolites are present which act as insecticides or antimicrobial agents.

The evaluation of plant extracts to ensure efficacy and safety is followed by identification of active principles, dosage formulations, efficacy and pharmaco-kinetic profile of the new drug. Many plants have been used because of their antibacterial, antifungal, anti-biofilm, antioxidant, cytotoxic, anti-inflammatory, antidiabetic, anti-cholinesterase, anti-nociceptive, anti-proliferative, genotoxic, anti-genotoxic, neuroprotective, herbicidal, enzyme inhibition, anti-Alzheimer properties and have been investigated by a number of researchers worldwide. Ethnopharmacologists, botanists, microbiologists, natural product chemists, and many others are searching for phytochemicals which could be developed for some applications (Das et al., 2010). It has to be pointed out that biological activity data for majority of chemically investigated wild growing plants are still missing (Čavar Zeljković & Maksimović, 2014).

This study gives an overview of current status and trends in research of potential of wild-growing aromatic plants of different biological aspects and methods *in vitro*.

Extracts and Essential oils

Plants are complex matrices, which produce a wide range of secondary metabolites with different functional groups and polarities. Categories of natural products commonly encountered include waxes and fatty acids, polyacetylenes, terpenoids (e.g. monoterpenoids, iridoids, sesquiterpenoids, diterpenoids, triterpenoids), steroids, essential oils (lower terpenoids and phenylpropanoids), phenolics (simple phenolics, phenylpropanoids, flavonoids, tannins, anthocyanins, quinones, coumarins, lignans), alkaloids and glycosidic derivatives (e.g. saponins, cardiac glycosides, flavonoid glycosides). For preliminary investigation of biological properties the most important is to test complex mixture as total plant extracts. Several approaches can be employed to extract the plant material. Although water is used as an extractant in many traditional protocols, organic solvents of varying polarities are generally selected in modern methods of extraction to exploit a solubility of plant constituents. Solvent extraction procedures applied for the initial extraction plant natural products include maceration, percolation, Soxhlet extraction, pressurized solvent extraction, ultrasound assisted solvent extraction, and steam distillation. Terpenoids and essential oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. The most abundant terpenes in essential oils are monoterpenes (C10) and sesquiterpenes (C15) while diterpenes (C20), as well as aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones (Seidel, 2006; Bohlin et al., 2012) are often present.

Antimicrobial activity

Current standard antimicrobial methods approved by various organizations such as Clinical and Laboratory Standards Institute (CLSI), and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) exist, for guidelines of antimicrobial susceptibility testing of convenient drugs, these might not be exactly applicable to plant extracts and modifications have to be made. In general, antimicrobial methods are broadly classified into diffusion and dilution methods. Diffusion tests include agar well diffusion, agar disk diffusion, poison food technique, and bioautography, while dilution methods include agar dilution, broth microdilution and broth macrodilution technique (Das et al., 2010).

Agar well diffusion and disk diffusion have been applied in the first screening, where extract or test substance are placed into agar or applied on the filter disc. Zone of inhibition have been measured. Bioautography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components. Bioautography is a very convenient way of testing plant extracts and pure phytochemical compounds for their effect on both human pathogenic and plant pathogenic microorganisms. It can be employed in the target directed isolation of active constituents (Choma & Grzelak, 2011).

However, the most preferred method is broth microdilution assay (or micro-well dilution assay), using microtitration plates, by making the serial two-fold dilutions of test substances in corresponding medium. Using this method, minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) are recorded. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of the samples inhibiting visible growth. Minimal bactericidal/fungicidal concentration (MBC/MFC) is defined as the lowest sample's concentration that kills 99.9% of bacterial/fungal cells. The main advantages of broth methods, especially microdilution which is carried out in microtiter trays are lower workloads for a larger number of replicates and the use of small volumes of the test substance and growth medium. In this method dilution of the oil is better and there is no agar in the medium, which both enable better diffusion through the liquid medium (Soković et al., 2011).

Antimicrobial activity of wild growing Apiaceae species

The *in vitro* antimicrobial activity of essential oils and various extracts of wild growing Apiaceae has been investigated by the microdilution method, where a panel of pathogenic bacteria and microfungi was tested (Milosavljević et al., 2007; Soković et al., 2009; Stojković et al., 2009;

Glamočlija et al., 2011; Matejić et al., 2012, 2013, 2014, 2015; Marčetić et al., 2014a; 2014b; Mileski et al., 2014, 2015; Popović et al., 2015a, 2015b) (**Tab. 1**). Essential oil analyses of inflorescence and aerial parts of endemic species, *Ferulago macedonica* showed α-pinene as the main compound (43% and 23%, respectively). Both oils demonstrated strong antibacterial activity. The essential oil of *Echinophora spinosa*, containing δ-3-carene (60.86%) as the dominant component, was the most prominent against *Escherichia coli* and *Pseudomonas aeruginosa* while the most resistant bacterial species against the oil was the *Staphylococcus aureus* (Glamočlija et al., 2011). Essential oil of *Echinophora sibtorpiana* (characterized by presence of methyl eugenol (60.40%), followed by *p*-cymene (11.18%) and α-phellandrene (10.23%) demonstrated considerable antimicrobial activity. The lowest antimicrobial activity was detected for aqueous extracts. The most resistant were fungus *Aspergillus niger* and bacteria *Micrococcus flavus* and *Escherichia coli* (Mileski et al., 2014). Essential oil of *Seseli rigidum*, with dominance of α-pinene (48.5%), showed antimicrobial activity. The most resistant bacterial strains were *Micrococcus flavus* and *Staphylococcus epidermidis,* and the most resistant micromycete was *Aspergillus niger* (Stojković et al., 2009). Methanol extracts of various *Seseli* taxa revealed activity against different bacteria and yeast *Candida albicans* (with MICs of 0.78-12.5 mg/ml) (Matejić et al., 2012). Methanol and ethyl acetate extracts of *Cacrys cristata* - rare and critically endangered species in the flora of Serbia, exhibited similar antibacterial activity. Methanol extract from aerial parts, inflorescence and fruits of *Opopanax hispidus* showed significant antimicrobial effect (Matejić et al., 2014, 2015).

Antimicrobial activity of wild growing Lamiaceae species

The *in vitro* preliminary antimicrobial tests were carried out by disc diffusion method. The results of the antimicrobial activity obtained by disc-diffusion assay showed that the diethyl ether extracts of *Stachys* inhibited the growth of all the tested bacteria, and *S. plumosa* exhibited significant antimicrobial activity against pathogens *E. coli, P. aeruginosa* and *S. aureus* (comparable to antibiotics used as the positive controls (Lazarević et al., 2010). In last decade majority of antimicrobial experiments used microdilution method. It is well known, that essential oils from Lamiacae family showed high antimicrobial potential. Antibacterial and antifungal activity of essential oils of wild growing *Thymus*, *Mentha*, *Mellisa*, *Hyssopus*, *Salvia*, *Satureja*, *Sideritis* and *Stachys* species were analyzed (**Tab. 1**). Essential oils of *Salvia sclarea* (linalyl acetate and linalool) and *Mentha longifolia* with dominance of *cis*- and *trans*-dihydrocarveol and piperitone, were analyzed for their antifungal potential. The most susceptible was phytopathogen *Phomopsis helianthi*, while the most resistant was *Trichoderma viride* (Džamić et al., 2008, 2010). Essential oil of *Satureja kitaibelii* characterized by dominance of *p*-cymene showed strong antimicrobial activity (MIC values of 0.10-25 mg/ml) (Kundaković et al., 2011; Mihajilov-Krstev et al., 2011), while essential oil of *Hyssopus officinalis* subsp. *pilifer* (1,8-cineole, β-pinene and isopinocamphone) showed lower antimicrobial potential (Džamić et al., 2013). Methanolic extract of *Satureja kitaibelii* and its major compound rosmarinic acid possessed strong antimicrobial activity against tested bacteria and microfungi (Stanojković et al., 2013).

The components with phenolic structure are known to be highly active antimicrobial agents. Thymol, a main constituent of various *Thymus* essential oils, was responsible for wide spectrum of antibacterial and antifungal activity (Soković et al., 2009; Vladimir-Knežević et al., 2012; Petrović et al., 2016). Carvacrol and thymol are recognized as strong antimicrobial agents able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane. Antimicrobial activity is associated with chemical structure of compounds. The most important is lipophilic character of hydrocarbon skeleton and hydrophilic character of functional group. Antimicrobial activity should be arranged in this order: phenols > ketones > alcohols > ethers > aldehydes > hydrocarbons (Dorman & Deans, 2000).

Antimicrobial activity of wild growing Rosaceae species

Antibacterial activity of three wild fruits, red wild berry fruit (*Cornus mas*), blackthorn (*Prunus spinosa*) and wild blackberry (*Rubus fruticosus*) extracts was analyzed using disc-diffusion and microdilution methods against 12 bacterial strains (**Tab. 1**). The antimicrobial activity of wild berry fruits extracts was high against almost all the tested bacterial strains (Radovanović et al., 2013).

Antimicrobial activity of wild growing Asteraceae species

Essential oil of *Helichrysum arenarium* in bioautography assay showed clear inhibition zones which were indication of strong antimicrobial activity Gram (+) (*Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and Gram (-) bacteria (*Escherichia coli*, *Pseudomonas tolaasii*, *Salmonella enteritidis*, *Salmonella typhimurium*) and yeast *Candida albicans* (Rančić et al., 2005). The most rarely used was micro-atmosphere assay. The essential oil evaporated and the activity by vapor contact has been measured. Due to the high lipophilic nature of mycelia coupled with a large surface area relative to the volume of a fungus, vapours of essential oils may act mainly by accumulation on mycelia than in the agar (Inouye et al., 2000). Stupar et al. (2014) investigated antifungal activity of the essential oil of *Helichrysum italicum* using micro-atmosphere method. The main components of the oil were γ-curcumene, α-pinene, and neryl acetate. The essential oil of *H. italicum* showed moderate antifungal activity against fungi isolated from cultural heritage objects. The most susceptible fungi to oil treatment were *Epicoccum nigrum* and *Penicillium* sp., while the most resistant was *Trichoderma viride*.

Pathogenic strain *Pseudomonas aeruginosa* causes pathological changes on vegetables, and it is considered as conditional parasite of plants, but also as a human parasite because it causes infections of urinary and respiratory tract. The effect of the water and ethanol plant extract was tested against pathogenic strains *Pseudomonas aeruginosa* and *P. fluorescens* using disc diffusion method. Water and ethanol extract of *Achillea millefolium* and *Salvia officinalis* showed the greatest inhibitory effect on *Pseudomonas aeruginosa*, (the water extract of *Achillea*). Great inhibitory effect of the ethanol extract had *Helichrysum arenarium*, *Rosmarinus officinalis*, *Satureja montana* (Petrović et al., 2003).

In general, microdilution assay is the most rewarding method for different analyses. The several strains of microorganisms should be tested on one plate, and small amount of tested sample could be used. Nowadays this technique is the most frequently used. The chemical composition and the potential to have antimicrobial activity depend highly on the plant species, habitat and ontogeny phase as well as of methods of processing, extraction solvent and extract dose. Many times the values of the inhibition zones were proportional to the concentration of the extracts and essential oils. Sometimes dominant compounds are crucial for the biological activity. Syinergistic effect of the compounds are also important.

Antioxidant activity

Compounds with antioxidant activity are mainly phenolic acids, flavonoids and polyphenols (Dillard & German, 2000). Phenols provide the plants with defense mechanisms to neutralize reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores. Flavonoids show a wide range of biological activities such as inhibition of cell-proliferation, induction of apoptosis, inhibition of enzymes and other antibacterial and antioxidant effects (Pandey & Gupta, 2014). Antioxidants may directly react with the reactive radicals to destroy them by accepting or donating electron(s) or they may indirectly decrease the formation of free radicals by inhibiting the activities or expressions of free radical generating enzymes or by enhancing the activities and expressions of other antioxidant enzymes. Many research models have been established in chemical and/or biological systems for studying the mechanisms of action of antioxidants and for identifying new antioxidants (Lu et al., 2010) and it is suggested to test potential antioxidants by different methods. Important step for determination of the antioxidant capacity is quantification of phenolic compounds in samples by using Total phenol content (TPC) assay with Folin–Ciocalteu reagent, as well as quantification of total flavonoids (TFC).

The most frequently used *in vitro* antioxidant methods are: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) radical scavenging activity, Ferry reducing power (FRAP) assay, Total reducing power (TRP) assay, β-carotene-linoleic acid assay etc.

Available literature data of *in vitro* antioxidant activity of wild growing species from Central Balkans are given in **Tab. 1**.

Antioxidant activity of wild growing Apiaceae species

Methanolic extract of *Seseli* taxa tested for its antioxidant potential (total phenols, total flavonoids DPPH and ABTS assays) revealed *Seseli libanotis* ssp. *libanotis* for its highest reducing power (Matejić et al., 2012). Essential oil of *Echinophora sibthorpiana* possessed high radical scavenging capacity in DPPH and ABTS assays. Aqueous extract of aerial parts was the strongest tested extract. Extracts of aerial parts expressed higher radical scavenging activity in comparison to the root extracts. All extracts had lower antiradical activity compared to BHA and vitamin C (Mileski et al., 2014). In the case of various extracts of *Ferulago macedonica*, it was found that there is no strong relationship between total phenolic and flavonoid contents and radical scavenging activities

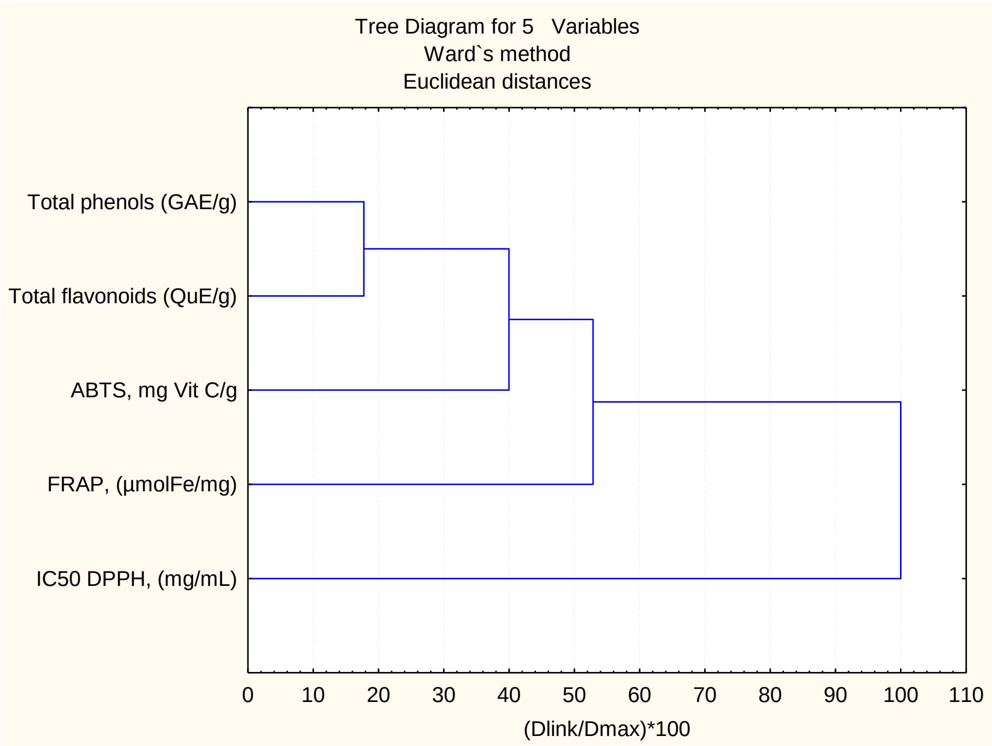

Fig. 1. Dendrogram of applied assays for antioxidant characteristics evaluation for *Melittis melissophyllum* extracts (Grujić et al., 2014).

(DPPH, ABTS). These results can be explained since the antioxidant property of a plant extract is generally considered as the result of the combined activity of a wide range of compounds including, not only phenols, but also peptides, organic acids and other components. Both, phenols and flavonoids were present in higher amounts in *F. macedonica* extracts of inflorescence in comparison to the aerial parts (Mileski et al, 2015).

Antioxidant activity of wild growing Lamiaceae species

Extracts of waste water after hydrodistillation of *Satureja montana* and *S. cuneifolia*, showed better ability to reduce stable DPPH radical than the standards essential oils or thymol, carvacrol, and thymoquinone. This is probably due to the high concentration of other active compounds, such as (*E*)-coniferyl alcohol. To compare these results, total antioxidant activity of these two *Satureja* species was also tested using the phosphomolybdenum method. This test is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. Total antioxidant activity of the phosphomolybdenum model evaluates both water-soluble and fat-soluble antioxidants, i. e. total antioxidant capacity. The highest activity in this method was found for essential oil of *S. cuneifolia* (Čavar et al., 2013).

For scavenging ability on DPPH radicals, various extracts of *Hissopus officinalis* subsp. *pilifer* were effective in the order of: deodorized aqueous extract > deodorised metanol extract > deodorised ethyl acetate extract. The total phenol content, determined by Folin–Ciocalteau reagent was in the same order. Essential oil of *H. officinalis* subsp. *pilifer* possessed the lowest activities compared to other extracts and control substances (Džamić et al., 2013). Antioxidant potential of *Melittis melissophyllum* various hydro alcoholic, ethanol and methanol extracts revealed a high scavenging activity by DPPH and ABTS methods. Reducing power of extracts were evaluated through the FRAP assay. The highest phenol and flavonoid content were identified in the 96% ethanol extract and the lowest were in the ethanol 10% solution. Also, the highest correlation was observed between total phenol and flavonoid contents. Statistically significant correlations were registered for DPPH and ABTS assays which is logical due to their similar mechanism of action as radical scavengers. A hierarchical cluster analysis (HCA) was performed on the total phenols and total flavonoids content DPPH, ABTS, and FRAP assay. The dendrogram (Fig. 1) shows that total phenols and total flavonoids content are quite homogeneous, while DPPH was placed in separate cluster (Grujić et al., 2014).

Table 1. Antimicrobial and *in vitro* antioxidant activity of extracts and essential oils of wild-growing species from Central Balkans

Fam	Species	Extracts	EO composition	Antimicrobial methods	Antioxidant methods	References
Apiaceae	*Cachrys cristata* DC.	Methanol, ethyl acetate, acetone, aqueous	Aerial parts, fruits	Antibacterial Antifungal Microdilution	Total phenols Total flavonoids DPPH ABTS	Matejić et al.,2012, 2014
	Daucus carota L.		Fruits, flowers, root, leaves and stem	Antibacterial Antifungal Microdilution		Soković et al., 2009
	Echinophora sibthorpiana Guss. *Echinophora spinosa* L.	Methanol, ethanol, aqueous	Inflorescence aerial parts	Antibacterial Antifungal Microdilution	Total phenols Total flavonoids DPPH ABTS	Mileski et al., 2014 Glamočlija et al., 2011
	Eryngium palmatum Pančić and Vis.	Methanol chloroform	Root	Microdilution Antibacterial Anticandidal	Total phenols DPPH FRAP	Marčetić et al., 2014a; 2014b
	Ferulago macedonica Micevski and E. Mayer	Methanol, ethanol, aqueous		Antibacterial Antifungal Microdilution	Total phenols Total flavonoids DPPH ABTS	Mileski et al., 2015
	Laserpitium latifolium L. *L. zernyi* Hayek *L. ochridanum* Micevski	Chlorophorm	Underground parts aerial parts fruits	Antibacterial Antifungal microdilution antibiofilm		Popović et al, 2015 a,b
	Opopanax hispidus (Friv.) Griseb.	Methanol, ethyl acetate		Antibacterial Anticandidal Microdilution	Total phenols Total flavonoids DPPH ABTS	Matejić et al., 2015
	Seseli pallasii Besser, *Seseli libanotis* (L.) Koch ssp. *libanotis* *Seseli libanotis* ssp. *inter-medium* (Rupr.) P. W. Ball *Seseli rigidum* Waldst. & Kit. *Seseli globiferum* Vis. *Seseli annuum* L.	Methanol	 Aerial parts fruits	Antibacterial Antifungal Microdilution	Total phenols Total flavonoids DPPH ABTS FRAP	Matejić et al., 2012 Ilić et al., 2014; 2015 Janacković et al, 2011 Stojković et al, 2009 Milosavljević et al., 2007
	Tordylium maximum L.	Methanol, aqueous		Antibacterial Anticandidal Microdilution	Total phenols Total flavonoids DPPH ABTS	Matejić et al., 2013
Lamiaceae	*Hyssopus officinalis* ssp. *pilifer*	Deodorized aqueous, methanol and ethyl acetate	Aerial parts	Antifungal Microdilution	Total phenols DPPH	Džamić et al., 2013
	Mentha longifolia L.		Aerial parts	Antifungal Microdilution	DPPH	Džamić et al., 2010
	Mellisa officinalis L.		Aerial parts	Antibacterial Antifungal Microdilution	DPPH OH radical	Mimica-Dukić et al., 2004
	Melittis melissophyllum L.	Methanol, Ethanol (hydroalcoholic)			Total phenols Total flavonoids DPPH ABTS FRAP	Grujić et al., 2014
	Salvia nemorosa L. ssp. *nemorosa* *Salvia sclarea* L.		Aerial parts	Antibacterial Antifungal Microdilution	DPPH Phosphomolybd enum	 Božin et al., 2011 Džamić et al., 2013
	Satureja montana L. *Satureja cuneifolia* Ten *Satureja montana* ssp. *pisidica* (Wettst.) Šilić *Satureja montana* L. ssp. *montana*		Aerial parts	Antibacterial Antifungal Microdilution	DPPH ORAC TRP APF	Čavar et al., 2013 Kundaković et al., 2014 Mihajilov-Krstev et al., 2014; 2011

Table 1. continuation of the table

Fam	Species	Extracts	EO composition	Antimicrobial methods	Antioxidant methods	References
Lamiaceae	*Satureja kitaibelii* Wierzb. ex Heuff. *Satureja horvatii* Šilić					Bukvički et al., 2014 Lakušić et al., 2008
	Sideritis montana L.		Aerial parts	Antibacterial Microdilution		Miladinović et al., 2012
	Stachys germanica ssp. *heldreichii* (Boiss) Hayek *Stachys iva* Griseb. *Stachys plumosa* Griseb. *Stachys scardica* Griseb.	Diethyl ether, ethyl acetate		Antibacterial Antifungal Disk-diffusion	Phosphomolybdenum	Lazarević et al., 2010
	Thymus praecox Opiz ssp. *polytrichus* *Thymus longicaulis* C. Presl *Thymus serpillum* L. *Thymus tosevii* Velen	Supercritical extracts Methanol, ethanol, aqueous	Aerial parts	Antibacterial Antifungal Microdilution Macrodilution Disk-diffusion	DPPH	Petrović et al., 2016 a, b Vladimir-Knežević et al., 2012 Soković et al., 2009
Rosaceae	*Crataegus oxyacantha* L.			Antibacterial Disk-diffusion	Total phenols Anthocyan content Flavonoid content DPPH	Kostić et al., 2012
	Cornus mas L. *Prunus spinosa* L.	Acidified methanol solution		Antibacterial Disk-diffusion Microdilution	DPPH	Radovanović et al., 2013
	Rosa canina L.	Methanol		Antibacterial Antibiofilm Microdilution		Živković et al., 2015
	Rubus caesius L. var. *aquaticus* Weihe and Nees *Rubus fruticosus* L.	Methanol, ethanol, acetone, aqueous Acidified methanol solution		Antibacterial Disk-diffusion Microdilution	Total phenols Total flavonoids DPPH ABTS FRAP TRC	Veličković, et al., 2015; Radovanović et al., 2013
Asteraceae	*Achillea collina* Becker ex Heimerl s.l. *Achillea pannonica* Scheele		Aerial parts	Disk-diffusion	DPPH	Božin et al., 2008
	Ambrosia artemisiifolia L.	70% aqueous acetone extracts			Total phenols Total flavonoids DPPH FRAP	Maksimović, 2008
	Artemisia alba Turra *Artemisia absinthium* L.	Ethanol	Aerial parts	Antibacterial Anticandidal Microdilution	Total phenols Total flavonoids DPPH FRAP ABTS	Đorđević et al., 2013 Mihajilov-Krstev et al., 2014
	Helichrysum arenarium (L.) Moench *Helichrysum italicum* (Roth) G. Don		Aerial parts	Antibacterial Antifungal Microdilution Bioautography		Rančić et al., 2005 Stupar et al., 2014
	Hieracium pillosela L.	Methanol, dychlorometane, ethyl acetate, dychlorometane:methanol		Antibacterial Disk-diffusion		Stanojević et al., 2008
	Xeranthemum annuum L	Methanolic acetone ethyl acetate		Antibacterial Antifungal Microdilution	Total phenols Total flavonoids DPPH	Stanković et al., 2011

Antioxidant activity of wild growing Rosaceae species

Radovanović et al. (2013) found that in polyphenolic extracts of fruits from tree Rosaceae species main compounds were gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, (+)-catechin, procyanidin B2, (-)-epicatechin, quercetin, rutin and quercetin-3-glucoside. All extracts showed high scavenging effect on DPPH radical. Extracts obtained from *Hieracium pilosella* have significant free radical scavenging activity on stable DPPH• and high reactive hydroxyl radical. The data suggest that aqueous, ethanolic and methanolic extracts of *H. pilosella* from Southeastern Serbia are a potential source of natural antioxidants. Chlorogenic acid was detected in the highest quantities in all investigated extracts (Stanković et al., 2009).

Antioxidant activity of wild growing Asteraceae species

According to Božin et al. (2008) the main detected compounds in essential oil of *Achillea callina* were β-pinene, chamazulene and *E*-caryophyllene, while in essential oil of *A. pannonica* 1,8-cineole and camphor were the dominant components. Authors represented that both oils possess strong antioxidant effects *in vitro* and *in vivo* assays.

Antioxidant capacities are influenced by many factors, which cannot be fully described with a single method. The most commonly used method for assessment of antioxidant properties of natural products is DPPH radical assay. The DPPH radical assay overcomes the limitations of monitoring the activity of the numerous samples over a specified period of time. It is reproducible and strongly correlated with phenolic compounds (Miliauskas, 2004).

Cytotoxic activity

Brine shrimp, *Artemia salina*, is the test organism that is used for evaluation of possible cytotoxic activity of plant extracts or essential oils. Brine shrimp assay is simple, inexpensive method and results are obtained quickly. In the study represented by Janaćković et al. (2008) a brine shrimp assay was used to examine potential cytotoxic activity of different *Centaurea* species, measuring lethal concentration (LC). Cytotoxic activity of methanol and ether extracts was tested. The methanol extract of *C. arenaria* showed very significant activity while the lowest activity was found with *C. chrysolepis* methanol extract. Among ether extracts, the most active was *C. splendens* (and the lowest activity was recorded for the ether extract of *C. scabiosa*. Toxicity test on *Drosophila melanogaster* showed that the essential oil of *A. absinthium* is toxic for developing insect larvae. Starting with the concentration of 0.38% of essential oil in medium, significant mortality of larvae exposed to the oil was noted when compared to the control. The essential oil also affected the development of *D. melanogaster* larvae and significantly delayed achievement of the pupa stadium (Mihajilov-Krstev et al., 2014).

Many plant extracts and natural products, especially phenols, with high antioxidant activity have shown cytotoxic effects in different cell lines. Flavonoid anticancer activities include inhibition of cell growth, inhibition of protein kinase activities, and induction of apoptosis. Popular and broadly used for cytotoxity examination is the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay which is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Methanolic extract of *Origanum vulgare* showed significant antiproliferative activity in both colon cancer (HCT-116) and breast cancer (MDA-MB-231) cell lines. Inhibitory activity appeared to be particularly conspicuous in treated HCT-116 cell lines, whereas at the highest concentration (500 μg/mL), only 10% of the cells remained viable. The results indicate that *O. vulgare* is considered to be a particularly valuable source of effective anti-proliferative and cytotoxic substances (Grbović et al., 2013). The methanol extract of *Satureja kitaibelii* exhibited strong activity against Fem-x human malignant melanoma cells, and moderate activity against breast cancer cell lines (Stanojković et al., 2013). The cytotoxic effect of the essential oils from *Satureja montana* ssp. *pisidica* from two localities (mountains Korab and Galičica) was tested against cancer cell lines MDA-MB-361, MDA-MB-453, HeLa, LS174 and MRC5 cells. The essential oil from Korab demonstrated significantly better results than the oil from Galičica, particularly against HeLa and MDA-MB-453 cell lines, while the oil from Galičica was the most active on the human epithelial cervical cancer HeLa cells (Kundaković et al., 2014).

Other activities

Most studies describe the action of essential oil and extracts for other biological activities. Strong inhibition of human serum cholinesterase by essential oil of *Satureja montana*, suggests this as a natural source of compounds that can be used in the treatment of foodborne and neurological diseases, wound and other infections, as well as for general health improvement (Mihajilov-Krstev et al., 2014). Considering human and horse serum cholinesterase inhibition, both essential oils acted as strong inhibitors, even higher than effect of their dominant constituent α-pinene, leading to the conclusion that the other constituents of the essential oil manifested their action toward cholinesterase (Ilić et al., 2015). Thyme essential oil, as well as its major phenolic constituent's thymol and carvacrol were researched for application in Alzheimer's disease treatment or as remedies for cognitive disorders. It was noted that phenolic chemotypes possess higher antioxidant activity than non-phenolic ones, as well as acetylholinesterase inhibitory activity (Čavar Zeljković & Maksimović, 2014).

Conclusion

The reported results from *in vitro* studies hint the potential antimicrobial and antioxidant value of the wild growing plants from Central Balkan. It was prepared based on plenty literature search of biological activity of extracts and essential oils. The microdilution method was found as the mostly used in antimicrobial assay. DPPH method is the most frequently used in antioxidant assay. The traditional use of many aromatic species leads to discovering the unknown biological potential of related species. The most important approach is combination of phytochemical investigation and bioassay. Essential oil and extracts without identifying of compounds responsible for this activity are also insufficient for preclinical trials. In spite the fact that a lot of studies have been reported so far, many of wild-growing plants are still waiting to be explored.

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